Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in Ribonucleotide Reductase Ia

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“Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will”

- Galileo Galilei
DECLARATION

I hereby declare that this thesis has been written independently and with no other sources and aids than quoted.

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ABSTRACT

Ribonucleotide reductase connects the RNA and the DNA world via strictly controlled radical chemistry that reduces all four essential ribonucleotides to deoxyribonucleotides. In RNR Ia, the starting point is the µ-oxo diiron cofactor, where a “stable” tyrosine radical (Y_{122} \cdot) is formed from a nearby tyrosine in the β subunit. Successive studies showed that Y_{356} \cdot(β) Y_{731} \cdot(α) and Y_{730} \cdot(α) are intermediate steps of an intersubunit radical pathway, before a putative catalytic cysteine radical (C_{439} \cdot) is formed in the α subunit. Conformational gating hinders the direct observation of these transient radicals. A well-characterized mutation strategy by site-specific incorporation of the unnatural 3-amino-tyrosine (NH₂Y) was successfully used to omit conformational gating. To analyze electrostatic effects and hydrogen (H) bond networks, all three Ys (Y_{356}, Y_{731} & Y_{730}) were successively mutated. Seminal studies revealed an exceptional difference between the tyrosine radicals formed within the radical propagation and the Y_{122} \cdot at its beginning. The stepwise oxidation and reduction of these amino acid radicals is directly linked to a proton-coupled electron transfer (PCET). Therefore, the investigation of electrostatics and H bonds is fundamental to understand this important process in biology.

Pulsed 263-GHz EPR spectroscopy as well as ENDOR spectroscopy delivered insight based on closely characterized mutation approaches into the electronic and H bond structure of the NH₂Ys•. It could be shown that an electropositive surrounding of moderate to strong H bonds are a common feature in α and β subunits. In the α subunit, double mutant approaches delivered insight into the effect of the removal of an H bond donor on the radical transfer efficiency and supported the assignment of the ENDOR studies. Deuteron nuclei (²H) ENDOR spectroscopy revealed 2, 1 and 0 H bonds perpendicular to the ring plane of NH₂Y_{730}•, NH₂Y_{731}• and NH₂Y_{356}•, which is consistent with a "π-stacking" between Y_{731} and Y_{730}. Three structural DFT models for NH₂Y_{731}• based on optimized crystal structures have been discussed in terms of H bonds and environment. A perpendicular strong H bond (1.6 Å) and a weak H bond (≥1.9 Å) was consistent with the electrostatics observed at NH₂Y_{731}•. NH₂Y_{356}• showed the lowest gx value, typical for a polar electrostatic environment. Due to the limited structural data, no active model of NH₂Y_{356}• could be obtained. The possible influences on the gx value were discussed based on small model DFT calculations. Experimentally, one weak to moderate H bond (1.9±0.1 Å) could be resolved in the forward radical transfer to a wild type-β Y_{356}• environment using a different mutation strategy. DFT models consistent with the obtained g values proposed another weak H bond (>2.1 Å). All moderate H bonds found at residue β-356 were in-plane of the tyrosine π system. Overall, this illustrates that different H bond networks in the α and β subunit are used to promote this long proton-coupled redox chain.
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LIST OF ABBREVIATIONS AND ACRONYMS

§ section
A Alanine
A hyperfine coupling coupling value
a\(_{iso}\) isotropic hyperfine coupling value
C(•) cysteine (radical)
cf. confer
DFT density functional theory
Dopa (S)-2-Amino-3-(3,4-dihydroxyphenyl) propanoic acid
E. coli Escherichia coli
ELDOR electron-electron nuclear double resonance
ENDOR electron nuclear double resonance
EPR electron paramagnetic resonance
ET electron transfer
Exp. experimental
HAT hydrogen atom transfer
H bond hydrogen bond
HF hyperfine
MW microwave frequency
(d)NDP (2'-deoxy)nucleotide diphosphate (nucleotide = A, adenosine; G, guanosine; T, thymidine; U, uridine; C, cytidine)
NH\(_2\)Y(•) 3-amino tyrosine (radical)
PAS principle axis system
p. page
P phosphate
PDB ID protein database identification code
PELDOR pulsed electron-electron double resonance
ref. reference
RF radio frequency
PS photosystem
PT proton transfer
RMSD root mean square deviation
RNR ribonucleotide reductase
RT radical transfer (translocation)
SRT shot repetition time (delay between two pulse sequences)
S/N signal to noise ratio
SPP shots per point
\( T \) temperature
\( T_{\perp/\parallel} \) orthogonal/parallel anisotropic hyperfine coupling
\( T_1 \) spin-lattice relaxation time
\( T_2 \) spin-spin relaxation time
UAA unnatural amino acid
wt wild type
XRD X-ray crystal structure diffraction
\( Y(\bullet) \) l-tyrosine (radical)

\( g_e \) 2.002329304, g-factor
\( \nu_{1H} \) 142.63 MHz, \(^1\)H Larmor frequency \((B_0=3.35 \text{ T})\)
\( \nu_{2H} \) 21.895 MHz, \(^2\)H Larmor frequency \((B_0=3.35 \text{ T})\)
\( \nu_{19F} \) 134.16 MHz \(\cdot\) \(^{19}\)F Larmor frequency \((B_0=3.35 \text{ T})\)
\( \gamma_e \) -1.760 s \(\cdot\) \(\text{T}^{-1}\), gyromagnetic ratio for an electron \((g\mu_e/\hbar)\)
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1 INTRODUCTION

RIBONUCLEOTIDE REDUCTASES

Decades after their discovery, Ribonucleotide Reductases (RNRs) remain at the frontier of science in diverse disciplines. With the first tyrosine radical ever found in an enzyme,¹ RNRs opened up a complete new field of radical enzymes.² ³ Regarding oxygen storage and transformation, the RNR cofactor structure has sparked interest in atomistic molecular mechanisms.⁴ Furthermore, orally administered compounds targeting ribonucleotide reductases were found useful in cancer therapy.⁵ Therefore, a short overview of this class of essential enzymes for higher life forms is given.⁶ ⁷

1.1 Ribonucleotide Reductases: Bridging the RNA and DNA World

With the evolution from RNA to DNA, the necessity to form deoxyribonucleotides from ribonucleotides—under the retention of configuration—fostered the need for a specialized radical enzyme, the ribonucleotide reductase.⁸ This enzyme is entirely responsible for providing 2'-deoxyribonucleotides (as dNPDs or dNTPs) from all four ribonucleotides (as NPDs or NTPs; Figure 1-1);⁹ thereby it plays a strictly controlled central metabolic role in regulation of DNA precursors.¹⁰ ¹¹ Depending on the target organism, this essential role has been used successfully for anticancer, antibacterial and antiviral therapies.¹²-¹⁵
Diverse Classes of Ribonucleotide Reductases

1.2 Diverse Classes of Ribonucleotide Reductases

RNRs are divided into three enzyme classes. From the evolutionary view point, the different classes of RNRs can be linked to the change from a reducing to an oxidizing atmosphere. The different classes of RNRs are connected to aerobic and anaerobic life forms. In all classes a cysteine radical is proposed to induce a 3’ abstraction on the nucleotide side, as illustrated in Figure 1-1. Hence, this radical has to be generated. The three classes of RNR can be distinguished by their cysteine radical generation, see Figure 1-2. The cysteine radical is located on the tip of a loop within a structurally conserved ten-stranded α/β barrel protein, the α subunit. The structural motive belongs to the super family of glycyl radical enzymes. RNR class III indeed produces a glycyl radical by an activase. This additional enzyme utilizes iron sulfur chemistry (Fe₄S₄⁺/⁻) in a complex with S-adenosylmethionin. Glycyl radicals are oxygen sensitive; therefore RNR III works strictly under anaerobic conditions. Here the catalytic cycle is closed by a chemically simple formate as reductant, supporting the idea that class III is closely related to the ancient form of RNRs.

Figure 1-1: Chemical reaction catalyzed by RNRs. The 3’ hydrogen (marked red) abstraction leads to the irreversible loss of the 2’ hydroxyl (marked blue) in the form of water. The reaction is catalyzed by a bound cysteine radical (C•). The C• is initially formed by several radical precursors as shown in Figure 1-2. Proton(s) and two electrons of two neighboring thiols (RNR I and II) or from formate (RNR III) reduce the nucleotide. For RNR I and II the rereduction is performed by thioreoxin (TR) coupled to thioredoxin reductase (TRR) using NADPH as cofactor.
Introduction

Class II RNRs can tolerate oxygen, but are also independent from oxygen. They are activated by a radical cofactor formed from adenosylcobalamin and have been found to include the only functional monomeric RNRs.\textsuperscript{21} RNR class II and III proteins are common in bacteria and archaea, but rare in eukaryotes.\textsuperscript{29} A comparison of the α/β barrel has found a higher structural similarity between RNR II and RNR I compared to RNR III. The root mean square deviation (RMSD) increases from 1.0 Å to 1.7-1.8 Å (based on 70 C\textsubscript{α}s).\textsuperscript{20-22}

Class I RNR’s are common in eukaryotes and rare in bacteria and archaean.\textsuperscript{29,30} This class I harbors an μ-oxo-di-metallo cofactor, which induces a tyrosine radical (Y•) in the β subunit.\textsuperscript{27,31-33}

Depending on the environmental conditions, one RNR class can be better suited than another, as in anaerobic conditions RNR III, iron limiting or facultative oxygen supply conditions RNR II and in aerobic oxygen rich environments RNR I.\textsuperscript{6} Therefore, often several enzymes are found in an individual organism, as in \textit{Escherichia coli} (\textit{E. coli}).
The class I RNRs are further divided into three subclasses: (i) class Ia harbors an Fe\textsuperscript{III}\textsubscript{2} site, (ii) RNR Ib has a Mn\textsuperscript{III}\textsubscript{2} cofactor and (iii) RNR Ic has a mixed Mn\textsuperscript{IV} and Fe\textsuperscript{III} \textmu-oxo-\textmu-hydroxy complex. Despite the huge diversity of RNRs, some structural aspects are highly conserved, for instance, the nucleotide reduction mechanism, the activity and the specificity binding site.\textsuperscript{33-35} It is noteworthy that the location of the glycyl radical (RNR III), \textsuperscript{5}deoyadenosyl radical (RNR II) and two tyrosines (RNR I) occupy the same structural space in all RNRs. RNR Ia and Ib form a \textsuperscript{Y}\textsuperscript{•} in direct vicinity to the metal cofactor,\textsuperscript{20-22} whereas RNR Ic forms a \textsuperscript{Y}\textsuperscript{•} in \textbeta under a similar radical propagation mechanism.\textsuperscript{36-38} In RNR Ib, however, the formation is dependent on a cosubstrate (NrdI).\textsuperscript{32, 39, 40} Whereas for RNR Ia and Ic the active state resembles an \textalpha\textsubscript{2}:\textbeta\textsubscript{2} complex,\textsuperscript{33, 41} RNR Ib shows a variety of active encounters.\textsuperscript{42} All class I enzymes must transfer the electron from the \textalpha to the \textbeta subunit. This radical translocation and its mechanism based on putatively conserved radical intermediates within RNR Ia (cf. §1.4, p. 6) is still part of ongoing research and studied within this thesis.\textsuperscript{22, 38, 43-49}

1.3 Differences and Similarities of *Escherichia Coli* and Eukaryotic RNRs

This thesis focuses on the prototypical RNR Ia from *E.coli*. However, class Ia is most prominently found in eukaryotic organisms.\textsuperscript{29} RNR structures of yeast, mice and humans have been studied.\textsuperscript{50} Therefore, structural differences and similarities should be mentioned. The \textalpha subunits of several RNRs can be compared based on existing crystal structures (Figure 1.3).

Although the sequence homology of approximately 27\%, is quite small, the RMSD of *E. coli* RNR structure is small compared to that of eukaryotic RNR.\textsuperscript{50} For example, the differences in all common \textalpha\textsubscript{a} positons between *E. coli* and human RNR is 1.3 Å\textsuperscript{50} and to yeast it is 1.7 Å.\textsuperscript{50} Moreover, conserved residues, which are intended to take part in the radical translocation, allocate the same structural space for all found class I \textalpha structures (cf. Figure 1-3). In *E. coli* RNR Ia numbering, they are Y\textsubscript{730} and Y\textsubscript{731} (dark gray in Figure 1-3).
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

Introduction

Comparing eukaryotic RNR β subunits to E. coli, several differences were found: the so-called stable Y• (Figure 1-2) has been found to be far more accessible in eukaryotes compared to the prokaryotic structure. This also had implications on the electron paramagnetic resonance (EPR) spectra of this radical site. The Y• in eukaryotic RNRs is hydrogen (H) bonded, whereas that of E. coli is isolated; however, both are expected to be in a hydrophobic environment (cf. Figure 1-5, inset). The structural differences to E. coli in the β subunit are larger. For example, based on the C-α overlay 389 atoms fit between the hp53R2 dimer and the E. coli (1PFR) structure with an r.m.s. deviation of 1.67 Å. The α2/β2 subunit interaction is weak with a Kd of 0.4-0.5 µM in pro- and eukaryotic RNR Ia. So far, no structure of the active state could be found in any RNR.

Figure 1-3: Similarities between the different RNR Iα subunit structures from four organisms. Important conserved residues (Y-Y-C) for the radical transfer are shown in dark gray. The occupancy of the nucleotide reduction side or the effector side is shown in white spheres. The crystal structures used can be found in the protein data base under PDB ID: 1PEQ, 2X0X, 3S87 and 3HND left to right, up to down.

Figure 1-3: Similarities between the different RNR Iα subunit structures from four organisms. Important conserved residues (Y-Y-C) for the radical transfer are shown in dark gray. The occupancy of the nucleotide reduction side or the effector side is shown in white spheres. The crystal structures used can be found in the protein data base under PDB ID: 1PEQ, 2X0X, 3S87 and 3HND left to right, up to down.
In the following part of the chapter, the current knowledge and the aim of this thesis is further defined.

1.4 Escherichia Coli RNR Ia

1.4.1 Structural Basis of the Radical Transfer

1.4.1.1 The Tertiary and Quaternary Structure

To understand the activity of RNR Ia enzymes, several features have to be considered. Two diferrous sites are located in the obligate dimeric $\beta_2$ subunit with 87 kDa.$^{46,60}$ The $\beta_2$ subunit forms the $\mu$-oxo-diferic $Y\cdot$ cofactor using molecular oxygen.$^{60}$ UV-vis spectroscopy has shown that this tyrosine cofactor is remarkable in terms of stability. $Y_{122}\cdot$ is exceptionally stable with a half-life of $t_{1/2} \approx 14$ d at 4 °C,$^{61}$ whereas tyrosine radicals in solution are reduced within $\mu$s.$^{62}$ The crystal structure of the oxidized form revealed that $Y_{122}$ is 10 Å away from the surface and embedded in a closed hydrophobic region.$^{46}$ EPR crystal studies showed only a slight tilt ($\approx -3^\circ$ ring dihedral) of $Y_{122}\cdot$ after reduction.$^{63}$ On the other hand, stability of the radical implies that the $\beta$ subunit is able to trigger catalysis in $\alpha$ over a long reaction time.

Due to its central role in DNA synthesis and repair, the enzyme has to be tightly regulated. Beside the regulation in transcription,$^{64}$ the subunit interaction controls the formation of the $\alpha_2/\beta_2$ active complex. This is regulated by the large $\alpha_2$ dimer, with 172 kDa (Figure 1-4). Binding of ATP and dATP in the activity site increases and reduces the activity, respectively. Furthermore, allosteric control insures for the four different substrates ($S =$ CDP, ADP, GDP, UDP) by nucleotide binding to the effector site ($E =$ ATP, dGTP, TTP and dATP) a balanced pool of dNTPs. Overall, the binding of nucleotides intensifies the inter subunit binding by a factor of 2-8.$^{56,59}$ Moreover, the equilibrium between the active $\alpha_2\beta_2$ complex and the inactive $\alpha_4\beta_4$ is regulated by the activity site.$^{65}$

The active complex envisioned in silico based on shape complementary of the individual subunits $\alpha_2$$^{22,66,67}$ and $\beta_2$$^{46,63,68}$ is shown in Figure 1-5. The inactive ($\alpha_4\beta_4$) complex has been characterized by low-resolution methods such as cryo-EM$^{69,70}$ and small angle X-ray scattering data.$^{71}$ Interestingly, the distance between the substrate binding site and the $Y_{122}\cdot$ increases from about 40 Å to 55 Å between the active to the inactive form based on these models.$^{65}$ This regulatory process, however, tells us nothing about how this distance can be overcome to form the catalytic $C\cdot$. 

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Introduction
The Proposal of the Radical Translocation Pathway

In the docking model, as mentioned in the previous section, a rigid α/β barrel structure interacts with a buried Y122•. Ridged-body docking models could not further reduce this distance between Y122• and the catalytic site. This in silico model implied a radical transfer (RT) over 38 Å. Uhlin and Eklund concluded in 1994, that a long-range radical transfer takes place to overcome the distance between Y122• and nucleotide reduction side. The proposal of an electron transfer (ET) over more than 35 Å was unprecedented at that time. Four residues were suggested to form radical intermediates as W48 and Y356 in β, whereas Y731 and Y730 were assigned to take part in this RT in α (cf. Figure 1-5 left).

The participation of β-W48 in RT was suggested based on two arguments. First, it is a strictly conserved residue at the interface. Second, W48 was in a local environment similar to a W in cytochrome c oxidase, where W• has been found as an intermediate. However, no experimental evidence has been shown for the participation of W48• in the ET of RNR up to today.
Escherichia Coli RNR Ia

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Figure 1-5: *E. coli* RNR α₂β₂ docking model.²² The β₂ subunit (red and yellow) harbors the diferric Y₁₂₂• cofactor (large inset).⁴⁶ The α₂ dimer (green and blue) has the catalytic side (GDP in white) with the nearby catalytic C₄₃⁹• (small inset).²² The distance in this model between β₂-Y₁₂₂ and α₂-C₄₃⁹ is 38 Å. Strictly conserved residues along the radical translocation pathway are shown in black. 32 C terminal residues are not observed due to thermal lability including β₂-Y₃₅⁶ (marked with an ellipsoid).

Despite the absence of structural information (Figure 1-5), residue β₂-Y₃₅₆ was proposed to form a Y•⁷₅ and bridge the gap between β₂ and α₂ subunits. Seminal studies demonstrated that, although binding of the subunits is not perturbed, Y₃₅₆F mutation renders the protein inactive.⁴⁵, ⁵⁶ In α₂, the phenylalanine mutants of Y₇₃₀ and Y₇₃₁ were revealed to be inactive,⁴⁷ although the inter-subunit binding and crystal structures of the mutant were unperturbed.⁴⁷ Despite the success in assigning putative intermediates, the detection of radicals along the pathway remained elusive. A slow conformational step has been postulated as the main reason for the absence of pathway radicals in the wild type (wt) enzyme and mutants mentioned so far.³⁴, ⁴⁷, ⁷⁶
1.4.2 Unnatural Amino Acids to Study the Radical Translocation

In RNR Ia, two things have to be considered in studies of the RT: local structural or kinetic changes. First, RNR has an apparent half-site reactivity, but the active model is completely symmetric. Half-site reactivity is otherwise known from pseudo-symmetric molecular machines like the photosystem II (PS II). In RNR both half-sites are proposed to be active successively. A slow conformational arrangement occurs before the first α/β RT takes place and the second conformational step during or after product formation, but before the other half-site is triggered. This clearly complicates a stepwise investigation of the formed intermediates. The radical transfer and nucleotide reduction steps are not directly observable. Therefore, unnatural amino acids (UAA) were incorporated into E. coli RNR.

1.4.2.1 Evidence for the Active Role of β-Y356

First experimental evidence for an active role of β-Y356 within RT was obtained by turnover studies with unnatural amino acids. Catalytic rates changed by changing the redox potential (compared to Y -> Y• + e⁻ + H⁺) and the pKa at position 356. Fα-Y’s and 4-NH₂-phenylalanine (Figure 1-6 B) were incorporated at this position. It could be shown that redox potential differences from -50 to 50 mV are tolerated without loss of activity. When the pH of the buffer was changed stepwise at redox potential differences above 120 mV, only 30% of wt activity was observed. The differences in pKa values also revealed that RNR does not require a protonated tyrosine at 356 to be active. These studies suggested that a proton transport (PT) is not a prerequisite for ET from and to Y356.

Notably, this was the first indication that in the β subunit, due to the higher pKa of tyrosines, the proton travels orthogonally to the ET. Here a strongly conserved E350 in β has been proposed to be involved. Mutation of β2-E350 to alanine leads to a catalytically inactive enzyme, despite the ability to bind αe.

1.4.2.2 Observation of Radical Intermediates

Three main procedures were developed to introduce a new rate limiting step in RNR and to observe radical intermediates. First, an unnatural amino acid (Figure 1-6, 2&3) was incorporated to reduce the electron potential. This method can be applied to all RT pathway residues. Second, the potential of Y122 can be increased by introduction of an unnatural amino acid. This method will be discussed in Chapter 5 in more detail. Furthermore, a
radical can be produced via photoexcitation, for instance with a Re(I) complex. This complex is apolar and can alter the local environment at the interface. 

Introducing more stable radical intermediates to form radical “sinks” or “traps” could be performed site selectively in various ways. The first example is the incorporation of (S)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid (Dopa) as unnatural amino acid. It has a 260 mV lower reduction potential compared with tyrosine under similar conditions. Hence, incorporated for Y356-β2 the protein becomes inactive, but a radical can be observed at Dopa356-β2 after reaction with excess substrate, effector and α2 (single turnover conditions).

Figure 1-6: Unnatural amino acids (UAA), which could be incorporated into RNR. A) UAA 1, 3-7 have been incorporated by the AMBER Stop codon in vivo nonsense suppression. UAA 1, 2, and 4-7 have been incorporated in position 356 of β2 by expressed protein ligation. B) Positions within the pathway were UAA have been incorporated by the AMBER stop codon (in color) and protein ligation (in gray). Phenylalanine (F) or alanine (A) are shown in black incorporated by site specific mutagenesis. As summarized in ref. 41.

The second example stabilizes the radical on the pathway and still allows residual activity of the mutant protein complex with 3–12%. Additionally, the pKₐ is nearly identical to that of Y. 3-Amino tyrosine (NH₂Y) is 190 mV easier to oxidize to its corresponding radical (NH₂Y•) than Y based on the peak potentials. Under single turnover conditions it competitively “traps” the radical along the pathway. Here crystal structures of α2 revealed an undisturbed environment for the NH₂Y incorporation at 731 and 730, as displayed in Figure 1-7. These mutants were incorporated at all pathway tyrosine residues, as summarized in Figure 1-6. Kinetic studies revealed that the radical formation is kinetically competent compared to the overall rate of wt RNR of 2-10 s⁻¹. The biphasic behavior of all
radical formations was assigned to a conformational step. This step is prior to radical formation, thus between Y_{122} and Y_{356}. After formation of the radicals in ~30–40% yield, they are stable up to several minutes. This offered the possibility for their spectroscopical (§1.4.3 §1.5 and Chapter 4) and biochemical investigation.

Figure 1-7: Overlay of crystal structures of NH$_2$Y$_{730}$ (yellow), NH$_2$Y$_{731}$ (blue) and wt-$\alpha_2$ (green). Three RT pathway residues are shown as sticks and oxygen nuclei of water molecules in red spheres.

1.4.3 Experiments on the $\alpha_2\beta_2$ RNR Complex

The first experimental evidence of the “active” complex was obtained from pulsed EPR spectroscopy of radical pairs. The coupling between radical pairs can be measured by pulsed electron double resonance (PELDOR) spectroscopy and a distance can be assigned (see Section §2.1.4, p.35). After observation that both $\beta$ subunits carry a Y$_{122}$ radical, the half-site reactivity was used to measure the distance between $\beta$-Y$_{122}$ and radicals in $\beta$ or $\alpha$, as shown in Figure 1-8A. First, in a PELDOR experiment with solely wild type enzyme only one distance from Y$_{122}$-Y$_{122}$ could be observed. Then a nucleotide analog inhibitor forming a stable radical in $\alpha$ was used. A diagonal distance of 48±1 Å was obtained.
Figure 1-8: Diagonal distances of the active complex obtained with radical traps. A) PELDOR on β₂ revealed two Y₁₂₂• in β₂. Under reaction conditions with the substrate inhibitor 2‘azido-2’-deoxyuridine-5’-diphosphate only two distances are observed, 48 Å and 33 Å. B) In three consecutive reactions with three β₂/α₂ mutants diagonal distances gave the first experimental evidence for the proposed radical intermediates. All distances supported the docking model. Successive work also measured diagonal distances to Dop₃₅₆• and NH₂₃₅₆• at all suggested RT Y positions (Figure 1-8B). All distances supported the docking model. The “active” α₂β₂ complex is meanwhile generally accepted. These PELDOR studies were equally important as the first experimental evidence of radical intermediates between the subunits bridging the interface. And therefore they demonstrated the long distance electron transfer. Recently, the same distance to Y₃₅₆• has been determined in the millisecond time scale (8 ms) using another UAA (NO₂Y₁₂₂•) approach discussed in §1.4.5 (p.16). The “active” complex was only observed during the lifetime of a metastable transient radical (NH₂Y₇₃₀•) formed in α. Using this mutant, it had been demonstrated that in the “active” complex the subunit interaction increases by a kinetic factor of ~10⁴. This information along the interface could identify binding principles, which might be intercepted by inhibitors. Finding specialized inhibitors in RNRs is still an ongoing process. For instance β-peptides mimicking the C-terminal region of β₂ (see Figure 1-4) or nucleotide inhibitors operate at the interface. Structural information might improve these inhibitors. Currently, a 32 Å resolution structure of the “active” complex is available from cryo-electron microscopy reconstruction.
1.4.4 Proton-Coupled Electron Transfer

All radical transfer intermediates shown so far are amino acids. During each nucleotide turnover, they oxidize reversibly within the turnover rate of RNR (2-10 s\(^{-1}\)). Under physiological conditions, reversible oxidation requires release of a proton to an acceptor concomitant with oxidation. Otherwise, high energy intermediates are formed. More specifically, for tyrosines the difference between a stepwise transfer and a concerted proton electron transfer (CPET) is 540 mV in redox potential. A strong acidic (Y-OH\(^{•+}\)) transition state would be formed in a stepwise transfer, as illustrated in the parallelogram in Figure 1-9. To avoid high energy intermediates a variety of individual proton coupled electron transfer (PCET) steps are linked to the nature of the RNR radical enzyme. Studying individual PCET steps can be a paradigm to understand common principles, which control this fundamental process. Basic principles among PCET processes in biology are still hardly defined including but not limited to: photosynthesis, respiration and nitrogen fixation.

In RNR several amino acid oxidations take place over an unprecedented length. Here nearly every combination of possible PCETs has been discussed. The differences in mechanism should be briefly described in terms of forward radical transfer toward the active site (forward PCET). A recent proposal of the PCET mechanism in RNR is shown in Figure 1-10.

![Figure 1-9: PCET pathways between two Y radicals. An electron transfer (ET) and a proton transfer lead successively to charge separated transition states (double dagger). A concerted proton coupled electron transfer (CPET) avoids these states. A hydrogen atom transfer (HAT) is a special case of CPET in which the proton and the electron are transferred to the same orbital.](image-url)
Y_{122}^* and Y_{356}^* are reduced based on the current model (Figure 1-10) by a long range electron transfer and a short range proton transfer. The direction of ET and PT is different for the Y_{122}^* and Y_{356}^* redox reaction this is coined a bidirectional PCET. This step is strongly linked to the intrinsic quantum mechanical nature of the proton and electron movement. Electrons with their light mass can travel over large distances (in biology up to 20 Å), whereas PTs are limited to short distances (< 1 Å). Experimentally, exponential distance decay parameters have been estimated with $\beta_{ET} = -1.4$ Å$^{-1}$ and $\beta_{PT} \approx -27$ Å$^{-1}$ for ET and PT, respectively (see §2.4.1 p.54). The different nature of these transfers is also the background for a recent finding. Y_{122}^*$ is first activated by a proton transfer from an iron cofactor ligand, only then does the electron transfer (most probably to Y_{356}^*) take place.

The proton acceptor for β-Y_{356}^* has been proposed to be β-E_{350}. The assignment of E_{350} as proton acceptor, however, is still elusive. Experimental evidence is absent for participation of any of the conserved glutamates within β. It is generally accepted that the
electron to reduce $\beta\cdot Y_{356}^{3-}$ comes from $\alpha\cdot Y_{731}$. The proton acceptor of $\alpha\cdot Y_{731}$, however, is again undefined.

The adiabatic CPET between $\alpha\cdot Y_{730}$ to $\alpha\cdot Y_{731}\cdot$ is generally postulated, represented by a purple arrow in Figure 1-9 and Figure 1-10. Spectroscopic evidence is still missing. A prerequisite for this CPET is a parallel displaced "$\pi$-stacking" between $\alpha\cdot Y_{730}$ and $\alpha\cdot Y_{731}\cdot$ (cf. Theory §2.4.2, p.57). Open questions remain, because some crystal structures showing either a T-shaped "$\pi$-stacking" (see Figure 1-3) or even distant conformations between the reduced $\alpha\cdot Y_{731}$ and $\alpha\cdot Y_{730}$ amino acids (see Figure 1-12B). Moreover, it is still not clear if the proton travels as hydrogen atom (HAT) or if the electron interacts with the $\pi$ system of the nearby aromat (CPET). The former is often defined as a transfer from and to the same acceptor orbital. The CPET case describes the process in which the proton and the electron travel to two different acceptor orbitals (cf. §2.4.2.1). Common pitfalls in the assignment and term discussion are explicitly stated in a recent review. Additionally, it is unclear if a water can participate in this transfer step, or which hydrogen bond (H bond) interactions can modulate the PCET (cf. water in Figure 1-7).

The interaction between $\alpha\cdot Y_{730}$ and $\alpha\cdot C_{439}$ has been questioned to occur over an additional water species that generates a double PCET step. This has been postulated based on a QM/MM study. Results from EPR and density functional theory (DFT) a postulated direct transfer above, as discussed in §1.5.3. Here the authors assigned the proton acceptor of $\alpha\cdot Y_{730}$ to $\alpha\cdot Y_{731}$ and $\alpha\cdot C_{439}$, for forward and reverse radical transfer, respectively. The central difficulty is to find, locate and finally to assign interactions within the PCET. Our approach is to use the still spectroscopically observable stabilized radical state and resolve the interaction of protons in the environment after each PCET step, as introduced in §1.5 (p.19).

In most discussions of mechanism, calculations have always played a central role. Siegbahn et al. have demonstrated that the mechanism of PCET can be investigated without prior knowledge of the complete surrounding. Therefore various studies investigated the transfer between $Y_{731}$ and $Y_{730}$ solely by modeling a dipeptide. However, already in

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* The models were set up with and without an intervening water molecule present between the two Ys.
1998 Siegbahn clearly stated that at least the H bond interaction has to be known in order to obtain an accurate model, which is in part the scope of this thesis.  

Beside the proximity, energetics play a major role in PCET reactions. For short electron transfers (14 Å) it has been calculated that endogenous transfer steps of 450 mV can be overcome. At 10² s⁻¹, the rates remained within the range observed in RNR. In RNR, an endogenous step of more than 150 mV has been theoretically found for the forward radical transfer between Y₇₃₀ and C₄₃₉ in α. If the potential is not the rate limiting factor in electron transfers, how does RNR then control the remarkable specificity of its PCET? In RNR, a change in one residue renders the whole electron transfer inactive as discussed before.

ET rates in oxidoreductases are typically faster than observed in RNR with a catalytic rate of 2-10 per s. These ETs are reported to be in the order of μs. Therefore it was interesting to investigate which fast processes are hidden under the slow conformational step(s). Fast rates as 10⁵ s⁻¹ could be found for the PCET within α by photo activation, using a deprotonated UAA tyrosine analog (2,3,5-F₃Y) at residue β₃₅₆.

1.4.5 Rates and Thermodynamics during PCET

The discussion of PCET energetics is usually either based on bond dissociation energies (BDE) or redox potentials. The former can lay out the general picture of an endogenous forward PCET. BDE of PhO–H, RS–H, and HOCH₂–H are ~86, 91, and 94 kcal·mol⁻¹, respectively. The redox potential discussion tries to consider not only the solution redox potential, but also the potential in the individual protein environments. Initial studies could show that the pKₐs are within one subunit similar at Y₇₃₁ and Y₇₃₀. Only Y₁₂₂ has a >1.5 units larger pKₐ shift compared to the three on pathway tyrosines. Therefore it was postulated that by incorporation of different tyrosine analogs the redox potential could be estimated over the whole pathway. Up to now, studies have reported two major indications. Both use mutants, which increase the oxidation potential of Y₁₂₂, to omit the conformational gating, as shown on the left side of Figure 1-11. When 2,3,5-F₃Y₁₂₂ is incorporated, Y₃₅₆• is formed first up to 50% in the ms time scale, then it reduced to 25% in comparison with 2,3,5-F₃Y₁₂₂•. Therefore it was suggested that the 2,3,5-F₃Y₁₂₂ has a similar redox potential as Y₃₅₆ for radical formation (cf. Chapter 5). Furthermore studies incorporating NO₂Y₁₂₂ could demonstrate, that Y₃₅₆• is formed in equilibrium in a ratio of
10:1:1 with Y_{731}\cdot and Y_{356}\cdot. This equilibrium can be interpreted thermodynamically. The following redox potentials are obtained relative to Y_{122}, as shown in Figure 1-11.\cite{41} However, there is evidence for a non-Nernstian behavior between Y_{122} and Y_{356} positions.\cite{28, 41} This is suggestive for a conformational gating step present between these Y’s.\cite{28, 41} Additionally, the peak potentials reported here are under revision.\cite{15, 22} Reversible redox potentials are reported for the 3,5-F_{2}-Y up to now.\cite{28, 41, 121, 122}

![Figure 1-11: Redox peak potential changes relative to Y_{122}.\cite{41, 110} The potentials are evaluated for the reaction Y \rightarrow Y\cdot + e^{-} + H^{+}. Local pK_{a} considerations have been taken into account for the UAA in position β-122.\cite{41}](image)

1.4.6 PCET through the Interface of the Subunits

Information of the interaction between α-Y_{731} and β-Y_{356} is essential to understand the function of this enzyme class. The information from the individual crystal structures should be briefly summarized. As already mentioned, the C-terminal tail is unstructured in the β dimer, however it becomes ordered in the active state as PELDOR data and NMR work has revealed.\cite{43, 123} From an electrostatic point of view, the sequence of the C-terminal β tail reveals the presence of three negatively charged amino acids but zero positively charged ones. A plot of the expected electrostatic potential of an individual α at the interface to β is shown in Figure 1-12A. Thus not only the nucleotide binding site, but also the larger region is dominated by positive electrostatic charges.
Figure 1-12: Electrostatic and local structural information found for α-RNR. A) Electrostatic surface potentials were calculated using the program APBS\textsuperscript{124} with the nonlinear Poisson–Boltzmann equation and contoured at -4 to 5 kT/e. A dielectricity of water (ε=80) and ε=4 for wt protein α subunit was used.\textsuperscript{67} B) The crystal structure of NH$_2$Y$_{731}$ (blue, PDB ID 2XO5) is compared to the third monomer of NH$_2$Y$_{730}$ (yellow, PDB ID 2XO4). The O-O distance in Å and the ring dihedral is given.\textsuperscript{67}

The ET rate decays with distance. Considering this, the distance of the ET is limited to insure a selective transfer step across the interface (see Theory §2.4, p.54).\textsuperscript{99} The current proposed step of 33 Å between β$_2$-Y$_{122}$ and α$_2$-Y$_{731}$ is long, even including the β$_2$-Y$_{356}$ intermediate.\textsuperscript{125} However, a flexibility present in one monomer of the X-ray structure shows Y$_{731}$ changing its conformation and distance to NH$_2$Y$_{730}$ by 6 Å (Figure 1-12B). A conformational change at β-Y$_{356}$ cannot be excluded and could also reduce the distances for an individual PCET steps. Conformational changes could be too fast to be observed even using rapid freeze quench (>5 ms)\textsuperscript{126} techniques or other spectroscopic assays (>10 ms).\textsuperscript{127} However, a conformational change at a Y has been reported by infrared (IR) spectroscopy in β.\textsuperscript{128} They compared the non-oxidized to the oxidized state within minutes reaction time.\textsuperscript{128} IR spectroscopy normally need well characterized ring dihedrals and backbone dihedrals in order to assign IR shifts precisely (cf. Figure 1-12B).\textsuperscript{129–131} Barry et al. propose based on their Y,T di-peptide model\textsuperscript{129–131} a conformational change of Y$_{122}$•,\textsuperscript{128} however, the apparent contradiction between the ring dihedral from EPR spectral simulation (43°)\textsuperscript{132} and their assigned ring dihedral (80°) is currently not resolved.\textsuperscript{133} Generally, information on ring dihedrals within the PCET of RNR is reported only at one additional position. The ring dihedral of NH$_2$Y$_{730}$• was assigned by EPR spectroscopy (see next section).\textsuperscript{134} Spectroscopic
investigation in frozen solution and in the second time scale can deliver complementary information to interpret results on a common foundation.

1.5 EPR Spectroscopy for PCET Pathways

1.5.1 Overview

EPR spectroscopy on biomolecules is an elegant way to study selectively active sites around radicals and paramagnetic ions. Radicals and paramagnetic ions have an unpaired electron, which can be probed in a magnetic field \( B_0 \). Pulsed EPR techniques like double resonance techniques can meanwhile routinely be applied. One example is PELDOR (§1.4.3, p.11) spectroscopy, which delivers structural information based on the magnetic interaction between two unpaired electron spins. Another technique, Electron nuclear double resonance (ENDOR) spectroscopy, can be applied to retrieve structural information if an electron spin interacts with a nuclear spin (hyperfine interaction), i.e., protons of H bonds around a \( \bullet \).

EPR techniques brought valuable insight into PCET systems in general\(^{135-138} \) and particularly to RNR.\(^{41,139} \) EPR spectroscopy applied in combination with UAA is able to characterize trapped radicals in the “active” RNR complex.\(^{41,44} \) Radical intermediates (i.e., \( \text{NH}_2\bullet \)) were assigned in the PCET of RNR for the first time.\(^{43,44} \)

EPR spectra provide information about the radical species observed, for instance the resolved \(^{14}\text{N} \) hyperfine (HF) coupling in \( \text{NH}_2\bullet \).\(^{134} \) In organic radicals (as \( \bullet \) and \( \text{NH}_2\bullet \)) only at high-field (> 3 T) another interaction becomes resolved the \( g \) tensor. This \( g \) tensor is often essential for the identification of the organic radical species.\(^{140} \) In the principle axis system, the \( g \) tensor has three \( g \) values \( (g_x, g_y, \text{and } g_z) \). The orientations of the principle axis are directly connected to the molecular frame (cf. Figure 1-14A). \( g \) Values can be viewed as an electronic finger print of a radical species. At high accuracy, however, they are also a function of the oxygen spin density population (see §2.1.2, p.31). Therefore they are affected by electrostatic interactions in the surrounding of the phenoxy oxygen. In \( \pi \) radicals as tyrosines especially \( g_x \) and \( g_y \) values are affected by local electrostatics as charges or protons around the phenoxy oxygen nuclei.\(^{141-146} \)

In biomolecules with several paramagnetic species as in the PS II or RNR it is often necessary to selectively probe one paramagnetic species. The literature shows
examples for the separation of EPR spectra at very high fields (> 9 mT),\textsuperscript{147} by relaxation filtering\textsuperscript{148} or by advanced pulsed methods.\textsuperscript{149} A general procedure cannot be given, because the paramagnetic species, their surrounding and their relaxation properties have to be considered. In RNR, Y\textsubscript{122}\textsuperscript{•} is always present in samples investigating PCET radical intermediates (cf. §1.4.3, p.11). However, Y\textsubscript{122}\textsuperscript{•} has a short spin lattice relaxation $T_1$ due to the interaction to the ferric diiron site in its vicinity.\textsuperscript{55, 150, 151} Therefore the contribution of Y\textsubscript{122}\textsuperscript{•} in the EPR spectra could be efficiently removed at elevated temperatures (70 K) in combination with pulsed EPR spectroscopy.\textsuperscript{55, 148, 152}

1.5.2 High-Field ENDOR and EPR in Other PCET systems

The effect of electrostatic interaction on the g value can depend on an H bond distance to a Y\textsuperscript{•} as reported experimentally in a recent publication by Chatterjee \textit{et al.}\textsuperscript{153} Here the authors could demonstrate that the combination of high-field ENDOR and EPR can bring unprecedented spectroscopic evidence for a PT from Y\textsubscript{0}\textsuperscript{•} in PS II. In this study, a proton transfer could be demonstrated between a cold temperature state (7 K, “tensed”) and an elevated temperature state (70 K, “relaxed”) by ENDOR spectroscopy (the study is illustrated in Figure 1-13). The hyperfine (HF) interaction decreases from the “tensed” to the “relaxed” state according to the dipolar coupling strength of the nuclei spin and the electron spin (see §2.1.4, p.35). The dependence on the H bond distance is clearly visible in this example. By contrast, the g value increases by 1 ppt concomitant with the increase in H bond length of 0.25 Å. This is in agreement with the reduction of electrostatics and has been predicted by DFT calculations before for Y\textsubscript{5}\textsuperscript{•}.\textsuperscript{143, 154}
Introduction

1.5.3 High-Field ENDOR and EPR in RNR

In the last sections, it could be shown that incorporated NH₂Y’s are useful to observe the radical in the “active” complex. Section §1.4.4 (p. 13) highlighted the need for a well-defined H bond network in order to calculate energetic landscapes via for instance DFT. Hyperfine (HF) interactions from intermolecular protons or deuterons can be probed precisely by modern high-field ENDOR spectroscopy. Thus information about H bonds can be derived. Additionally, the electrostatic interaction can be investigated by high-field EPR spectroscopy. The investigation on NH₂Y• intermediates at residues β-356, α-730 and 731 was started by T. Argirević in our research group.⁹², ¹¹⁰, ¹³⁴ At NH₂Y₇₃₀• it was demonstrated that at higher field/frequencies the principal g values can be partially resolved at 94 GHz and fully resolved beyond ≈180 GHz, as shown in Figure 1-14A.⁹² Below the spectrum, the individual principal axis orientation of the NH₂Y• g tensor toward the magnetic field are shown. Orientations can be selectively probed, if the excitation bandwidth of the pulse is much smaller than the spectral width of the EPR line.⁶ Orientation selection in combination with high-field ENDOR was employed. In this case, T. Argirević could assign three intermolecular HF couplings as highlighted in Figure 1-14B (yellow, red and blue) in a ²H Mims ENDOR spectrum. With the aid of a DFT

¹ And hyperfine couplings can be neglected in size. (cf. §2.1.3).
structures HF couplings obtained from NH$_2$Y$_{730}$ were assigned to three protons in the surrounding. The assignment is illustrated by red, blue and yellow dotted lines between the oxygen (O-Y$_{731}$) and the individual H bond donor nuclei in Figure 1-14C.

The tensor shape found in NH$_2$Y$_{730}$ for the nearly perpendicular H bonds (red and blue, Figure 1-14 B&C)\textsuperscript{110} has not been described before by orientations selective ENDOR on Ys$. Several other orientation selective HF spectroscopy studies were performed (a selection is summarized in Table 1-1). One has investigated the yeast RNR Y$_{122}$ analog.\textsuperscript{55} Where Y$_{122}$. is not H bonded, its yeast counterpart (Y$_{127}$) has an H bond with a distance of 1.8 Å and the O-H vector is nearly collinear to g$_x$.\textsuperscript{55} The H bond of Y$_{s}$ is also within the ring and its direction is displayed in Figure 1-13.\textsuperscript{155} It is noteworthy that for these H bonds nearly within the ring plane the HF coupling were described by a dipolar tensor shape (§2.2.5, p.45). Interestingly, this seems to be independent of the estimated distance.\textsuperscript{153}

By contrast, the study of NH$_2$Y$_{730}$ showed a tensor, in which the so-called Fermi contact interaction plays a role. This can be seen in Figure 1-14B, by an increase of isotropic coupling ($a_{iso}$). Such couplings cannot be explained by simple point dipolar interaction approximations. Therefore quantum chemical calculations have shown to be useful.

A joint EPR and DFT structural representation of the active state could be obtained. It linked the information of the inactive crystal structure to the active state observed via these mutants.\textsuperscript{110}

In a multi-frequency EPR investigation in H$_2$O and D$_2$O buffer Argirević assigned g values and HF couplings for all three NH$_2$Ys (Table 1-2, p.25 and Table A.1, p.191). Results showed at least one stronger perpendicular $^2$H-HF coupling to NH$_2$Y$_{731}$ as to NH$_2$Y$_{730}$.\textsuperscript{92} Controversially, the g values were identical between NH$_2$Y$_{730}$ and NH$_2$Y$_{731}$ (Table 1-2, bottom), although different ENDOR spectra indicated a change in the environment. In order to resolve this, 263 GHz spectra were recorded in all three NH$_2$Ys in this thesis.

Furthermore Argirević reported the highest electrostatic interaction at β-NH$_2$Y$_{356}$, but did not investigate the local structure of this mutant (cf. §1.4.1.1). Notably, ENDOR at NH$_2$Y$_{356}$ is more challenging, because Y$_{122}$. content per β$_3$ in all studied mutants is reduced by a factor of 2. Moreover, the NH$_2$Y$_{356}$ radical yield is reported to be $\approx$38%, which is lower than reported for NH$_2$Y$_{730}$ or NH$_2$Y$_{731}$ with $\approx$50%\textsuperscript{67}.
Figure 1-14: High-field EPR and ENDOR spectra combined with DFT calculations derive a structural model of the active structure of $\text{NH}_2\text{Y}_{730}^\bullet$ in the PCET of RNR. A) Multi-frequency EPR characterization of $\text{NH}_2\text{Y}_{730}^\bullet$. The $g$ values are not resolved at 9 GHz (orange), but have a contribution to the line shape at 94 GHz and are resolved at 180 GHz (red). Adapted from ref. 92. Each $g$ value ($g_x, g_y, g_z$) corresponds to an orientation of the molecule in the magnetic field. B) If the spectral width of the absorption signal is larger than the excite width of a microwave pulse (MW), then a single orientation can be excited. Three excitation bandwidths along a 94 GHz spectrum are shown in blue, red and green. By applying a Mims ENDOR sequence $^2\text{H}$ couplings can be probed for each molecular orientation. In $\text{NH}_2\text{Y}_{730}^\bullet$ three contributions beside the amino deuterons were found. A weak H bond in the ring plane (yellow), a weak to moderate perpendicular H bond (blue) and a moderate perpendicular H bond (red). C) These couplings were interpreted with an DFT structure and an “active” state model (gray) has been formulated with 0.2 to 0.6 Å shorter H bond distances than found in the crystal structure (golden sticks, green cartoon). D) By exchanging successively the Y residues by $\text{NH}_2\text{Y}$s each position could be probed. The additional double mutants prepared for the present investigation of this thesis are highlighted in yellow.
1.5.3.1 Electrostatic Effect of H Bonds on the g Value

In order to understand the effect of electrostatic interaction from H bonds on g values of \( \text{NH}_2\text{Y}^* \) (Table 1-2 left) several DFT models were set up.\(^{110}\) The effect from an isolated \( \text{NH}_3\text{Y} \) over one H bond to two H bonds was successively studied by models. In the DFT models the g value decreases by about 0.5 ppt per weak to moderate H bond (2.0 to 1.8 Å). The g value increases taking the second sphere into account in this case by 0.3 ppt (entry 4 and 5). Interestingly, if the electrostatics of the second sphere are considered the removal of the weak H bond (2.0 Å) changes the g value only by 0.2 ppt. The effect of the second shell is likely a consequence of the polarization of the surrounding and steric effects. Notably, the calculations did not treat any continuum polarization or gauge origin correction. Hence, the uncertainty was estimated with 0.5 ppt for these models. However, most DFT uncertainties are systematic shifts. Therefore, it is reasonable to compare relative changes within the models.\(^{110}\)

Table 1-1: HF couplings of tyrosine to exchangeable intermolecular deuterons (D) for two examples. The results of Y\(_6^*\)\(^{153,155}\) and the Y\(_{127}^*\) analog of yeast RNR (Y\(_{127}\))\(^{55}\) are shown. The corresponding proton couplings are shown in parenthesis.

| \( \text{g} \text{D} \text{D} \) | \( A_x \) [MHz] | \( A_y \) [MHz] | \( A_z \) [MHz] | Euler angles \( \alpha, \beta, \gamma \) | | Qx [MHz] | Qy [MHz] | Qz [MHz] |
|---|---|---|---|---|---|
| \( 1.49 \) Å | -0.68 | -0.91 | 1.59 | 0, 90, 108 | 0, 90, 126 | -0.074 | -0.066 | 0.14 |
| \( 1.75 \) Å | -0.48 | -0.58 | 1.06 | 0, 90, 120 | 0, 90, 142 | -0.07 | -0.04 | 0.11 |
| \( Y_{127}^* \text{-D} \) \( 1.8 \) Å | -0.6 | -0.6 | 1.2 | 0, 110, 155 | 0, 110, 155 | -0.02 | -0.06 | 0.08 |
| \( Y_{127}^* \text{-D} \) \( 1.84 \) Å | -0.51 | 1.10 | -0.59 | -26, 16, -9 | -48, 30, 29 | -0.09 | -0.15 | -0.06 |

\( ^{a)} \) The Euler angles are defined in respect to the principal axis frame of the g tensor (\( \mathbf{A} \rightarrow g \)). A positive rotation is anti-clockwise. \( A_x \), \( A_y \), and \( Q \) are defined as largest values.

For Y\( ^* \) several high-field EPR studies are reported with various H bond environments (Table 1-2 right). Here the change from zero to one and to two moderate H bonds decreases the g value by \( \approx 1.4 \) ppt and 1.0 ppt, respectively. A strong H bond, defined here with a length between 1.5-1.6 Å, leads to a decrease of about \( \approx 2.4 \) ppt. Three H bonds around a tyrosine have only been reported by DFT calculations for functional essential Y\(_2^*\) in PS II.\(^{156}\) This theoretical value is in agreement with the change of more than 1 ppt per moderate H bond.
Table 1-2: The $g$ values as a function of the environment are tabulated for NH$_2$Ys and Ys. The $g$ values decrease with an increase of H bond interactions in number and/or strength. Left: For NH$_2$Ys it has been demonstrated by DFT model calculations. Right: For tyrosines several H bond situations have been found experimentally so far. Three H bonds to a Y have only been reported by DFT calculations.

<table>
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<th>NH$_2$Y• DFT models</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
<th>No.</th>
<th>Experimental Ys•</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
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<td>Free NH$_2$Y•</td>
<td>2.0061</td>
<td>2.0045</td>
<td>2.0022</td>
<td>1</td>
<td>Isolated Y$_{122}$•&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.00912</td>
<td>2.00457</td>
<td>2.00225</td>
</tr>
<tr>
<td>Y$_{31}$•+NH$<em>2$Y$</em>{30}$• (1 H bond: 1.77 Å)</td>
<td>2.0055</td>
<td>2.0044</td>
<td>2.0020</td>
<td>2</td>
<td>1 H bond ($&gt;$1.9 Å) Y$_{31}$•</td>
<td>2.0075&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.0044&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.0022&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td>Y$_{122}$• in eukaryotes&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.0076&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.0043&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.0023&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y$<em>{31}$•+NH$<em>2$Y$</em>{30}$•+C$</em>{439}$ (2 H bonds: 1.77/2.03 Å)</td>
<td>2.0050</td>
<td>2.0040</td>
<td>2.0018</td>
<td>3</td>
<td>1 H bond ($&gt;$1.5-1.6 Å)</td>
<td>2.0067&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.00453&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.00232&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y$<em>{31}$•+NH$<em>2$Y$</em>{30}$•+C$</em>{439}$+Wat1 (3 H bonds: 1.80/2.04/1.78 Å)</td>
<td>2.0046</td>
<td>2.0039</td>
<td>2.0017</td>
<td>4</td>
<td>2 H bonds (1x 1.6 Å)</td>
<td>2.00661&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.00418&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.00244&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
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<td>Model including second sphere&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0049</td>
<td>2.0041</td>
<td>2.0018</td>
<td>5</td>
<td>3 H bonds Y• (1.6 Å, 2x 1.8 Å)</td>
<td>2.0055&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.0043&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2.0023&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Model including second sphere (without WAT1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0051</td>
<td>2.0041</td>
<td>2.0019</td>
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<td>From simulations of the experiment</td>
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<tr>
<td>α$_2$-NH$<em>2$Y$</em>{30}$&lt;sup&gt;•n&lt;/sup&gt;</td>
<td>2.0052</td>
<td>2.0042</td>
<td>2.0022</td>
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<td>2.0042</td>
<td>2.0022</td>
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<td></td>
<td></td>
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<tr>
<td>β$_2$-NH$<em>2$Y$</em>{16}$&lt;sup&gt;•n&lt;/sup&gt;</td>
<td>2.0050</td>
<td>2.0041</td>
<td>2.0021</td>
<td>9</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

a) 2-amino-4-methyl-phenol radical (2-AMPR) model; b,c) All residues within 5 Å in the surrounding of NH$_2$Y$_{30}$• have been modeled with (b, model 4) and without (c, model 6) WAT 1. d) Taken from ref. 157. e) Median value of several Y$_{30}$• organisms reviewed in 136. f) Taken for the relaxed and tensed state from ref. 153. g) Values reported for yeast<sup>155</sup> mouse<sup>158</sup> and HSV1<sup>159</sup> Y$_{122}$• RNR corresponding amino acid. h) H bonded Y radical in prostaglandin H-Synthetase from ref. 160. i,k) γ-irradiated L-Y-HCl crystal with two H bonds from ref. 161. j) DFT study of Y• in the S2 state of photosystem II, ref. 156. m) The values are taken from ref. 134 and 92.

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**Introduction** 25
1.5.3.2 Investigation of the PCET in α with H Bond and Electrostatic Information from NH$_2$Y$_{730}$.\textdagger

Detailed DFT models could be set up that considered the proposed H bond interactions, as illustrated in Figure 1-14C. Taking into account all H bond interactions and a large model size, a more reliable energetic representation of the PCET in α could be calculated. The necessary electrostatic information and minim model size from the successive models for NH$_2$Y$_{730}.\textdagger$ were also considered (Table 1-2). The model was expanded to the radical positioned at residue 731, to calculate the PCET transition state between the Y$_{731}.\textdagger$ and Y$_{730}.\textdagger$. However, the H bond environment at Y$_{731}.\textdagger$ was not considered here.$^{110}$

The assignment of H bonds at NH$_2$Y$_{731}.\textdagger$ in agreement with the observed polarity at this site is a core focus of my thesis. It has still to be understood if two H bonds also cause the observed polarity at this site. Two double mutants (α-NH$_2$Y$_{730}$/C$_{439}$A and α-NH$_2$Y$_{731}$/Y$_{730}$F) have been characterized to test the assignments made in Figure 1-14D (see §4.3, p. 87).

1.6 Motivation of the Work

Before unnatural amino acids (UAA) could be incorporated, it was possible to study any of the detailed chemistry of the PCET in RNR. The incorporation of UAA is an expanding field and many more expansions of the genetic code$^{162-164}$ but also organisms$^{165}$ adapted to the UAA incorporation have been or will be developed. The development of these methods as well as the identification of its advantages and shortcomings is important.$^{84,120,134,166}$ Here, I have the chance to explore the utility for EPR of a NH$_2$Y as a competitive radical trap for an interesting and still not understood PCET.$^{41}$ It requires three tyrosine stepping stones over a distance of more than 35 Å. This is an ideal test case to compare these three NH$_2$Y$\bullet$ with each other and relate the findings to their function in the forward PCET.
CHAPTER 4:

The assignment and the active state model, as shown in Figure 1-14C, should be spectroscopically tested.

- Is the difference in H bonding geometries between NH$_2$Y$_{730}$• and NH$_2$Y$_{731}$• resolvable by pulsed 263 GHz EPR spectroscopy?\(^92\)
- Can double mutations be used to test these interactions?
- Can flexibility of α-Y$_{731}$, postulated based on different conformers in X-ray structures, be observed by high-resolution 263 GHz spectra of NH$_2$Y$_{731}$?
- Are interactions from β-Y$_{356}$ to α-NH$_2$Y$_{731}$• and from α-Y$_{731}$ to β-NH$_2$Y$_{356}$• observable?

CHAPTER 5:

In the second part of the thesis, another approach was used to circumvent conformational gating by the incorporation of β-2,3,5-F$_3$Y$_{122}$ forming β-2,3,5-F$_3$Y$_{122}$•.\(^{28,120}\)

This recently developed radical species has to be characterized by high-field EPR to clarify:

- Is conformer state and the \(g\) value of β-2,3,5-F$_3$Y$_{122}$• unperturbed compared to β(wt)-Y$_{122}$•?

This β-2,3,5-F$_3$Y$_{122}$• combined with a PCET blockade mutant (α-Y$_{731}$F) can be used to study a forward PCET. β-Y$_{356}$• could be formed.\(^{28,84}\)

- The number, distance and orientation of H bonds to β-Y$_{356}$• can give evidence for the type of PCET mechanism present at this position.
Tyrosines and UAA neutral radicals are studied in this thesis. From the EPR point of view, they resemble organic radicals with light first and second row elements of the periodic table. This theory chapter will therefore be restricted paramagnetic systems with an electron spin \( S = \frac{1}{2} \). Pulse EPR offers a variety of techniques to probe different interactions of the electron spin with its environment. Long-range interactions as the dipole-dipole coupling between two electron spins are studied routinely with PELDOR. Local interactions can be probed by ENDOR or other suitable HF detecting methods (§2.2). Changes in the CO bond charge distribution and influence on the spin density population can be studied as shown in the introduction.

The different sizes of these magnetic interactions can be effectively evaluated in the framework of the spin Hamiltonian (§2.1). EPR DFT calculations (§2.3) and EPR simulations use these effective Hamiltonians.\(^{167-169}\)

### 2.1 Spin Hamiltonian and Magnetic Interactions

The energies within the EPR treatment are generally small compared to the other terms of the electronic Hamiltonian. Therefore they can be often separated in the static spin Hamiltonian (2-1). This form of Hamiltonian describes magnetic resonance behavior without going into physical details.\(^{170,171}\) They were constructed to describe the interaction
influencing magnetic resonance spectra. Therefore specific properties of the system have
often to be related to these observables by quantum mechanical calculations (cf. §2.3.4. p.
52).167 The introduction to the spin Hamiltonian is described in several textbooks.170-172

For an organic radical the typical contributions for this effective Hamiltonian are:

\[ \hat{H}_e = \hat{H}_{eZ} + \hat{H}_{nZ} + \hat{H}_{HF} + \hat{H}_{NQ} \] (2-1)

The contributing terms are:

The electron Zeeman interaction \( \hat{H}_{eZ} \),

the nuclear Zeeman interaction \( \hat{H}_{nZ} \),

the hyperfine couplings between the electron spin and the nuclear spins \( \hat{H}_{HF} \) and

the nuclear quadrupole interaction \( \hat{H}_{NQ} \) for spins with a nuclear spin quantum number

\( I > \frac{1}{2} \).

Several contributions of the spin Hamiltonian have been neglected here, either

because the typical line broadening in EPR is larger than their contribution or due to the

restriction to organic radicals with \( S=1/2 \) systems.

For all these contributions a separate treatment can be performed, if the

contributions are clearly separated in energy from each other. To visualize this, Figure 2-1

shows the relative sizes of these contributions at low- and high-field.
2.1.1 The Zeeman Contribution

The Zeeman interaction is the sole directly field dependent contribution for the nuclei and the electron. The energy of this quantity can be expressed in terms of the spin Hamiltonian for a free electron as:

$$\hat{H}_{zz} = g_e \mu_B \vec{S} \cdot \vec{B}_0.$$  \hspace{1cm} (2-2)

Where $g_e$ is the $g$-factor of the free electron (2.002319.) and $\mu_B$ is the Bohr magneton. If the magnetic field is large and defined along the $z$-direction, the Hamiltonian simplifies to:

$$\hat{H}_{zz} = g_e \mu_B S_z B_0.$$  \hspace{1cm} (2-3)

For an $S = \frac{1}{2}$ system the two states are separated by the energy given by:

$$\Delta E = g_e \mu_B B_0.$$  \hspace{1cm} (2-4)

The energy difference $\Delta E$ between two nuclear spin states can be written in an analog equation as:

$$\Delta E = g_n \mu_B B_0.$$  \hspace{1cm} (2-5)
Where $g_n$ is the nuclear g-factor and $\mu_n$ the nuclear magnetic moment. The combination of both energy splittings (Equations 2-3 & 2-4) with the field is illustrated in Figure 2-5 (p.38). The difference in $\Delta E$ between nuclear Zeeman and electron Zeeman term, originates from the difference in mass of the two particles. For example, the proton and electron magnetic moments are compared in following equation:

$$
\mu_p = \frac{e}{2m_p} \hbar \quad \text{versus} \quad \mu_e = \frac{e}{2m_e} \hbar
$$

(2.6)

2.1.2 Anisotropic g Tensors in Organic Radicals: The Spin-Orbit Contribution

Approaching a real system, a resonance is seldom defined exactly at the value of the free electron. To explain the deviation several effects have to be taken into account. Some are small like the relativistic mass correction; a particularly large one is the spin-orbit coupling. For the latter contribution, an effective g value has been introduced that satisfies the resonance condition $h\nu = g_e \mu_e B_0$. This value is orientation dependent and forms a 3x3 matrix, which is diagonalizable; it is commonly called g tensor. The Hamiltonian as a function of orbital angular momentum $L$ is

$$
\hat{H}_{E(\lambda)} + \hat{H}_{ls} = g_e \mu_e \tilde{B}_0 \left( L + g_s \hat{S} \right) + \lambda \hat{L} \hat{S}.
$$

(2.7)

Whereas $\hat{H}_{ls}$ is the spin-orbital interaction with the spin-orbit coupling constant $\lambda$. For organic radicals with nuclei with small atomic number $Z$, the second order perturbation treatment is a good approximation, obtaining

$$
g = g_s 1 + 2 \lambda \Lambda
$$

(2.8)

where $\Lambda$ is a symmetric tensor, with elements defined by Eq. (2.9).

$$
\Lambda_{ij} = \sum_{n\neq 0} \frac{\langle \psi_n | L_i | \psi_n \rangle \langle \psi_n | L_j | \psi_n \rangle}{\epsilon_n - \epsilon_0}
$$

(2.9)

The electronic wave function of the single occupied ground state is $\psi_0$; it has the energy $\epsilon_0$. Any other state $n$ has the wave function $\psi_n$ and the energy $\epsilon_n$.

With this angular momentum contribution, the g values differ depending on the spin-orbit coupling of the individual nuclei bearing a part of the free electron $\left( | \psi_n | \neq 0 \right)$. A

§ Bold letters numbers are indicate a matrix or a tensor and $\tilde{B}_0$ is the transposed form of $B_0$.
relation taking into account the relative spin density population $\rho_{\pi}^{\nu}$ of the individual nucleus can be formulated for instance for oxygen. In Ys* the oxygen atom is the sole nucleus with a high spin-orbit coupling and orbitals contributing strongly to the single occupied orbital. It has in this organic radical not only the highest spin-orbit coupling $\lambda_{\text{nuclei}}$ with $\lambda_{\pi}=151$ cm$^{-1}$ (\(\lambda_{\nu}=76$ cm$^{-1}$, $\lambda_{\eta}=28$ cm$^{-1}$). Notably, $^{19}$F also has a high spin-orbit coupling with $\lambda_{\text{F}}=270$ cm$^{-1}$ ($\S$5.2). However, the spin density population of oxygen is larger compared to all other individual atoms in tyrosines and their analogs. Thus Eq. (2-10) has a substantial influence on the small spin-orbit-couplings resolved here. Due to the geometry of the individual orbitals (cf. Figure 2-2A) not all orbitals can mix. The in-plane contribution of closest lying non-bonding p$_\pi$ orbital in is denoted by $c_{\text{aby}}$. The mixing is governed by the orbital angular momentum as shown in Figure 2-2B. Electrostatic effects are by far not as strong as in ligand field complexes. Small charge dependent differences, however, can be realized. The effect on the frontier orbitals is illustrated in Figure 2-2C-D.$^{140}$

$$g_{O2,\pi}(\nu)=\frac{2\lambda_{\pi}\rho_{\pi}^{\nu}c_{\text{aby}}}{\epsilon_{\nu}-\epsilon_{ab}}$$  \hspace{1cm}  (2-10)

For interactions along the p$_\pi$ orbital as perpendicular H bonds, partial covalent bond character of the interaction can be assumed based on the increasing Fermi contact interaction.$^{110}$ In protonated organic radicals, it could be shown that protonation leads to a lower participation of the high lying non-bonding orbital.$^{174}$ Therefore, lower lying HOMOs contribute to the spin-orbit coupling. The energy difference between the excited state and lower lying HOMOs increases, which reduces the spin-orbit coupling along $g_x$ and $g_y$. The $g_{\text{O2}}$ value decreases to a slightly larger extent than for H bonds along the non-bonding orbital.$^{154,174,175}$

For the organic radicals investigated in this thesis, the differences are small. Differences can still be resolved, however, by considering the proportionality of the $g$-factor with magnetic field $B_0$. Taking the resonance condition into account, the difference in the field spectrum is

$$\Delta B=\frac{\hbar \nu}{\mu_B} \left( \frac{1}{g_1} - \frac{1}{g_2} \right)$$  \hspace{1cm}  (2-11)

Thus the separation of the signals scales with the used microwave frequency.
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

2.1.3 Hyperfine Interaction

To understand the factors that govern the hyperfine (HF) interaction, it is typically separated into two contributions

\[ \mathcal{H}_{HF} = \mathcal{H}_{FC} + \mathcal{H}_{DD,HF} = a_{iso} \hat{S} \hat{I} + \hat{S} \hat{T}_{Dip} \hat{I}. \]  

(2-12)

For the Fermi contact interaction \( \mathcal{H}_{FC} \), the probability density function \( \psi_0(r) \) of the electron density at the nucleus \( r = 0 \) is considered. Thus, this is a spherical isotropic value and therefore the \( a_{iso} \) is defined as isotropic part of the hyperfine interaction.\(^{172}\)

\[ a_{iso} = - \frac{\mu_e^2}{3\hbar} \hat{S} \hat{G}_s \hat{G}_s \hat{\mu}_n \psi_0(0)^2 \]  

(2-13)

Strictly defined, only s orbitals have electron density at the nucleus; however a polarization mechanism is defined explaining how higher angular momentum orbitals (p, d and f) can contribute to the spin density at \( r=0 \).\(^{167}\)

A coupling of protons in plane of the aromatic systems has been described by McConnel.\(^{176}\)

Assuming that a partially singly occupied molecular orbital (SOMO) of nucleus C is hybridized with the bonding orbitals as shown in Figure 2-3, the energetic degeneracy forces parallel spins in near environment of these spins based on the Hund’s rules. In the bonding

Figure 2-2: Illustration of the spin-orbit coupling and effects on the g value. In a tyrosyl radical observed along the C-O bond (A) the effect of the orbital angular momentum \( L \) is shown (B). The mixture of the non-bonding orbital and the antibonding SOMO increase the \( g \) value. C and D) For the non-bonding orbital, the electrostatic effects can be considered. A positive charge will stabilize the orbital and a negative charge will lead to an energy increase. E) Interactions along the SOMO cannot be treated electrostatically anymore. Adapted in part from ref. 140.
Spin Hamiltonian and Magnetic Interactions

orbital the states are occupied following the Pauli principle. Therefore the spin far away from nucleus C is oriented antiparallel to the SOMO spin. This is a polarization mechanism of the electron spin at the nucleus. For protons this spin has s orbital character; thus the spin density is negative at the nucleus H.\textsuperscript{171, 176-178} For in-plane H bonds the polarization mechanism is weakly contributing to the HF interaction, whereas for perpendicular H bonds a similar polarization mechanism applies as for covalently bond protons (Figure 2-3 C&D).

![Figure 2-3: Spin polarization mechanism in an aromatic CH-fragment. The proton orbital cannot directly interact with the electron spin in the \( p_z \) orbital. Correlation energy description point out that the energy of case A is lower than case B in a magnetic field along the z axis. For an H bond within the ring plane a polarization is weak C, along the oxygen \( p_z \) orbital polarization is a non-neglectable contribution.\textsuperscript{110}](image)

After the consideration of spin density at the nucleus and polarization, the second contribution to the HF interaction is the dipole-dipole interaction between the electron and the nucleus in distance \( r \). It is given by\textsuperscript{172}

\[
\hat{\mathcal{H}}_{\text{DD,HF}} = -\frac{\mu_0}{2\hbar} g_e \mu_s \mu_n \left( \frac{\mathbf{S} \cdot \mathbf{I}}{r^3} - \frac{\mathbf{I} \cdot \mathbf{S}}{r^5} \right) \quad (2-14)
\]

The integration over the spatial electron distribution result in the following anisotropic dipolar Hamiltonian (2-15).

\[
\hat{\mathcal{H}}_{\text{DD,HF}} = \mathbf{S} \cdot \mathbf{T}_{\text{Dip}} \mathbf{I} \quad (2-15)
\]

\( \mathbf{T}_{\text{Dip}} \) is a traceless symmetric 3x3 matrix of the ground state wave function with the elements:

\[
T_{ij,\text{Dip}} = -\frac{\mu_0}{2\hbar} g_e \mu_s \mu_n \left( \frac{3\mathbf{r}_i \cdot \mathbf{r}_j - \delta_{ij}}{r^5} \right) \left| \psi_0 \right|^2 . \quad (2-16)
\]
In which, $\delta_{ij}$ is the Kronecker symbol ($\delta_{ij} = 0$ for $i \neq j$ and $\delta_{ij} = 1$ for $i = j$). The diagonal elements of the matrix in a principle axis system (PAS) are often defined as $T_\perp$, $T_\perp$, and $T_\parallel$.

The equation can be simplified, considering a $^1$H in hydrogen bond distance $r$ to an oxygen, i.e., of a tyrosine within a range of 2.5 Å. For the proton the predominantly dipolar contribution scales with spin density population of the oxygen $\rho_O$ and thus the single electron contribution on the oxygen. In the point dipole approximation the Eq. (2-16) can be then approximated with Eq. (2-17).

$$
\rho_O \approx \frac{11.86}{T_\perp} 
$$

(2-17)

2.1.4 Dipolar Interaction

As in the nuclear to electron spin case the dipolar interaction between an electron spin and a second electron spin leads to a detectable dipolar frequency. Within this thesis the exchange contribution ($J$ coupling) as scalar contribution is neglectable, because long-range distances are probed, thus Eq. (2-18) is obtained for $\theta = 90^\circ$. The angle $\theta$ is defined between the magnetic field and the interspin vector with the length $r_{AB}$ as shown in Figure 2-4. Solving for the distance and assuming a $g$ value of organic radicals near the value of the free electron it can be further simplified (2-19).

$$
\nu_\perp = -\frac{\mu_0 \mu_r^2 g_A g_B}{4 \pi \hbar} \frac{3(\cos^2 90^\circ - 1)}{r_{AB}}
$$

(2-18)

$$
\nu_\perp [\text{MHz}] \approx \frac{52.29 [\text{MHz}]}{\nu_\perp [\text{MHz}]}
$$

(2-19)

Figure 2-4: Dipolar coupling between two spins A and B in the magnetic field $B_0$. The coupling is dependent on the distance $r_{AB}$ and the angle $\theta$ between the magnetic field and the inter spin vector.
2.1.5 Quadrupole Interaction

Nuclear spins with $I \geq 1$ are distinguished by a non-spherical charge distribution described by an electrical quadrupole moment $Q_{eq}$ with the Hamilton operator $\hat{H}_\text{NQ}$:

$$\hat{H}_\text{NQ} = \sum_{k,l \neq \sigma} \hat{I}_k Q_{eq} \hat{I}_l$$  \hspace{1cm} (2-20)

The matrix of $Q$ is a traceless 3x3 matrix in its PAS, and can therefore, be written as:

$$Q = \begin{pmatrix} 0 & Q_{eq} & 0 \\ Q_{eq} & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}$$

with the asymmetry parameter $\eta = (Q_{xy} - Q_{yz})/Q_{eq}$ for $|Q_{eq}| \leq |Q_{xy}| \leq |Q_{yz}|$ and $0 \leq \eta \leq 1$. The elementary charge is $e$ and $q$ is the electric field gradient. Consequently, the quadrupole interaction $\hat{H}_\text{NQ}$ can be solved in the molecular frame through knowledge of $Q_{eq} = e^2 q Q / (2I(2I-1)\hbar)$ and $\eta$ as well as the three Euler angles in respect to the $g$ tensor in the PAS.

2.1.6 Analytical Treatment of the Spin Hamiltonian

To demonstrate the analytical solutions for the hyperfine interactions a model system with $S = 1/2$ and a nucleus with a spin of $I = 1/2$ will be discussed. Additionally, an isotropic $g$ tensor and an anisotropic hyperfine interaction are presumed. Thus, the static Hamiltonian in the PAS can then be written as:

$$\hat{H}_\text{HFI} = \omega_S S_z + \omega_I I_z + \hat{S} \hat{A} \hat{I}$$

$$\approx \omega_S S_z + \omega_I I_z + A_S S_z I_z + B_x S_z I_x + B_y S_z I_y$$  \hspace{1cm} (2-22)

The resonance frequency of precession in angular frequency units is the Larmor frequency with $\omega_S$ and $\omega_I$ for electron spin and the nuclear spin, respectively. Here the approximated Hamiltonian includes the high-field approximation ($\omega_S \gg |A|$). In this case, terms with $S_x$ and $S_y$, called non-secular terms, can be neglected. The non-secular terms of the HF interaction are still treated with $A = A_{xx}$, $B_x = A_{sx}$ and $B_y = A_{sy}$ described as pseudo-secular hyperfine couplings of the $A$ matrix in the PAS of the $g$ tensor. The coordinate system for the nucleus spin can be transformed from the $x$-axis to the $xz$-plane, to simplify the non-secular terms to $B = (B_x^2 + B_y^2)^{1/2}$ only dependent on $S_z$.  

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Theoretical Background
To set it all in the rotating frame the electron Zeeman frequency is substituted by the resonance offset \( \Omega = \omega - \omega_{mw} \), where \( \omega_{mw} \) is the microwave frequency. The combined transformation and substitution displays \( \hat{H}_0 \) as follows:

\[
\hat{H}_0 \approx \Omega S_z + \omega I_z + AS_\perp I_z + B S_z I_z
\]

with given spin matrices the diagonalized form of the Hamiltonian results to

\[
\begin{pmatrix}
\frac{\omega_x}{2} + \frac{\omega_y}{2} + \frac{A}{4} & B/4 & 0 & 0 \\
B/4 & \frac{\omega_x}{2} - \frac{\omega_y}{2} - \frac{A}{4} & 0 & 0 \\
0 & 0 & -\frac{B}{4} & B/4 \\
0 & 0 & -\frac{B}{4} & \frac{\omega_x}{2} - \frac{\omega_y}{2} + \frac{A}{4}
\end{pmatrix}
\]

(2-24)

The corresponding eigenvalues of the Hamiltonian in nuclear frequencies can be obtained with:

\[
\omega_{\beta/\alpha} = \left| \omega_{1/2, 3/4} \right| = \sqrt{\left( \frac{\omega_x + A^2}{4} \right) + B^2/4}
\]

(2-25)

The frequencies \( \omega_{\beta/\alpha} \) are given for the NMR transitions \( 1 \rightarrow 2 \) and \( 3 \rightarrow 4 \) for the two spin manifolds \( \alpha \) and \( \beta \), as shown in Figure 2-5A. Within the high-field approximation the B term is negligible as long as \( \omega_x \ll \omega_y \). This has been fulfilled throughout this thesis at frequencies of \( \omega_x/2\pi \geq 94 \text{ GHz} \) except for the \( ^{19}\text{F} \) HF couplings of \( 2,3,5\text{-F}_3\text{Y*} \) (§5.3, p.135).

To consider the interaction of the electron spin with \( I = 1 \) nuclei like \( ^{3}\text{H} \) \( (I = 1) \), the resonance conditions change. Within the high-field limit the resonances \( \omega_{\eta} \) (see Figure 2-5B) of the allowed transitions are given by

\[
\omega_{\eta} = A/2 \pm \omega_x \pm \frac{3}{2} Q
\]

(2-26)

With these approximations one finally arrives at the energy level diagram shown in Figure 2-5 for two cases typical cases within this thesis.
2.2 High-Field Spectroscopy for Small Hyperfine Couplings

2.2.1 Detection of Hyperfine Interaction

The HF interactions were introduced in §2.1.3 are as closely related to the electronic structure of the individual radical. The local structure information is connected to the HF interactions of individual nuclei surrounding an electron spin. The typical interaction range is given with 0-10 Å, for high γ nuclei (2-14). Due to the transient nature of the radicals studied here the measurements are performed in frozen solution. This connected to the g anisotropy, which helps to retrieve the direction of the dipolar part of the coupling. This can always be used if the spectral width due to g anisotropy is larger than the excitation.
bandwidth of the spectrum. For organic radicals this is only fulfilled by operating at high- fields.

2.2.2 Nuclei Accessible in Hyperfine Spectroscopy

A typical system investigated within this thesis should be introduced from the magnetic interaction point of view, as shown in Figure 2-6. The 3-amino tyrosine has many internal couplings as presented in Figure 2-6 A, without isotope labeling $^{13}$C is seldom detected. For nitrogen $^{14}$N couplings are observed. In a non-isotopically labeled buffer the $^1$H nuclei will be observed, by buffer exchange to D$_2$O external deuterons can be distinguished from $^1$H as $^2$H nuclei (cf. Figure 2-6 B). However, the amino protons will also be exchanged. Thus, even in a deuterated protein these contributions cannot be separated.

![Figure 2-6: Nuclear spins coupled to a NH$_2$Y. The electron spin (e-, red) is here the 3-amino tyrosine radical. A) Several magnetic nuclei isotopes are in the interaction sphere for the tyrosine analog, displayed are $^1$H, $^{17}$O, $^{14}$N. B) Buffer exchange can introduce $^2$H at exchangeable sites. C) By separating the different nuclei due to different sizes of nuclear Zeeman couplings, one can select $^2$H HF couplings.](image)

In the introduction, it was shown how resolution can be improved according to the electron Zeeman term for electron spin part. The same applies for the nuclear Zeeman contribution. Taking additionally common hyperfine interactions into account, the separation between low frequencies as X-band and high frequencies like W-band are depicted in Figure 2-7. Thus, several magnetic coupling nuclei can be specifically detected, i.e., exchangeable protons by their $^2$H resonance (Figure 2-6 C).
Theoretical Background

2.2.3 Mims-ENDOR Spectroscopy

Electron-nuclear double resonance (ENDOR) in general is a double resonance technique resolving small couplings due to the selective pumping of nuclear transitions ($\Delta m_I = 1$ & $\Delta n_S = 0$) or NMR transitions. The name NMR transition might be misleading. Typically, transitions detected in an ENDOR spectroscopy experiment have sensitivities higher than those of conventional nuclear magnetic resonance (NMR) detection and higher selectivity because of detection through the electron spin. The higher Boltzmann
polarization of the electron spin is advantageous here. On the contrary due to this interaction broad lines are observed.

ENDOR was first introduced to the scientific community by Feher,\textsuperscript{181} shortly after two techniques were developed and named after their inventor Davis\textsuperscript{182} and Mims ENDOR\textsuperscript{183}. Already in the 1970s it could be shown that Davis ENDOR suffers from broad blind spots near the Larmor frequency of each nuclear spin.\textsuperscript{184} Thus Mims ENDOR was used throughout this thesis for small couplings up to $a_{\text{HF}} \approx 2$ MHz. The Mims pulse sequence consists of a stimulated echo sequence on the electron transitions and an inversion pulse swept over nuclear frequencies. The stimulating echo sequence has three microwave pulses generating an echo after third pulse and the delay $\tau$, as shown in Figure 2-8.\textsuperscript{183}

![Figure 2-8: Mims ENDOR sequence for the detection of small HF couplings. The microwave frequency of the pulses stays constant detecting a stimulated echo. By varying the radio frequency of a $\pi_{\text{RF}}$ pulse different NMR transitions (black, red and blue) are probed. If a nuclear resonance is met, the echo amplitude will be reduced (red and blue echo).\textsuperscript{183}](image)

Ideally the detection with the last $\pi/2$ only reads out the $M_z$ magnetization. Therefore the mixing is acting on the $M_z$ magnetization. For small couplings microwave irradiation is in most cases not selective enough. Furthermore the echo width increases beyond the detection limit with an increase in pulse length or selectivity. In the classical picture for an non-selective mw pulse Mims ENDOR is described as phase offset dependent on the nuclear frequency ($\Delta \approx A_{\text{HF}}$), as illustrated in Figure 2-9B. The part unaffected by nuclei offset will interfere destructively with the magnetization pattern with the additional phase shift, which then decreases the echo size. Overall an increase of sensitivity for small offset $\Delta$ is achieved. Therefore, the Mims ENDOR is superior if a selective excitation of the
nuclei spins is prohibited due to the small coupling sizes by Davis ENDOR. Davis ENDOR has a different magnetization pattern as indicated by the original line shape in Figure 2-9A.

![Illustrative picture of the pattern created in the preparation sequence $\pi/2-\tau-\pi/2$.](image)

The inset shows the pulse sequence acting on a broad line. Enlarged (A) shows an artificial pattern imposed on the line. B) Displacement by a resonant nuclei with an interaction energy of $\hbar\Delta$. Adapted from references 172 & 183.

The population $P$ and its population difference $P_i$ between the energy levels $i$ (=1, 2, 3 & 4), assigned in Figure 2-5A (p.38), is given by Eq. (2-27).183

$$
P_{(1,2)-(3,4)}(\omega) = P_{(1,2)-(3,4)}(\omega) \left( 1 - \frac{3}{2} \cos\left(\frac{\omega}{2\pi\tau}\right) \right) - \frac{1}{2} P_{(1,3)} \cos\left(\frac{\omega + \Delta}{2\pi\tau}\right) - \frac{1}{2} P_{(2,4)} \cos\left(\frac{\omega - \Delta}{2\pi\tau}\right)
$$

A drawback of Mims ENDOR lies in the constructive interference of the patterns generated. In the spectrum this leads to regions were the echo cannot be diminished, Mims holes are generated. These Mims holes depend on the following hole function (Figure 2-10A)172, 183

$$
I_{\text{Mims ENDOR}} \propto 1 - \cos\left(2\pi A_{1\text{eff}}\right).
$$

(2-28)
To avoid holes within the spectrum an upper limit can be set for each investigated coupling with the largest hyperfine coupling \( A_{\text{max}} \) Eq. (2-29) can be used. Measurements to average out blind spot effects are often prohibited by the long measurement time for low concentrated biological samples (\(< < 50 \mu\text{M})\).\(^{172}\)

\[
A_{\text{max}} = \frac{1}{(2\tau)}
\]

The efficiency of Mims ENDOR \( F_{\text{Mims ENDOR}} \) (2-30) increases tremendously for small couplings with the interpulse delay \( \tau \).\(^{180}\) One could think about increasing the Mims holes to a frequency in the order of the Nyquist frequency, but this is prohibited by fast \( T_2 \) relaxation in the xy magnetization plane (Figure 2-10A).

\[
F_{\text{Mims ENDOR}} = \frac{1}{4} \left(1 - \cos(A_{\text{HF}}\tau)\right)
\]

Practically the distortions are often too severe in powder spectra. Therefore, the largest \( \tau \) in agreement with Eq. (2-29) is chosen. The center line for \( A_{\text{HF}} \) approaching zero is only theoretically completely suppressed. For protons it is often the most intense line due a large number of distant protons.\(^{185}\) The matrix line is observed due to the finite bandwidth of the RF pulse.\(^{185}\)

Figure 2-10: Mims hole functions in dependence of the interpulse delay \( \tau \) (A). B) ENDOR efficiency according to multiplication of Eq. (2-29) with the hole function and the \( T_2 \) relaxation function (green) for two different small HF couplings 2 MHz (red) and 0.8 MHz (green).

2.2.4 Comparison to Other Hyperfine Detection Methods

HF detecting techniques characterize couplings too complex or too small to be resolved in conventional EPR spectra. There are several other techniques that allow for the detection of HF couplings. One of them is called ESEEM, which is most efficient at low fields and will
Another technique, called Davies ENDOR, has already been partially introduced. An emerging technique is called electron double resonance (ELDOR) detected NMR, here instead of another RF pulse a highly selective microwave pulse is used to drive transitions. Because both techniques are polarization transfer techniques these two can readily be compared. The efficiency of both techniques can be described by their polarization transfer capabilities as shown in Figure 2-11. Here the character of each technique becomes evident; the excitation bandwidth in a typical RF pulse is much narrower than available in any MW ELDOR pulse. Thus, Davies can be much more selective in terms of orientation selection and omitting line broadening of HF patterns, but ELDOR detected NMR is able to excite more spins and has therefore advantages in the sensitivity. A 30-times higher sensitivity has been reported on organic, ubiquitously used nitroxide labels. Even more could be achieved by newly developed detection schemes. A drawback is that the lines can be broader compared to a standard ENDOR setup.

The relaxation behavior is also different. The high turning angle ELDOR pulse acts directly on the initial $M_z$ magnetization. Especially for small couplings, Davies ENDOR requires long preparation pulses turning the magnetization into the xy-plane; here faster $T_2$ relaxations can diminish the signal for fast relaxing paramagnetic centers. ELDOR detected EPR was meanwhile successfully applied to several high spin systems, so it seems to be robust in terms of $T_1$ relaxation. Here especially low $\gamma$ nuclei such as oxygen $^{17}$O have been detected. One example even shows a narrow central line of a second shell water molecule. Although a forbidden transition is used, it seems it can detect even small HF couplings. Notably, this has not been investigated thoroughly up to now.

A big disadvantage of ELDOR detected NMR is the large blind spot around the detecting microwave frequency, which is influenced by the ELDOR pulse length. Here the detectable magnetization is reduced to 0. However, this blind spot does not depend on the coupling size as in ENDOR techniques therefore these techniques can be complimentary. Selective Mims ENDOR employs an analogous polarization scheme as Davies ENDOR in Figure 2-11A.
2.2.5 Hyperfine Tensors and Origin

Based on the energy level diagram (Figure 2-5, p. 38) and the hyperfine coupling mechanism (§2.1.3) the appearance of these interactions should be illustrated. Typical hyperfine spectroscopy pake patterns found in tyrosine systems should be introduced. Quadrupole splittings and rhombicity of the HF tensor are neglected for simplicity.

The simplest case is a distant coupling, which is only governed by the dipolar interaction. The full pake pattern is seen in Figure 2-12A. A good example of this would be distant protons as found in yeast RNR or even nearby protons non-interacting with the p$_z$ orbital.152, 153
The second case occurs if the orbital directly interacts with a spin bearing nucleus. Good examples are protons on electron negative nuclei like the amino protons in the 3-amino tyrosine. Here the tensor shape is strongly governed by the opposite signs between $a_{iso}$ and $T_{||}$, as illustrated in Figure 2-12 C.\textsuperscript{110,134}

The H bond axial to the ring plane as described by Argirević et al. is an intermediate case as illustrated in Figure 2-12B.\textsuperscript{110} In this case the $p_z$ orbital interferes directly with the $s$ orbital of the nuclei. Negative spin density at the nuclei is produced slight (bonding) overlap of the wave functions of the nuclei (cf. HF interaction §2.1.3).\textsuperscript{110} The $p_z$ orbital has no spherical symmetry. Therefore the interaction $a_{iso}$ is a function of distance and angle.

The last case is a nearly isotropic coupling with an $a_{iso}>0$. This case can be observed for β-methylene couplings (Figure 2-12D).

Figure 2-12: Hyperfine powder patterns for different ratios of $a_{iso}$ to $T$. A) $a_{iso}=0$; B) $a_{iso}=-1/4\cdot T_{||}$; C) $a_{iso}=-1/3\cdot T_{||}$; D) $a_{iso}=5\cdot T_{||}$. On the left side examples are shown where these types of couplings had been found. The proton couplings $H_X$ corresponds to case $X=A$, $C$, $B$ or $D$. 
2.3 Density Functional Theory: Limitations and Advantages

The idea behind the determination of HFC and g values as presented in the previous sections is one part of the information and the understanding of structural restraints, the visualization of the geometry is another. DFT has been found to be a valuable tool for correlating spectral parameters with structure and comparing different spectroscopic methods. To understand the advantages of this comparison and its limitations, the following section will introduce some basics of DFT theory. EPR and NMR parameters can be treated in DFT by taking the effective Hamiltonians on the bases of the “occupied orbitals only” ground state. The focus will lie on certain DFT methods used in this thesis. The interested reader is referred to a didactically written perspective of the origin of DFT and the development of their density functional approximations.192

The core idea was formed by Hohenberg, Kohn, and Sham in 1964-65.193, 194 The nuclear potential \( v_{\text{ext}}(r_i) \) defines the Hamiltonian \( \hat{H} \) (2-31) at the wave function \( \Psi_0 \) and the wave function defines the electron density \( \rho \). This has been shown to be reversible,193 thus from the electron density everything of the ground state system will be known for the given coordinates [Eq. (2-32)].

\[
\hat{H} = -\frac{1}{2} \sum_i \nabla_i^2 + \sum_i v_{\text{ext}}(r_i) + \frac{1}{2} \sum_{i \neq j} \frac{1}{|r_i - r_j|}
\]  

(2-31)

For simplicity atomic units are used here, where \( \hbar, m, e, \text{ and } 4\pi\varepsilon_0 \) are set to be 1.

\[
v_{\text{ext}} \rightarrow \Psi_0 \rightarrow \rho \rightarrow \frac{\rho(r_{ij})}{\varepsilon_0} \rightarrow \frac{1}{\varepsilon_0} \rightarrow \Psi_0
\]  

(2-32)

The function \( F(\rho) \) connecting the spin density to \( v_{\text{ext}}(r_i) \) is unknown. However, it could be shown by the variation principle that only the correct spin density will lead to the energy minimum. Thus, searching for the correct spin density is a minimization problem.193 Non-representative variational densities will collapse, thus it was necessary to separate the known total kinetic energy \( T_0(\rho) \) and classical Coulomb \( V_{\text{e}}(\rho) \) self-exchange from the unknown smaller exchange correlation \( E_{\text{EX}}(\rho) \). Although the exchange correlation is a small contribution to the total energy, it is the essence for covalent bonds and attractive non-electrostatic interactions. Otherwise, the theory to this point is complete. Electrons in atoms, molecules, and solids can be viewed as independent particles moving in effective potential \( v_{\text{KS}} \).192
2.3.1 Explaining Tendency within the Functionals

The theory laid out how everything depends on exchange correlation $E_{XC}$, but how is it calculated? Here density functional approximations have been formulated.

In the main, three ways to calculate the Kohn Sham exchange term $E_{XC}$ have been developed:

i. The local density approximation (LDA) has been applied. It assumes that the exchange-correlation energy has the local volume $\rho(r)$ of the spin density. This is a bold assumption for molecules originating from the idea of an ideal (uniform) electron gas (similar as found in metals). Based on the adiabatic (constant density) approximation the coupling strength can be integrated over the individual couplings. Localized pairs of non-exchanging spin densities can be separated as correlation only cases. These are called exchange “holes.” Generally this approximation leads to overbinding, thus shorter internuclear distances.

ii. Generalized gradient approximations (GGA) should reduce this overbinding effect. Here not only the local density is considered, but also the gradient at the local position. Subtracting from the local density exchange $E_{xLDA}$ this gradient to a certain order is the basic idea, albeit fitting the ratio and exponent of the local gradient to the uniform electron gas or benchmark sets; the functional is still LDA with a correction. A weak overbinding tendency was thus retained due to the localization of delocalized exchange “holes.”

iii. The so-called hybrid functionals mix Hartree Fock exchange correlation with LDA and GGA exchange correlations in order to fit atomic absorption data. For the first time, delocalized ”holes” are taken into account due to the Hartree Fock exchange. The exact Hartree Fock exchange is underbinding due to the unconsidered electron correlation.

Known pitfalls of these GGA and hybrid functionals are the following: an overestimation of molecular radicals, poor treatment of charge transfer processes and the inability to account for dispersion interactions (methods are introduced in the next section).

These interatomic effects cannot be treated by the density functionals made to describe a tightly packed uniform electron gas. For an unpaired electron the localized approximations fail to localize this highly delocalized exchange minimum or “hole.” This over stabilizes the radical state. The delocalization increases also for lighter atoms.
Local DFT approximations had been shown therefore to have a barrier too low for hydrogen-atom transfers, especially in $\text{H}_2 + \text{H} \rightarrow \text{H} + \text{H}_2$. Visually the problem can be shown for the dissociation curve of $\text{H}_2^+$ in Figure 2-13. DFT functionals with N4 scaling will have a certain underestimation of barrier heights. PBE0 as functional is within this set the best choice by a comparison shown by Becke with a mean error of -3.6 kcal/mol. It is notable that this has been chosen by C. Riplinger from Neese group to calculate the PCET between radical intermediates in α-RNR Ia.

2.3.2 Dispersion Correction

Dispersion interaction is especially important for intramolecular interactions. In this thesis, several complexes will contain dispersive interactions. For instance, the interaction between two tyrosines in close proximity is governed by electrostatics and London dispersion interactions. After the development of local DFT approximations, non-local effects should also be treated. Therefore dispersion correction was applied to functionals and basis sets. This can become quite complex. A straightforward implementation is the addition of the empirical London forces scaling with $r^{-6}$ and $r^{-8}$. The empirical potential has influence in the local field, for instance, below 3 Å for an argon gas interaction as shown in Figure 2-14. The method is reported to be robust and has not shown any basis set or functional dependent errors.
Figure 2-14: Dispersion correction for two argon atoms. The dispersion correction (--) takes sixth- and eighth-order terms into account, in contrast an un-damped $C_6R^6$ term (⋯) and zero (—) Becke and Johnson damping is shown. Obtained from ref. 200.

The absence of considered dispersion interaction has been shown to have an impact on geometry optimization of two phenols. In this study the stacked orientation of the phenol rings could only obtained with wave function theory or by dispersion corrected DFT. The energetics of the barrier heights for proton transfers were not significantly improved upon dispersion correction.\textsuperscript{113} For these barrier calculations the PBE0 functional has shown better capability in obtaining values comparable to the QM gold standards.\textsuperscript{192,202}

In this thesis we use the robust correction method\textsuperscript{203} shown in Figure 2-14 in combination with the well-known and often tested B3LYP functional. Also we used effective triple zeta diffuse basis sets (see 2.3.3, p. 51) operating near the basis set limit. Recently, this combination has been tested for its performance and offered the best performance to cost result.\textsuperscript{204}
2.3.3 Basis Sets

In current DFT applications, Gaussian type orbitals are nearly always employed. These approximated orbitals are faster to compute than more realistic Slater type orbitals. Compared with each other, they have different exponential behaviors with \( e^{-\alpha r} \) and \( e^{-\zeta r^2} \) for Slater type orbitals and Gaussian type orbitals respectively. Additionally, Gaussian type orbitals (GTO) have typical for Gaussian functions a broad area around \( r=0 \), whereas Slater type orbitals (STO) are cusp, as seen in Figure 2-16.205

The fit of three Gaussian type orbitals to one Slater type orbital for a single atomic orbital is the minimal basis set, as would be applicable to H and He. For an oxygen there would be five basis functions 1s, 2s and 2p_x,y,z. The difference in the \( r \to 0 \) dependence effect especially EPR parameter as HF couplings. An effect caused by the HF couplings dependency on the core shell. However, an early recognized beneficial compensation of errors is reported to overcome this effect in DFT geometries.167

In the triple zeta case, three basis sets are used for each atomic orbital. The first approach was to segment these additional contracted orbitals into valance shell and core shell, as in well-known 6-311G basis sets. Meanwhile they are coefficient optimized for loose and tight discrepancy of valence and core shell orbitals, respectively. This is also called correlation consistent. These basis sets offer the same quality, but a better calculation performance.
2.3.4 Effective Hamiltonian and Perturbation Theory for DFT

The effective Hamiltonian as the spin Hamiltonian (§2.1, p. 28) can be incorporated into DFT. Based on the Breit-Pauli approximations\textsuperscript{209} the spin-orbit coupling contribution is treated as a second order perturbation in a one-component relativistic treatment.\textsuperscript{210-212}

In the one-component treatment, all-electron contributions have a single origin (gauge origin), and no other relativistic effects beyond the spin-orbit coupling are treated, as current dependence in the exchange functional or admixture of the exact exchange. The sum over all occupied orbital and virtual orbital states of the spin-orbit coupling contribution on
the wave function is formed. The mean field approximation used for the sum over states contribution to the \( g \) tensor is similar to the high-field approximation used in analytical treatments of EPR interactions (cf. §2.1.6, p.37).

Further relativistic effects are small and have been neglected in the default ORCA\textsuperscript{214} (§ 3.6, p.73) EPR property calculation. Due to this one-component treatment the \( g \) tensor is not gauche invariant. Methods were reported to compensate this gauche dependence.\textsuperscript{167} Methods such as the gauche invariant atomic orbitals correction\textsuperscript{213} have not been implemented up to now in ORCA.\textsuperscript{214} However, scalar relativistic effects were introduced via certain basis sets (ZORA\textsuperscript{215}) recently.\textsuperscript{156} Especially for the small spin-orbit couplings treated here, these errors are often well encompassed by the uncertainty of the structure determination.\textsuperscript{167} For the determination of \( g \) values the combination of UB3LYP and EPR-II has proven to be effective and has been applied on various \( \pi \) radicals.\textsuperscript{110, 216, 217}

The solutions for the HF couplings in DFT connect the wave function of the before mentioned spin Hamiltonian to the obtained spin densities (§2.1, p. 28). The HF calculation at least on the same geometry is only minor affected on the DFT functional set used.\textsuperscript{167} Due to the mismatch in core potential with Gaussian functions often very flexible core basis sets are used (see last section). Here flexible double \( \zeta \) functionals for first and second row elements as EPR-II are often advantageous.\textsuperscript{167, 218-220} For certain nuclei IGLO III offers a flexible core in combination with a triple \( \zeta \) basis set.\textsuperscript{167, 218-220} The combination of UB3LYP and TZVPP has been shown to be efficient for precise \( g \) tensor calculations.\textsuperscript{221}
2.4 Proton Coupled Electron Transfer

To understand the different regions and dynamics of proton coupled electron transfer first a pure electron transfer will be considered. In principle one can show that if the rate limiting step is a long distance electron transfer the PCET kinetic is determined by $k_{ET} = k_{ET}^{1} + K_{PT} k_{PT}^{1}$. The index denotes proton transfer PT and electron transfer ET, respectively. The forward reaction kinetic constant is $k^{1}$ and the equilibrium constant is $K$. Here the electron transfer term can dominate, thus an electron transfer could be the rate limiting step in a PCET.\textsuperscript{97} In the $\beta$ subunit such a long-range electron transfer could be envisioned based on the current models (>16 Å).\textsuperscript{22}

2.4.1 Electron Transfer

The electron transfer is generally described by the Marcus theory\textsuperscript{222} as a transfer in the limits of the Born Oppenheimer (BO) approximation. At the transition state the Landau Zener semi-classical integration of the dynamical problem can be taken into account.\textsuperscript{223, 224} A time-dependent solution of the Hamiltonian is then analytical possible with a time-dependent perturbation as a linear function of time. A coupling between the diabatic states is fixed and the energy difference is also linearly time dependent. Two main contributions are most frequently discussed the reorganization energy $\lambda$ and the free Gibbs energy $\Delta G$ of the reaction.\textsuperscript{222} The general form is\textsuperscript{222}:

$$k_{ET} = v_{n} \kappa_{el} \exp \left[ \frac{-(\Delta G_{B}^{+} + \lambda)^{2}}{4\lambda k_{B}T} \right]$$ \hspace{1cm} (2-33)

Where $v_{n}$ is an effective nuclear frequency for the motion along the reaction coordinate that allows the transition state to be reached and $\kappa_{el}$ is the electronic transmission coefficient, given by:

$$\kappa_{el} = \frac{1 - \exp \left( \frac{-v_{el}}{2v_{n}} \right)}{1 - \frac{1}{2} \exp \left( \frac{-v_{el}}{2v_{n}} \right)}$$ \hspace{1cm} (2-34)

with:

$$v_{el} = \frac{V_{IF}^{2}}{\hbar} \sqrt{\frac{\pi}{\lambda k_{B}T}}.$$ \hspace{1cm} (2-35)
For the non-adiabatic limit the prefactor of the exponential term reduces to \( v_{el} \), whereas for the adiabatic case \( v_{n} \) is the prefactor (Figure 2-17b). A special case is the Marcus-inverted region, where a decrease of \( \Delta G_{R}^{\circ} \) the rate \( k_{ET} \) slows down. This is caused because the dependence of \( k_{ET} \) on \( \Delta G_{R}^{\circ} \) is an inverse parabolic function with an optimum for \( \lambda = -\Delta G_{R}^{\circ} \).

In Figure 2-17 the relevant parameters are shown by an ET between the initial state I and the final state F. The crossing of potential surfaces is assumed here for small \( V_{IF} \ (< k_{B}T) \) in the non-adiabatic regime. The case of an adiabatic regime is shown in Figure 2-17b.96

This equation has been substantiated tremendously in the meantime. And some general aspects are known about electron transfers in biology. In oxidoreductases most electron transfers take place between metal centers, shuttling one or two electrons. The most often found distance limit of pure electron transfer is 14 Å.99 Due to the short distances even endogenous steps of up to 450 mV can be tolerated.99 This was shown under the conditions of an ideal Marcus ET (\( \lambda = -\Delta G_{R}^{\circ} \)).99

![Figure 2-17: Cross section of the free energy surface along a nuclear reaction coordinate Q for ET. B) Motion on the effective potential surface is assumed to be a simple function of the potential energy (frictionless motion). The initial (I) and the final (F) electronic states are represented by diabatic (localized) parabolas. The equilibrium state of nuclear coordinates is denoted by \( Q_{I} \) and \( Q_{F} \), for initial and final state, respectively. At the lowest energy crossing of the nuclear coordinate the transition state \( Q_{t} \) is marked. The minimum splitting between the adiabatic states approximately equals \( 2V_{IF} \). The free reaction energy \( \Delta G_{R}^{\circ} \) and the reorganization energy \( \lambda \) are marked. The values of \( V_{IF} \) and \( \lambda \) are a function of coupling of the two electronic states. B) Adiabatic level crossing is shown for the case of \( V_{IF} \gg k_{B}T \). Here the system evolution proceeds on adiabatic ground states. The figure is from ref. 96.]
2.4.1.1 Distance Dependence of ET and Development of ET Theory

An electron transfer is distance dependent. This has been studied by artificial photochemical electron transfer reactions.\textsuperscript{100} In Figure 2-18 the result is presented. It could be shown that the distance dependence is influenced by the intervening medium. The dielectric packing between donor and effector for instance varies between proteins and water. A distance dependence was proposed on the modeled $\beta$ parameter. A general Arrhenius type law has been applied with $k \propto \exp\left( -\beta (Q_S - Q_I) \right)$.\textsuperscript{100,225} For proteins, the distance decay parameter $\beta$ has been found to be between 1.1-1.4 Å$^{-1}$, depending on the secondary structure of the protein.\textsuperscript{100}

![Figure 2-18: Distance dependence between acceptor and donor of an activationless electron tunneling $\tau$. Several intermediate media and oxidoreductases have been measured. A decay parameter $\beta$ for proteins is in the range of 1.0 Å$^{-1}$ to 1.4 Å$^{-1}$. The solid lines illustrate the tunneling-pathway predictions for coupling along $\beta$-strands ($\beta = 1.0$ Å$^{-1}$) and $\alpha$-helices ($\beta = 1.3$ Å$^{-1}$); the dashed line illustrates a 1.1-Å$^{-1}$ $\beta$. Distance decay for electron tunneling through glassy water is shown as a cyan wedge. Estimated distance dependence for tunneling through vacuum is shown as the black wedge. Data from ref. 100.](image)

Further development in the theory is ongoing, and meanwhile it has been pointed out that even structured water\textsuperscript{226} and small “gaps”\textsuperscript{227} along the electron transfer can be tolerated
without a significant loss in catalytic rate. Another recent investigation, considers fast movements and local quantum molecular effects. In this study it could be shown that the energy matching of the bridge to donor and acceptor can be improved by the molecular movement. A transfer path can be sustained unperturbed for electron transfer, if the time of ET is faster than the rearrangement frequency. Here several states are super positioned and can demonstrate a longer range flickering resonance transfer.

2.4.2 Expansion to an Electron Coupled Proton Transfer

To consider the coupling of the electron transfer to a proton transfer the larger masses in these quantum transfer processes have to be considered, as done early by Marcus et al. The two-dimensional ET coordinate becomes one cross section in the PCET energy landscape. The second proton transfer coordinate X is the third dimension as illustrated in Figure 2-19 A. Two coordinates can be seen in the square scheme introduced above. In this case, the proton transfer occurs along coordinate $X_p$, whereas the two possible electron transfer steps are along coordinate $Q_e$.

For the PCET in a especially the Soudackov-Hammes-Schiffer (SHS) theory is of interest. SHS has been applied to discriminate between HAT and a CPET. These two similar cases are proposed for di-tyrosine peptides by theorists. They especially discussed in the α subunit the PCET step between Y$_{731}$ and Y$_{730}$. Therefore, this section will focus on the SHS theory. However, many diverse theories will give rise to a statistically Arrhenius dependence either multiplied or summed with a dynamic prefactor. Nevertheless, they differ in the actual realization as reviewed in recent reviews.

The SHS theory is based on a VB description of the four possible steps in the consecutive processes of ET/PT and PT/ET. It uses for the environment a multistate continuum model instead of atomistic models for considering solvent effects. The proton donor-acceptor motion has been incorporated. For this motion linear response theory in combination with Fermi’s golden rule formulation were used here. In most of the modern models the vibronic coupling is taken into account by summation of the Boltzmann populations $P_k$ of the initial state. $S_{μν}$ is the overlap of the vibrational wave functions for the $μ$ and the $ν$ state. This is fully analog to the description of absorbance and fluorescence probabilities by the Frank Condon theory. A rate constant for an equilibrated system at each $X$ value can be obtained from these approximations (Eq. 2-36).
Proton Coupled Electron Transfer

\[ k_{\text{PCET}} = \frac{\pi}{\hbar} \sum_{\mu} \int_0^\infty dX \sum_{\nu} P_\mu (X) \left[ \frac{V_{\nu\mu} S_{\nu\mu}^{\text{p}} (X)}{k_B T} \right] \times \exp \left[ -\frac{\Delta G_R^+ (X) + \lambda_{\mu\nu} (X)}{4 \lambda_{\mu\nu} (X) k_B T} \right] \tag{2-36} \]

In the high temperature and or low frequency regime, the Eq. (2-36) for the X mode it is further simplified. Taken an equilibrium position at \( \Delta X = 0 \), the simplest form can be derived as Eq. (2-37).\(^{238, 239}\)

\[ k_{\text{PCET}} = \sum_{\mu} \sum_{\nu} \frac{V_{\nu\mu} S_{\nu\mu}^{\text{p}}}{\hbar} \left[ \frac{\pi}{\lambda_{\mu\nu} k_B T} \right] \times \exp \left[ \frac{2 \sigma_{\mu\nu}^2 k_B T}{M_p \omega_p} \right] \exp \left[ -\frac{\Delta G_R^+ + \lambda_{\mu\nu}}{4 \lambda_{\mu\nu} k_B T} \right] \tag{2-37} \]

Here the exponential decay is dependent indirectly on \( X \). \( M_p \) and \( \omega_p \) are the X-mode effective mass and frequency, respectively. \( a_{\mu\nu} \) is the decay parameter of the vibrational overlap. \( S_{\nu\mu}^{\text{p}} \) is here the vibronic overlap in the equilibrium state (\( \Delta X = 0 \)).

The model of Dogonadze, Kuznetsov and Levich has not only separated the electron from the proton movement (BO approximation), but also considered a second case where the proton movement is adiabatic to the solvent (frequency = \( \omega_0^S \)). To illustrate possible relative effects the following magnitudes were given: \( \omega_0^S \approx 10^{11} \) Hz \( \ll \omega_n \approx 10^{14} \) Hz \( \ll \omega_e \approx 10^{15} \) Hz. Here \( \omega_n \) describes the frequency of the bound reactive proton (I and F state) and \( \omega_e \) the electron frequency bound to the proton acceptor in an ionic PT step.

Both hydrogen atom transfer (HAT) and concerted proton coupled electron transfer (CPET) are usually vibronically non-adiabatic due to the small proton wave function overlap that produces vibronic couplings \( \ll k_B T \).\(^{109}\) Many biological PCETs are electronically non-adiabatic. For CPET reactions within these non-adiabatic reactions, the Eq. (2-37) is valid.\(^{240}\)
Figure 2-19: Extension from ET to a PCET A) The extension to a second coordinate X renders the ET to a two dimensional diabatic electron proton free energy surface connecting the vibronic states $\mu$ and $\nu$ as functions of two collective solvent coordinates. One coordinate is strictly related to ET ($Q_e$) and the other associated with PT ($X_p$). The equilibrium coordinates, the reaction free energy $\Delta G^R$ and reorganization energy $\lambda_{\mu\nu}$ are indicated similarly to Figure 2-17. Adapted from ref 241. B) Free energy along the reaction coordinate represented by the dashed line in the nuclear coordinate plane of panel A. Qualitative potential energy surfaces (PESs) and pertinent ground state proton vibrational functions are shown in correspondence to the reactant minimum, transition state and product minimum. ref. 242 C) Vibrational mode overlap in the diabatic PESs for the initial and final ET states and vibrational function: initial $\psi_D^{(i)}$ (blue) and final state $\psi_D^{(f)}$ (red). Small $V_{el}$ case is depicted. D) Large electronic coupling $V_{el}$ in an adiabatic ground PES. For an adiabatic system the vibronic coupling is half of the splitting between the energies of the symmetric (cyan) and antisymmetric (magenta) vibrational states of the proton. The excited vibrational state of the antisymmetric state is shifted up by 0.8 kcal/mol for a better visualization. Adapted from ref. 109.

2.4.2.1 CPET versus HAT

The comparison between HAT and CPET is difficult. Already the definitions are essential, whereas the HAT and the CPET is known to account for a single site and a multisite acceptor, respectively. This definition is fragile. Quantum effects hamper the knowledge of an exact position at a given time, thus superposition of different acceptors has to be treated. Thus, especially in the transition between a tyrosine radical stacked to a tyrosine, the electron acceptor orbital is not exactly defined.
A more vigorous definition follows from the nature of the transferred particle. For an HAT an electron neutral particle is transferred, leading to minimal reorganization energies. Thus, the electron is moving stringent to the adiabatic Born Oppenheimer approximation concomitant with the proton. In the CPET case a non-adiabatic transfer is present. By the comparison of a benzyl/toluene and a phenyl/phenoxy system it was revealed that the first case is an HAT and the later a CPET. A strong difference between the proton transfer $\tau_p$ and the electron transfer speed $\tau_e$ could be shown in the two cases. The ratio between $\tau_p$ and $\tau_e$ is the adiabaticity degree parameter $p$. Thus $p \ll 1$ are PCET reactions and $p \gg 1$ are HAT reactions. The transfer in the phenoxy/phenol couple occurred over a $\pi$-complex (proton donor-acceptor distance: 2.4 Å) with electrons 80 times faster than the proton movement.

In the benzyl/phenol case a $\sigma$ complex (proton donor-acceptor distance: 2.72 Å) was formed here the proton movement was calculated to be 3.5 times faster than the electron, thus the electron can respond instantaneously to the proton motion. Further analysis revealed that the electronic coupling $V_{el}^{II}$ is significantly different with 700 cm$^{-1}$ to 14300 cm$^{-1}$ (CASSCF calculations) between cases Figure 2-20 A and B, respectively. Figure 2-20 demonstrates the effect and clearly illustrates the differences between both cases. In general, the adiabaticity of a PCET reaction can be taken as a good indicator to discriminate PCET and HAT.
2.4.2.2 CPET between a di-Tyrosine Model

The interaction between two backbone connected Y groups has been also studied in the biological context for Y\textsubscript{730} and Y\textsubscript{731} in α by Kalia and Hummer.\textsuperscript{106} They could show that in the “π-stacked” arrangement in contrast to the linear geometry of Figure 2-20A, the ground state potential decreases and the electronic coupling increases. Nevertheless, they came to the conclusion that a PCET takes place between this geometry. Water participation is possible but the energy barrier increases for water molecule mediated PCET from 8.5 to 14.1 kcal/mol. Notably, their model revealed that by exchanging the Y with a NO\textsubscript{2}Y (cf. Figure 1-6, p.10) with a higher redox potential, this bias favors water mediated PCET. In this thesis, we will also apply bias on the natural di-tyrosine, by the introduction of a 3-amino tyrosine. The change from vacuum to water in a conductor like screening model increased the barrier by up to ≈4 kcal/mol. 

Over all, this also demonstrates the necessity for high level calculations. In general, it highlights that QM and MM calculations are important to understand the basics and common principles of PCET reactions.
2.4.3 Water Participation in ET and PCET

From initial studies of electron transfers in water it was estimated, that water is a slow ET media (cf. Figure 2-18). This in general has been shown to be an incomplete statement. From several studies working with a structural water environment have shown that here the ET kinetic rates are comparable up to 2 intervening water molecules to protein media. And for distances up to 12 Å, the kinetic effect of structured water was still detectable, compared to the rates of unstructured water shown in Figure 2-18 (p.56). It seems that electrostatic and van der Waals effects can reduce pathways, which interfere destructively with the donor accepter electron transfer. This triggered further investigations on how transfers are possible in fast changing mobile media. The delocalization of protons can be also advantageous for PCET transfers. However, as shown by studies on flickering resonance (up to 15 Å), the limit is the kinetic rate. As long as the kinetic rate is faster than the reorganization of ET pathways the transfer stays feasible. This does in part set an upper limit on distance and on the barrier height. The investigations for water participation in PCET have just started, by for instance pH dependent proof of principle investigations.
3 MATERIALS AND METHODS

3.1 Materials

D₂O (99.8% isotopically enriched) and ²⁵MgO (95.75% isotopically enriched) was purchased from Euriso-Top. Glycerol-D₃, di-sodium ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (TRIS) was purchased from Cambridge Isotope Laboratories, Fluka, and J.T.Baker, respectively. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), adenosine-5'-triphosphate (ATP), cytidine-5'-diphosphate (CDP), Sephadex® G-25, 2-methylbutan, hydrochloric acid solution (molecular biology grade, 36.5-38%) and sodium hydroxide were purchased from Sigma-Aldrich. Amicon® Ultra concentration device (30 kDa filter) was bought from Merck KGaA, whereas Glycerol and Magnesiumsulfate was used from Roth. Polyacrylamid Gels (PAGE, 7.5%, Tris-HCl) were bought from Biorad.

The following buffers were used:

i. A buffer consists of TRIS (50 mM), EDTA (1 mM) and glycerol (5% v/v) adjusted to pH 7.6

ii. Assay buffer consists of HEPES, MgSO₄ (15 mM), EDTA (1 mM) adjusted to pH 8.0

iii. Desalt buffer consists of TRIS (30 mM) and glycerol (5% v/v) adjusted to pH 7.6
The following EPR tubes were used:

i. 263 GHz: Vitrocom CV2033S/Q (Ø 0.33 mm)

ii. W band: Bruker E600-213/ST9O (Ø 0.9/0.5 mm) and Wilmad glass quartz tubes (Ø 0.9/0.5 mm); Bruker

iii. Q Band: Bruker quartz tubes ER221TUB-Q-10 (Ø 1.6/1.1 mm).

### 3.2 Sample Preparation

\( \alpha \)-NH\(_2\)Y\(_{730}\), \( \alpha \)-NH\(_2\)Y\(_{731}\), and \( \beta \)-NH\(_2\)Y\(_{356}\) were prepared and purified as previously described,\(^{44}\)\(^ {67}\) beside the absence of DTT in the final Sephadex column.\(^{253}\) These preparations were performed by T. Argirević form our group and for \( \beta \)-NH\(_2\)Y\(_{356}\) by E. Minnihan from the Stubbe lab at the MIT. For \( \alpha \)-NH\(_2\)Y\(_{731}\) the truncated form was removed by an additional anion exchange column (MonoQ, equilibrated in a-buffer) against a NaCl gradient (2.5-400 mM) over 50 mL. This step was performed with the help of Florian Brodhun in the Feussner lab (Georg August University, Göttingen). All NH\(_2\)Y mutated subunits were mixed in equimolar ratios with their corresponding wt (prereduced \( \alpha/\beta \)) and spin concentrated to 100-200 µM (\( \alpha_2\beta_2 \)) in D\(_2\)O (>99 %) and H\(_2\)O assay buffer.\(^{134}\) The concentration was checked by UV-vis spectroscopy on tyrosine and tryptophan absorption bands \( \varepsilon_{280\text{ nm}} \approx 320 \text{ mM}^{-1} \text{ cm}^{-1} (\alpha_2 \approx 189 \text{ mM}^{-1} \text{ cm}^{-1} + \beta_2 \approx 131 \text{ mM}^{-1} \text{ cm}^{-1}) \). The samples were stored in 0.5 mL Eppendorf tubes per 10 µL aliquots in liquid N\(_2\). The double mutant samples were directly obtained as a 1:1 complex by Wankyu Lee from the Stubbe lab at MIT.\(^ {254}\)

\( \beta \)-2,3,5-F\(_3\)Y\(_{122}\)• mixed with \( \alpha \)-wt or \( \alpha \)-Y\(_{731}\)F was prepared by Kanchana Ravichandran from the Stubbe lab at the MIT, as described previously.\(^ {120}\) The samples in D\(_2\)O (>99 %) and H\(_2\)O assay buffer were stored in 100 µL aliquots at 80K. pBAD-nrdB\(_{122TAG}\) and pBAD-FnYRS-E3 were co-transformed into E. coli TOP10 chemically competent cells and grown at 37°C on LB-agar plates containing 100 µg/mL ampicillin (Amp) and 35 µg/mL chloramphenicol (Cm). A starter culture (2 mL) supplemented with the antibiotics was inoculated with a single colony and grown until saturation (37°C, 12 h). This starter culture was diluted 100-fold into fresh 2X YT media containing Amp and Cm. After 16 h, the cultures were diluted 100-fold into 4 x 2 L of 2X YT with antibiotics and 0.7 mM 2,3,5-F\(_3\)Y (500 mM stock solution in water, NH\(_4\)OH solubilized). At an OD\(_{600}\) of 0.5, 100 µM o-phenanthroline (100 mM stock solution in 0.1 M HCl) was added to chelate iron. 30 min
later, 0.05% (w/v) L-arabinose (10% w/v stock solution in water) was added to induce the F$_2$YRS and NrdB. Growth was continued for an additional 5 h and the cells were harvested by centrifugation (3500 x g, 15 min).

Apo $\beta_2$-$\gamma_{122}(2,3,5)$-$F_3Y$ was purified using anion-exchange chromatography as previously described.$^{255}$ Typical yields of 10-15 mg pure protein/g cell paste were obtained.

**Reconstitution of Apo $\beta_2$-$Y_{122}(2,3,5)$-$F_3Y$**. Apo $\beta_2$-$Y_{122}(2,3,5)$-$F_3Y$ was deoxygenated and taken into an anaerobic chamber maintained at 4°C. 5 equiv. of Fe$^{II}$(NH$_4$)$_2$(SO$_4$)$_2$ was incubated with the protein for 15 min. The sample was brought out of the chamber sealed, and O$_2$ in the form of O$_2$-saturated 50 mM hepes pH 7.6, 5% glycerol was added to reconstitute the cluster. 250 µL of $\beta_2$-$Y_{122}(2,3,5)$-$F_3Y$ was frozen in an EPR tube immediately after reconstitution to quantitate radical content. Typical yields of 0.6-1.0 2,3,5-$F_3Y_{122}$•/β$_2$ were obtained for the reconstituted protein.

$^{25}$Mg$^{2+}$-samples were prepared by first washing the protein in 5 concentration (to 20% v/v) dilution steps with desalt buffer and buffer exchanged the sample with a $^{25}$MgCl$_2$ (15 mM) assay buffer with additional 5 steps. The $^{25}$MgCl$_2$ was obtained quantitatively by dissolving $^{25}$MgO (12.3 mg, 30 mM) in concentrated hydrochloric acid solution (98.4 µL, 120 mM) in test tube overnight, similarly as described previously.$^{256}$ Milli-Q® water was added and the HCl was allowed to evaporate in a desiccator and afterwards the product was dried under high vacuum. ESI-MS of the product dissolved in Methanol showed mass shift of 1 m/z compared to MgCl$_2$ in natural abundance. 4% of $^{24}$Mg(II) could be observed.

EPR samples were prepared by thawing each aliquot at 4°C and followed equilibration at 25°C for 10 min. The reaction was initiated by adding CDP and ATP in H$_2$O/D$_2$O assay buffer with final concentration 2 and 6 mM, into the reaction mixture (1 µL 263 GHz, 2.5 µL W band and 6 µL Q band) with final complex concentrations of 90-100 µM. Each reaction was allowed to proceed for 10-20 s and manually freeze quenched inside an EPR tube with liquid N$_2$. For Q-band PELDOR samples glycerol-D$_3$ (20 % v/v) was added after 10-20 s, and then the reaction was frozen in ice cold 2-propanol (≈185 K). The quench times were varied based on the individual kinetic rates of the different samples as measured by UV-vis stopped flow$^{44,67,254}$ or Rapid Freeze Quench.$^{28}$ This should ensure a maximum radical yield.
3.3 X/Q-Band Spectroscopy

Q-Band spectra were obtained by a Bruker Elexsys E580 spectrometer with a nominal output power of 3 W. The ESE traces and PELDOR traces were recorded in a Bruker (EN5107D2) cavity. The cooling of the cavity was achieved within a liquid Helium continuous flow cryostat (CF95550, Oxford Instruments). PELDOR\(^{257}\) \((\pi_{MW1}/2-\tau_1-\pi_{MW1}-[\tau_1+x]-\pi_{MW2}=[\tau_2-x]-\pi_{MW1}-\tau_1-echo)\) spectroscopy is a constant time 4 pulse experiment. PELDOR uses pulses at a pump (MW2) and detect microwave (MW1) frequency and was carried out by measuring the dipolar evolution over the time \(x\) in steps of 8 ns. Experimental details are given in the figure captions.

X-Band measurements were performed on a Bruker Elexys E500 spectrometer, with a HighQ CW-resonator (4122SHQE, Bruker) in an ESR900 (Oxford Instruments) cryostat.

3.4 W-Band Spectroscopy

The EPR and ENDOR spectra were recorded on an Elexsys\(^{680}\) with 400 mW output power and typical \(\pi/2\) pulse length of 16 ns at 70 K. The cooling was performed under continuous Helium flow in Oxford Instruments cryostat. A pulsed ENDOR probehead (1021H, Bruker) was used as a resonator.

Mims-ENDOR\(^{183}\) \((\pi/2-\tau-\pi/2-RF-\pi/2-\tau-echo)\) spectroscopy was carried out with a 40 \(\mu\)s RF pulse amplified by a 250 W RF amplifier (250A250A, Amplifier Research). All obtained ENDOR spectra were normalized to compare with simulations.

3.5 263 GHz Spectroscopy and Calibration

The spectra were recorded on a prototypical Elexys\(^{6}\) E780 from Bruker Biospin. The 263 GHz spectrometer works with a quasi optical front end. The front end produces a Gaussian beam that is focused to a corrugated waveguide. The typical output power of the bridge was 15 mW. The corrugated waveguide is coupled to a single mode (TE\(_{011}\)) cylindrical cavity (E9501610) with a typical quality factor (\(Q\)) of 500-1000. The electron spin echo \((\pi/2-\tau-\pi-\tau-echo, ESE)\) was recorded with a typical microwave field strength \(B_1\) of 10-17 MHz. The ESE spectra were recorded by 70 K, if not stated otherwise. The individual \(B_1\) is measured via the pulse length necessary for inversion of the magnetization \((\pi_p)\) by a nutation recorded by an inversion recovery experiment \((\pi_p-T-\pi/2-\tau-\pi-\tau-echo)\) scanning.
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over the pulse length of $\pi t_p$. A standard coal sample is used. The microwave field strength can be obtained by the turning angle $\theta$ of each pulse length $t_p$ by Eq. (3-1).

$$\theta = \sqrt{S(S+1)-m_s(m_s+1)} \frac{\beta B_i}{h} t_p$$  \hspace{1cm} (3-1)

Freeze quench samples in 0.33 mm EPR tubes were inserted under liquid nitrogen into the resonator surrounded by liquid nitrogen and then transferred into the precooled (80 K) EPR cryostat (Oxford Instruments).

To assess the accuracy of the $g$ values several error contributions have to be considered. As reported for other high frequency spectrometer\(^{258}\) the frequency change over a measurement is not a significant source of error. The spectrometer has a sweep coil with 250 mT range and the main magnet operating until 12 T. If the main magnetic field is changed a systematic change of all $g$ values has to be considered. Therefore, a reliable calibration of the field is necessary. Field calibration was originally performed based on a multiline Mn(II) standard sample, by Bruker. Normally, the Mn(II) (0.02% in MgO) has been used with a $g$ value of 2.001015(5) and the HF coupling $A = -243.9$ (1) MHz\(^{259,260}\). The Mn(II) in marble used by Bruker and Jeol has different values with $g = 2.0011$ and $A = -241.6$ MHz\(^{261,262}\). With this standard sample a single field point can be calibrated and the linearity of the sweep coil sweep is evaluated by the 6 lines of the $^{55}$Mn hyperfine interaction within the Kramers doublet. The non-linearity originates mainly from the self-inductance of the sweep coil, and the mutual inductance of the sweep and main magnet coil\(^{258}\). To compensate this non-linear behavior of the ratio between gauss to amp, Bruker implemented a linearization protocol for magnetic field sweeps in CW-EPR spectra. In this protocol the typically small measurement range for organic radicals (20-50 mT) is always measured by a full sweep range of the sweep coil. Here the curve difference to a linear behavior can be approximated by a second order fit (cf. Figure 3-2). Hence, the sweep coil has to be driven through the full (250 mT) sweep range. This extends the measurement time by a factor of 5 to 13 depending on the sweep range. To be able to omit this large drawback, the impact on the spectral accuracy of this linearization option was evaluated. For this reason, the CW-EPR spectra of the manganese standard sample in increasing field direction were measured with and without the linearization procedure. After aligning the first line (Figure 3-1 inset left) the shift was measured on each line position. Thus, the scan over the six lines performed with the linearization procedure is about 4.4 G narrower than one without linearization. This gives an estimate of the systematic error introduced by removing
the linearization procedure and reducing the measurement time. The additional error for a typical field sweep of 30 mT is 0.36 mT based on a fit of the data (Figure 3-2). Compared to the typical field of 9350 mT, this error is $3.9 \times 10^{-5}$ mT. Due to the $g$ factor of approximately two (for organic radicals in this thesis), a systematic error of $8 \times 10^{-5}$ is obtained. The overall systematic shift in the ampere to gauss ratio has to be re-adjusted from time to time, to align with the standard values with the setting. However, the standard sample used here could be only observed by CW-EPR with a sharp line width of $\approx 0.12$ mT. Therefore, another sample was necessary in order to test the pulsed set up.

Figure 3-1: Comparison of CW 263-GHz EPR spectra of Mn$^{2+}$ (in CaO, 0.02%) at room temperature with and without linearization. Exp. details: Field sweep range = 100 mT; modulation amplitude = 0.5 G; conversion time=100 ms; single scan.
Figure 3-2: Linearization improvement compared in CW 263 GHz EPR measurements. The difference in magnetic field between the linearized and the non-linearized field sweep as $\Delta B_0$ is plotted against the width of the Mn$^{2+}$ resonance lines. The points can be fitted with a second order polynomial (red line) with: $y = 0.148(1) \cdot x - 9.2(4) \cdot 10^{-6} \cdot x^2$ and $R^2 = 0.99994$.

To test the field accuracy in the pulsed mode the $\beta_{32}$-Y$_{122}^•$ E. coli RNR sample was used, which is well characterized at high-field EPR. An advantage of taking the Y$_{122}^•$ as a standard is that it can be used as an internal standard for the RNR samples studied. This internal standard is detectable at 10 K and is hidden at elevated temperatures (70 K). The derivative has to be formed to assign the principle axis values of $g_x$, $g_y$, and $g_z$ in the $g$ tensor broadened line. The spectrum of Y$_{122}^•$ has been recorded at 10 K. The spectrum was then compared to high-field powder data of Y$_{122}^•$. Högbom et al. have used as a calibration standard a narrow single line Li:F $g$-standard (Li in LiF, $g = 2.002293 \pm 0.000002^{263}$) measured at two different frequencies. Gerfen et al. used multiline Mn(II) (0.02% in MgO)$^{264}$ with a g value of 2.001015(5) and the HF coupling $A = -244.1 (1)$ MHz.$^{157,239,260}$ The Y$_{122}^•$ spectrum measured at our instrument was compared to a simulation based on these two literature values, as shown in Figure 3-3. The simulation parameters reported by Gerfen (gray) et al. show an agreement with the spectrum in terms of $g$ values and deviate from those reported by Högbom et al. (green) at $g_x$ and $g_y$ by $2 \cdot 10^{-5}$ and $6 \cdot 10^{-5}$, respectively. In both experiments an error of $4 \cdot 10^{-5}$ or $5 \cdot 10^{-5}$ was estimated.$^{63,84}$
Figure 3-3: Pulsed-EPR spectrum of the β₂-Y₁₂₂• as calibration standard. The experimental trace is shown in blue and the simulation gray and green with values from 157 and 63, respectively. Exp. details: ESE, 262.0109 GHz, T=10 K, π(π/2)= 52(90) ns, τ=319 ns, shots per point (SPP)=50, shot repetition time (SRT)=15 ms, scans = 43. The derivative was built by a Savitzky-Golay filter (second order, 3 points).

Another calibration with a N@C₆₀ sample (from A. Schneggs lab at the Helmholtz Zentrum, Berlin) was performed recently by I. Tkach in our group. He found a standard deviation within 8 resonance frequencies averaged over three line measurements to be in g 3.3·10⁻⁶. By reducing the sweep range from 60 mT by a factor of 10 a systematic shift of -1.5·10⁻⁵ could be observed, based on 3 observing frequencies and 12 measured resonances. The change of other parameters by a factor of ten, like a tenfold increase in sweep time gave no significant shifts. All of these errors are far below the errors reported within this thesis and are therefore neglected in the future discussion. Based on the measurements, the systematic error with or without linearization procedure in CW and pulsed EPR spectroscopy can be estimated. The error estimated by the differences observed here is below 5·10⁻⁵ in g scale. For experiments where field linearization has not been used a
systematic error of $9 \cdot 10^{-5}$ is estimated. However, due to low signal to noise ratios (S/N) and broad line widths, the g value uncertainty can vary in an individual spectrum.

3.6 Density Functional Theory Calculations

3.6.1 Set-up of the Models

DFT calculations have been performed with the ORCA 3.0.0 program package\textsuperscript{214}. DFT calculations were originally performed by Christoph Riplinger (ORCA 2.9.0) from the Neese group and had been performed as previously reported.\textsuperscript{110} The geometry-optimized large models are based on the crystal structure (wt-α, PDB ID 4R1R) and had shown by energy-optimized relaxed surface scans, along the reaction coordinate, energy barriers in agreement or lower than previously reported values with smaller models by DFT theorists.\textsuperscript{106,107,266} To compare these models to experimental findings, Simone Kossmann from the Neese group incorporated the amino group at Y731• and re-optimized the geometry. The EPR parameters were calculated by Simone Kossmann. The adaptation into magnetic resonance convention and the interpretation of the output was done by me. Small model calculations have been performed to test different environment dependencies of the structurally ill-defined region.

3.6.2 Geometry Optimizations

3.6.2.1 Large Models 1, 2 and 3

Initially, the coordinates of the large models 7 and 8 used in ref\textsuperscript{110} augmented by the amino group in the 3 position of Y731 and a water molecule between Y731 and Y730 for Model 3. These coordinates were first geometry optimized without further restraints. During the optimization the distance between C439 and Y730 increased constantly. It was supposed this results from the missing contact to the β subunit in the model, thus the coordinates were restrained for all Cα and for all Cβ. For Y730, NH2Y371 and C439 only the Cα were restrained. Additionally the Cartesian coordinates of the hydrogen atoms in the truncated GPD model replacing the bonds between C4 and C5 of the ribose as well as the bond between C1 of the ribose and the base were kept fixed.

The model structures were geometry optimized using a generalized gradient density functional the unrestricted BP86\textsuperscript{267,268} in combination with Ahlrich’s diffuse TZVP basis set
Density Functional Theory Calculations

of triple-ζ quality\textsuperscript{207,269}. Grimme’s dispersion correction\textsuperscript{200,270} has been now added on top of the SCF calculation. The Resolution of the Identity (RI) approximation with the corresponding auxiliary basis sets (def2-TZVP(P)/JK\textsuperscript{150}) has been employed throughout.

3.6.2.2 Small Models

In the small models the geometry optimization was performed on the B3LYP\textsuperscript{268,271,272} hybrid density functional in combination with the TZVPP basis set and def2-TZVPP/JK auxiliary basis set. In the models adapted from the large models only the dihedral angle of the peptide bond of Y\textsubscript{730} and Y\textsubscript{731} was fixed and the Cartesian restraints for all surrounding C\textalpha{}’s were kept. In order to compensate the electrostatics from the environment here a solvation model (COSMO\textsuperscript{273}) with polarity of ethanol (ε=24) was used. Otherwise Grimme’s dispersion correction\textsuperscript{199-201} and RIJCOSX\textsuperscript{274} approximations has been employed. The energy has been converged to 10\textsuperscript{-9} E\textsubscript{h}, if not stated otherwise.

3.6.3 EPR Calculations

The EPR calculations were carried out with the B3LYP\textsuperscript{268,271,272} hybrid density functional in combination with the RIJCOSX\textsuperscript{274} approximation. In the small models COSMO was retained for the single point calculations. Here Barone’s EPR-II (IGLO-II for sulfur) basis set of double-ζ quality has been used in combination with the def2-TZVPP/JK auxiliary basis set for all atoms.\textsuperscript{219,220,275} The g values were calculated\textsuperscript{210} using the tyrosine (analog) C\textsubscript{4} as gauge origin. In single amino acid models the def2-TZVPP basis set was held consistent with the geometry optimization step.\textsuperscript{207} The dihedral scans were performed with a geometry optimization for each restrained dihedral. The energy has been converged to 10\textsuperscript{-9} E\textsubscript{h}. 

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4 3-AMINO TYROSINE RADICAL INTERMEDIATES

To compare Ys in terms of electronic and molecular structure in enzymatic reactions like the radical propagation of E.coli RNR small differences in electronic and molecular structure have to be resolved. The multi-frequency characterization of radicals is commonly used to disentangle magnetic field dependent (for instance $g$ values) and independent parameters (for instance HFC’s).\textsuperscript{276} The spectral width of the EPR spectrum influenced by the $g$ tensor and the coupling strength of the nuclei in the surrounding are the factors governing the choice of a suitable frequency (cf. §2.2). Compared to Ys ($g_{xy}-g_{xz}$: 4.3-6.9 ppt) our amino tyrosine have generally a smaller spectral width ($g_{xy}$: 3 ppt) and were until now characterized by 9 to 180 GHz spectroscopy.\textsuperscript{92} Nevertheless, not all values were determined to a high precision ($<0.2$ ppt) and the question of possible underlying radicals remained open.

In order to extend the applicability of this radical probe and to answer open questions in the radical process of RNR, 263 GHz EPR spectroscopy was employed. The results will be first presented beginning with the last step of the forward radical process. Here previous EPR data had shown that NH$_2$Y$_{730}^\bullet$ hosts a well-defined H bond network (cf. §1.5.3, p.21).\textsuperscript{92,110} A part of this chapter has already been published.\textsuperscript{254}
4.1 Electrostatic Environment of 3-Amino Tyrosines in the α Subunit

4.1.1 263 GHz Spectra of NH$_2$Y$_{730}$•

The higher $g$ value resolution for NH$_2$Y$_{730}$• should determine accurately resolved $g$ values. Transient radicals were generated by adding to wild type β and α-NH$_2$Y$_{730}$ an excess of substrate (CDP) and the corresponding effector (ATP). The reaction proceeded 30 s, until the reaction was quenched in liquid N$_2$. To suppress line broadening from weak intermolecular $^1$H-couplings the protein samples were buffer exchanged with D$_2$O before the reaction. A $^2$H from a D$_2$O buffer has the advantage to provide a 6.5 ($\nu$-$^1$H/$\nu$-$^2$H) times weaker hyperfine (HF) interaction, scaling linearly with its gyromagnetic value (cf. §2.1.3, p.33). The half-site reactivity of this homodimer corresponds to the optimal yield, as reported to be around 50% based on all observed radical. The remaining contribution is the stable Y$_{122}$•. The relaxation times of Y$_{122}$• are rather short because of the proximal diiron cofactor (4.6 Å, Y-O:Fe$_1$).$^{55}$ Full suppression of the signal of Y$_{122}$• is accomplished even by recording the spectra at 70 K with short interpulse delays (200 ns).$^{55,148,152}$ In this delay time the phase memory time ($1/T_m$) influences the decay of the signal by three contributions: (i) spin-spin relaxation ($1/T_2'$), (ii) lifetime broadening ($1/(2T_1)$) and (iii) spin-lattice relaxation of spins in the vicinity ($1/T_1(B)$), i.e., iron spin states.$^{172}$ p.214 This relaxation filter will be applied in all ≥70 K experiments throughout this thesis to separate pathway radical contributions (i.e. α-NH$_2$Y$_{730}$•, α-NH$_2$Y$_{731}$ and β-NH$_2$Y$_{356}$) from the Y$_{122}$• signal (Figure 3-3). An absorptive electron spin echo (ESE) spectrum of pathway radicals is shown in Figure 4-1. The absorptive line (green) displays a rhombic $g$ tensor. A better resolution of the different orientations of the molecules along the magnetic field ($B_0||g_x, g_y, g_z$) is observed when the derivative (black) is formed.
Figure 4-1: 263-GHz echo detected spectrum of Y$_{730}$NH$_2$•, quenched after 30s reaction time. Green shows the absorption spectrum and black the enlarged derivative (obtained by a 5 points second order Savitzky-Golay filter). Exp. details: ESE, $\pi(\pi/2)=60(120)$ ns, $\tau = 300$ ns, shot repetition time (SRT)= 3 ms, shots per point (SPP)=50, 140 scans. Not linearized field sweep, difference to linearize in this case 0.00001 at $g_x$ (cf. Figure 4-3, Methods §3.5, p.66).

The spectral features contributing to the rhombic spectrum are clearly depicted in the first derivative (blue, Figure 4-1). The spectrum displays the three canonical orientations $g_x$, $g_y$, and $g_z$ parallel to $B_0$, marked as black dashed lines. The line shape around $B_0 || g_x$ shows a broaden doublet peak, the $g_y$ displays also a doublet and $g_z$ a quartet (doublet of triplet, 1:2:2:1). The $g$ values directly observed from this line shape are 2.0054, 2.0042, and 2.0022. The difference to the multi-frequency EPR study reported before for $g_x$ is subtle (0.2 ppt) but significant, compared to the error of 50 ppm (Methods §3.5). The major nearly isotropic splitting (29.8 MHz), consistent with the previous reports, is due to a $\beta$-methylene $^1$H HF coupling from hyperconjugated methyl protons (see Figure 4-2).\textsuperscript{110} The quartet arises from the overlay of this doublet and the triplet of the anisotropic $^{14}$N hyperfine interaction. An additional observation is the tiny doublet splitting observed on top of the quartet. It is a contribution not resolved along other canonical orientations and is 9-10 MHz in size at $g_z$ (Figure 4-3, inset). Generally HF tensors from protons bound to an aromatic ring have a slightly rhombic tensor shape, with ($A_1<<A_2\leq A_3$) the smallest value is along the bond direction and the largest along the $\pi$ orbitals of the aromatic ring (see §2.2.5).\textsuperscript{277} Due to spin
polarization, the HF coupling from C\textsubscript{6}-H would be strongest in the \textit{g}_z and \textit{g}_x plane, and smallest along \textit{g}_y. The second \textbeta-methylene coupling is reported to be mostly isotropic (cf. Figure 2-12 p.46).\textsuperscript{277} In Q-band (34 GHz) ENDOR spectroscopy it was shown that an upper limit for a \textit{^1}H hyperfine coupling is 8 MHz at \textit{g}_y and sharp lines were reported.\textsuperscript{92} Hence, this coupling is consistent with an isotropic \textbeta-methylene \textit{^1}H coupling from H\textbeta\textsubscript{2}, as shown in Figure 4-2.\textsuperscript{134}

Simulation of the spectra takes these HF couplings and \textit{g} values into account. The simulation (Figure 4-3) shows that previously reported parameters from a multi-frequency investigation are in a good agreement with the new spectra (red, Figure 4-3, Table 4-1). Two adjustments were made: the \textit{g}_x value had to be shifted and the small 9 MHz coupling had to be taken into account with a size comparable to the line width of the measurement. The small coupling at \textit{g}_z is enlarged in the inset in Figure 4-3.

![Figure 4-2: Ring dihedral \(\theta_{C\beta}\) comparison of the DFT/EPR model and the NH\textsubscript{2}Y\textsubscript{730} crystal structure (XRD, PDB ID: 2XO4).](image-url)
Figure 4-3: 263-GHz echo detected spectrum of Y$_{730}$ND$_2$•, quenched after 38 s reaction time, and simulation. Black shows the derivative and gray line the simulation. The inset shows the coupling pattern at $g_z$ enlarged. A quartet coupling pattern (~30 MHz) of 1:2:2:1 ratio and on top a doublet (9 MHz) are visible. The simulation was performed with the parameters of Table 4-1 and a line broadening of 1 G was used. Exp. details: ESE, π(π/2)=130(64) ns, $\tau=277$ ns, SRT=6 ms, SPP=500, 8 scans. The derivative was obtained by a 5 points second order Savitzky-Golay filter.

4.1.2 263 GHz Spectra of NH$_2$Y$_{731}$•

The large resolution of the g tensor for organic radicals offers also the possibility to search for conformers and different hydrogen bond environments.\textsuperscript{55, 110, 144, 278} Particularly at NH$_2$Y$_{731}$• two conformations were found in X-ray crystal diffraction structures (XRD, cf. Figure 1-12B\textsuperscript{67}), and preliminary work of T. Argirević showed time-dependent changes in the EPR spectra.\textsuperscript{92} However, measured PELDOR spectra only showed a narrow Gaussian distance distribution, which was found to be consistent with a defined conformation in a direct π stacking geometry.\textsuperscript{43, 67} Initially, different time points were measured within the second to minutes time scale at W-band (Appendix Figure A-2, p.193). The results were inconclusive, due to contributions supposed to arise from an insufficient separation of residual Y$_{122}$•, Mn$^{2+}$ lines and glass signals.

At 263 GHz unprecedented resolution could be obtained in Figure 4-4. The spectrum of NH$_2$Y$_{731}$• is displayed in blue. The reaction conditions were kept unchanged and the
reaction mixture was frozen at 18 s in liquid N$_2$. The spectrum shows clearly separated maxima of $g_x=2.00511(5)$, the second zero-crossing at $g_y=2.00399(5)$ and the last local minimum at $g_z=2.0022$. The first minimum resembles a triplet from two overlapping HF contributions in a 2:1 ratio. This and the doublet at $g_y$ as well as the quartet at $g_z$ were expected from previous 94 GHz spectra. The $\beta$-methylene HF coupling is here the isotropic contribution with $(a_{iso}=22$ MHz). The anisotropic coupling with a larger value at $g_x$ and $g_z$ has a typical tensor form of a ring proton coupling, with small HF coupling contributions along $g_y$. It is tentatively assigned to a ring proton at C$_6$ position (see §4.4). Notably, the spectral feature marked with an asterisk (*) at $g_z$ was overlapping with the $g_z$ line shape at W-band. At 263 GHz this contribution is resolved from the canonical orientation of $B_0\parallel g_z$ (Eq. 2-11, Theory §2.1.2). Due to the smaller C$_\beta$-H with $a_{iso}=22$ MHz smaller couplings can contribute to the line shape, which were not considered in the simulation. For instance a ring proton coupling becomes visible compared to NH$_2$Y$_{730}^\bullet$ (Table 4-1). The structural consequences of the assignment will be discussed in more detail together with the DFT models (§4.4 p.98). The $g$ values shifted by 0.1-0.2 ppt with respect to $g$ values previously reported. Notably, also the $g_y$ value shifted by 0.1 ppt, though this is within error of the 94 GHz data (0.1 ppt). All other values remained unchanged within the error of previous studies.

The spectra in protonated and deuterated buffer gave comparable $g$ values (Figure 4-5). It is worth noting that a difference in quench time of 1:52 min between both samples also does not affect the $g$ values, which is in agreement with the 94 GHz data (see Appendix Figure A - 2, p.193). In order to simulate the spectral line shape in protonated NH$_2$Y$_{731}^\bullet$ only the line broadening caused by the amino deuterons had to be taken into account. The simulation parameters for the $^1$H amino protons were taken from orientation selective $^2$H ENDOR spectra and scaled by their gyromagnetic ratio (cf. Table 4-6 p.104).
Figure 4-4: 263-GHz ESE spectrum ND$_2$Y$_{731}$• recorded for a sample quenched after 18 s reaction time. The red line shows the experimental derivative of the absorption spectrum (obtained by 8 points second order Savitzky-Golay smooth) and the gray line the simulation. For simulation the parameters in Table 4-1 were used and an isotopic line broadening of 2.8 G. Exp. details: ESE, $\pi/2=70$ ns, $\tau=270$ ns, SRT= 6 ms, SPP=100, 74 scans.

Figure 4-5: 263-GHz ESE spectrum obtained from ND$_2$Y$_{731}$• and NH$_2$Y$_{731}$• with a reaction time of 18 s and 2 min, respectively. The red line shows the derivative of the absorption spectrum (obtained by 7 points second order Savitzky-Golay smooth) and gray line the simulation. For simulation the parameters in Table 4-1 were used and an isotopic line broadening of 2.8 G. Exp. details: ESE, $\pi/2(\pi)=48(94)$ ns, $\tau=180$ ns, SRT= 2 ms, SPP=500, 200 scans. Parameter for ND$_2$Y$_{731}$• see Figure 4-4.
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Table 4-1: Parameters of the simulation for 263-GHz and 94-GHz EPR spectra for NH₂Y₇₃₁• and NH₂Y₇₃₀•.

<table>
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<tr>
<th></th>
<th>gₓ</th>
<th>gᵧ</th>
<th>gᵣ</th>
<th>A(Cₓ-Hₓ) [MHz]</th>
<th>Aₓ</th>
<th>Aᵧ</th>
<th>Aᵣ</th>
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<td>NH₂Y₇₃₀•</td>
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<td>2.0042</td>
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<td>29</td>
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<tr>
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<td>2.0040</td>
<td>2.0022</td>
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<td>22</td>
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</thead>
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<td>9</td>
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</tr>
<tr>
<td></td>
<td>NH₂Y₇₃₁•</td>
<td>13</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

a) The ¹⁴N hyperfine tensor of the NH₂Y• was not varied in the simulations and kept Aₓ = 2.4 MHz, Aᵧ = 1.6-5 MHz, Az = 30.7 MHz. Uncertainty in g values is about 0.05 ppt.

4.1.3 Comparing Simulation Parameters from 263 GHz EPR and 94 GHz EPR Spectra

In order to find a unique parameter set the parameters from the 263 GHz simulation and the 94 GHz spectra have to fit to the same parameter set. Therefore, the spectra reported earlier by T. Argirević were simulated again with the new parameters (Table 4-1). The spectra and the simulation are still in agreement with each other as shown in Figure 4-6. The spectrum in Figure 4-6 of NH₂Y₇₃₀• shows an agreement of the simulation based on the modified g values from the 263 GHz measurements. For the transient radical formed at the α interface, NH₂Y₇₁•, two regions indicate differences to the simulation as marked with an asterisk (*). The low field asterisk marks a deviation due to the sharp gᵧ feature (cf. Figure 3-3) of residual Y₁₂₂• and the lower field asterisk shows the resonance frequency of glass peaks observed due to quartz defects in the sample tubes. The impurity of Y₁₂₂• was estimated based on the gₓ feature height to be around 5%.

The consistent set of g values for NH₂Y₇₃₀• and NH₂Y₇₃₁• at 94 and 263 GHz is particularly interesting in its gₓ value. The observed gₓ value shift of 1 ppt is substantial compared to the value of a free NH₂Y•. The narrow line broadening and the high reproducibility up to the minute time scale of the spectra is indicative of a well-defined microenvironment and electrostatic environment. The increase in line broadening from 1 G to 2.5 G at ND₂Y₇₃₁• can arise from a distribution of g values due to flexibilities at this position. Since H bonds are expected as predominant effects on gₓ values, a possible correlation of the relative intensity and strength of HF couplings was examined.²⁴⁴


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Additionally, also 180 GHz data has been discussed by T. Argirević. A new alignment of the data could show consistency (data not shown) with the obtained $g$ values at 263 GHz. 180 GHz were recorded in protonated buffer. This prevents a highly accurate $g$ value determination, which has been observed before in other HF EPR studies in protonated buffer.

4.2 ENDOR Spectroscopy of NH$_2$Y$_{731}^\cdot$ Compared to NH$_2$Y$_{730}^\cdot$ in the α Subunit

4.2.1 ENDOR on NH$_2$Y$_{731}^\cdot$

After the EPR spectrum has identified NH$_2$Y$_{731}^\cdot$ as a single species within the available resolution, it is important to understand the H bond network present at this position. As just shown in the last part, the $g_e$ value is 0.3 ppt lower as compared to NH$_2$Y$_{730}^\cdot$ indicating a higher polarity. In proteins, this polarity is nearly exclusively associated with H bonding. Exchangeable protons are observed as deuteron (D) nuclei (2H) in Mims ENDOR spectra. To disentangle the contributions of different hyperfine couplings the spectrum was measured in a deuterated buffer. In D$_2$O the spectral contributions in 2H ENDOR are reduced to the large amino deuteron couplings (up to ± 2.3 MHz), the very weak couplings...
from the central line (matrix line region up to ±0.3 MHz) and H bond range in between absolute values of 0.4-0.8 MHz (cf. §2.2.2 p.39). The ND$_2$Y$_{731}^•$ ENDOR spectrum was recorded at B$_\parallel g_y$ in Figure 4-7B, and has a similar shape as already observed for ND$_2$Y$_{730}^•$.\textsuperscript{110}

By comparing the $^2$H ENDOR spectra in Figure 4-7 it becomes evident that the sharp feature has a larger resonance position increasing from ±0.62 MHz for NH$_2$Y$_{730}^•$ to ±0.78 MHz for NH$_2$Y$_{731}^•$. The splitting of the lines of this resonance position arises from an additional small coupling, i.e., the quadrupolar coupling, which is larger for ND$_2$Y$_{731}^•$ than observed for ND$_2$Y$_{730}^•$. Previously, two weak H bonds (∼1.8 Å) to ND$_2$Y$_{731}^•$ had been simulated to account for the line shape, as in the ND$_2$Y$_{730}^•$ ENDOR case.\textsuperscript{92} The necessity of both contributions for the sharp feature was spectroscopically not mandatory. Hence, another simulation approach was performed using a single stronger H bond with a larger quadrupole coupling instead of a second H bond. It is worth noting that the simulation is in agreement with the orientation selective data in Figure 4-8 and with the field dependent spectra simulated before (see Figure 4-4). The simulation parameters are collected in Table 4-6 (p. 104). The simulation has a reasonable fit, pointing out that NH$_2$Y$_{731}^•$ has a larger HF coupling contribution with a scalar ($a_{iso}$) contribution (see Figure 2-12 p. 46). This large $a_{iso}$ (i.e., $2\cdot a_{iso}=T_2^\perp$) originates from interaction with the p$_z$ SOMO (Theory §2.1.3). However, this does not lead to an assignment. For the assignment more precise angle dependence and control studies will be performed in the following sections.
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

Figure 4-7: 94-GHz $^2$H Mims ENDOR spectra at $B_0 \parallel g_y$ of trapped NH$_2$Y$\cdot$ intermediates in α. A) Spectrum of ND$_2$Y$_{730}$• at 10 K taken from Argirević et al.$^{92,110}$ The H bond pake patterns assigned to an H bond from Y$_{730}$ (red gradient) and C$_{439}$ (blue gradient) are highlighted below.$^{254}$ B) ND$_2$Y$_{731}$• ENDOR spectrum obtained at 70 K. The red gradient highlights the H bond resonance contribution (assignment on the right side). Exp. details: Mims ENDOR, $\pi/2 = 20$ ns, $\tau = 200$ ns, $\tau_{RF} = 40$ µs, SRT = 10 ms, random RF acquisition$^{279}$ at 1 SPP, acquisition time = 24 h. Excitation in the EPR line was at $B_0 \parallel g_x$. ENDOR spectrum is centered at the Larmor frequency $\nu_0$ of $^2$H, i.e., 21.9 MHz at a field of 3.3 T. C) Simulation of the ND$_2$Y$_{731}$• ENDOR spectrum. Individual contributions are shown in dashed/dotted lines below for the individual assignments of ND$^1$ (blue), ND$^2$ (green) and DO-Y$_{730}$ (red). The simulation is done based on the experimental parameters and parameters in Table 4-2 (p.86) with a line width of 55 kHz including a Mims hole function (Methods §3.4 p.66).$^{172}$

4.2.2 Direction of the H bond Observed at NH$_2$Y$_{731}$•

Evidence for an H bond interaction along the p, SOMO can be found by the orientation dependence of the $^2$H ENDOR resonances through the EPR line. Orientation selective measurements were performed at the canonical orientations of $B_0 \parallel g_{x,y,z}$ as shown in Figure 4-8A. Powder patterns are observed at each orientation, because the ratio between excitation bandwidth (FWHM ≈1.8 mT) and spectral width of (≈8 mT) is large. Still, each orientation shows clear differences in line shape. For NH$_2$Y$_{730}$• an HF tensor for a perpendicular H bond in the form of $|A_z| \geq |A_y| > |A_x|$ (using the definition $|A_x|<|A_y|<|A_z|$) has been reported previously.$^{110}$ This nearly axial tensor shape is also observed here. If the magnetic field is aligned along $g_x$ or $g_y$, the large roughly perpendicular $A_z$ component at
ENDOR Spectroscopy of NH₂Y₇₃₁• Compared to NH₂Y₇₃₀• in the α Subunit

± 0.7 MHz is present. The parallel HF component $A_∥$ is mainly along $g_z$ with roughly ±0.6 MHz. This already implies, that the isotropic HF has half the size but opposite sign with respect to the parallel component $(-2|d_{iso}| \approx |T_∥|$; cf. Figure 2-12, p.46). The spectra have different resonances of the H bond HF tensor in the upper and the middle spectrum of Figure 4-8A with ±0.7 and ±0.8 MHz, thus the HF tensor is slightly rhombic. It has been reported before that an H bond††, perpendicular to the tyrosine π plane has its smallest resonance along the H bond direction. Thus the H bond lies roughly along the $g_z$ molecular direction, consistent with the previous assignment based on the tensor shape. Simulations including the simulation of the EPR spectrum and excitation bandwidth were performed, as illustrated in Figure 4-8B. The simulation demonstrated that the HF tensor must nearly align with the $g$ tensor in order to reassemble three different resonances at $B_∥g_x, g_y, g_z$. Therefore, the angle between HF tensor and the $g$ tensor were not allowed to increase to more than ≈20°. Otherwise the contributions of the HF tensor were mixed at $B_∥g_x, g_y, g_z$ and the resonances were identical at each observed orientation. A preliminary fit was obtained with H bond dihedral angle (cf. Figure 4-8C) of 90° from the phenol plane. This would be in agreement with the C₅-C₄-OY₇₃₁-OY₇₃₀ dihedral of 90° found in the wt structure (2X0X). The early simulations were therefore simulating the H bond contributions with collinear principal axes of the HF and $g$ tensor. The structural restraints, however, do not fit to such a 90° perpendicular angle nor could they explain the rhombicity of the HF tensor. The interplay between a possible π stacking interaction and the H bond with orbital overlap introduce structural restraints. π Stacking in a di-tyrosine peptide would have an optimized H bond dihedral angle 50-60°, as has been reported by Siegbahn et al. without considering a protein environment. Here an H bond HF tensor with an angle $\angle(A_∥, g_z)$ equals 70° in Figure 4-8C reproduced the experimental spectrum. This is consistent with an H bond dihedral with the same 70° angle.

The small splitting on top of the resonances at ±0.6-0.8 MHz is assigned to the quadrupole interaction. As a function of the electric field gradient the largest value (defined $Q_z$) of the quadrupole interaction is along the H-X (X= O, N) bond of the H bond donor function. The splitting has its largest contribution (250 kHz) along $B_∥g_z$ indicating a nearly collinear quadrupole and HF tensor (i.e., $A_∥Q_z$). For this type of interaction the direct orbital overlap

†† The H bond at NH₂Y₇₃₁• has the assigned H bond donor Y₇₃₀-O-H.
with the \( p_z \) orbital does not lead to strong quadrupole contributions. This is in agreement with other H bonds observed in previous work\textsuperscript{110} and the DFT work shown in §4.4. An out-of-plane H bond has been mentioned before as a prerequisite of an effective HAT step in \( \pi \) radical as tyrosines.\textsuperscript{107} Therefore and due to the proximity of Y\textsubscript{731} and Y\textsubscript{730} in most crystal structures\textsuperscript{22,120} this interaction is assigned to the proton of Y\textsubscript{730}. The analog assignment in NH\textsubscript{2}Y\textsubscript{730}\textsuperscript{*} has been made for the moderate perpendicular H bond previously (§1.5.3, p.21). Additional spectroscopic evidence should be obtained by double mutant ENDOR measurements in §4.3.5 p. 95. At first these mutants have to be characterized (§4.3).

Figure 4-8: Orientation selective \(^{2}{\text{H}}\) Mims ENDOR spectrum of ND\textsubscript{2}Y\textsubscript{731}• in the ±1.5 MHz region from T. Argirević\textsuperscript{92}. A) Simulations of the spectra (red -) take into account ND\textsubscript{2} and H bond HF coupling, discussed so far (see Table 4-2). Contributions from the H bond are shown separately as red peaks. Field positions and excitation bandwidth (green, blue and red) for the individual measurements are illustrated in B (cf. Figure 4-6). C) The orientation of the H bond tensor is illustrated in the molecular frame. A line broadening of 50 kHz was used in the simulation. Exp. details: \( T=10 \) K, \( \pi/2=20 \) ns, \( \tau=320 \) ns, \( \pi_{RF}=40 \mu s \), SRT = 150 ms, random RF acquisition\textsuperscript{279}, acquisition time = 50 h/spectrum.
ENDOR Spectroscopy of NH₂Y₇₃₁• Compared to NH₂Y₇₃₀• in the α Subunit

Table 4-2: Simulation parameters for the ²H ENDOR spectra of ND₂Y₇₃¹•.

<table>
<thead>
<tr>
<th>NH₂Y₇₃¹•</th>
<th>Aₓ [MHz]</th>
<th>Aᵧ [MHz]</th>
<th>Aₗ [MHz]</th>
<th>α [°]</th>
<th>β [°]</th>
<th>γ [°]</th>
<th>Qₓ [MHz]</th>
<th>Qᵧ [MHz]</th>
<th>Qₗ [MHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y₇₃₀-OD</td>
<td>1.3</td>
<td>-1.43</td>
<td>-1.63</td>
<td>-160</td>
<td>110</td>
<td>80</td>
<td>-0.03</td>
<td>-0.09</td>
<td>0.12</td>
</tr>
<tr>
<td>ND₂ D(1)</td>
<td>-0.6</td>
<td>-2.9</td>
<td>-3.8</td>
<td>-86</td>
<td>98</td>
<td>90</td>
<td>-0.04</td>
<td>-0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>ND₂ D(2)</td>
<td>0.06</td>
<td>-3.1</td>
<td>-4.2</td>
<td>-96</td>
<td>93</td>
<td>-31</td>
<td>-0.06</td>
<td>-0.08</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The central matrix line has not yet been discussed. Here weak coupled deuterium nuclei are present. In the 10 K spectrum in Figure 4-8 also the HF couplings of Y₁₂₂• contribute to this matrix line. Y₁₂₂• has been shown to be only weakly coupled (± 0.25 MHz) to one proton.¹⁵²

To separate the effects a measurement was repeated at 70 K as shown in the Appendix Figure A - 4 (p.195). Compared to the previous work from T. Argirević⁹², still no HF coupling pattern was resolved, indicating at least one additional contribution in the line shape which is not present in the resolved central line at ND₂Y₇₃₀•. However, one can mention that the largest resonance of the matrix line is present at B₀∥gₓ and gᵧ with ±0.2 MHz and at B₀∥gₗ, it is ±0.15 MHz. At B₀∥gₗ, another feature is visible at about ±0.1 MHz. Under the assumption of a purely dipolar HF tensor this would be consistent with a dipolar tensor with diagonal elements of -0.1, -0.1 and 0.2 MHz. However, contrary to the previous interpretation for a moderate perpendicular H bond, in a purely dipolar tensor the parallel tensor component Tₚ is the largest value and along the H bond direction (cf. Figure 2-12).⁹²

Thus, an H bond in the gₓgᵧ plane would be indicated by the spectra observed. An analogous conclusion applies if the 0.1 MHz splitting resembles a quadrupolar splitting of the perpendicular HF tensor component. Then the H bond donor proton bond is within the ring plane ±40° (H bond dihedral, cf. Figure 4-8C). With a point-dipole approximation (Eq. (2-17), Theory §2.1.3, p. 33) the two cases resemble a distance from the phenoxy oxygen nuclei of 2.9 Å or 2.5 Å for an observed HF or quadrupole splitting, respectively. An oxygen spin density population of 0.21 was used in Eq. (2-17).¹¹⁰
4.3 Removal of one H Bond Partner by Double Mutants

4.3.1 The Concept of the Double Mutant Study

In an effort to assign the H bonds donor to $\alpha_2$-$\text{NH}_2\text{Y}_{730}^\bullet$ and $\alpha_2$-$\text{NH}_2\text{Y}_{731}^\bullet$ double mutants $\alpha_2$-$\text{NH}_2\text{Y}_{730}/\text{C}_{439}\text{A}$ and $\alpha_2$-$\text{NH}_2\text{Y}_{731}/\text{Y}_{730}\text{F}$ were expressed. It has already been shown that the removal of one PCET pathway amino acid renders the protein inactive (§1.4.1.2 p. 7). The 3-amino tyrosines show still residual activity. Therefore it was additionally interesting how the modification of the direct environment would change the individual radical intermediate. We proposed a structured H bond network at $\text{NH}_2\text{Y}_{730}^\bullet$ (§1.5.3, p.21) and $\text{NH}_2\text{Y}_{731}^\bullet$ (§4.2). Therefore the question arose if the absence of our assigned H bonds is detectable. Up to now our assignment was mainly based on proximities observed between the three amino acids $\text{Y}_{731}$-$\text{Y}_{730}$-$\text{C}_{439}$ in the inactive and reduced crystal structures. Here we hoped to see effects supporting our current assignment both kinetically and structurally. Scheme 1 explains the mutation strategy. The biochemical work and the SF-vis experiments were performed by Wankyu Lee, from the Stubbe lab, at MIT. It is reported here to examine the effect of the local perturbation due to the double mutation.

Scheme 1: Mutation strategy involves removal of an individual H bond for $\text{NH}_2\text{Y}_{731}^\bullet$ (A) and $\text{NH}_2\text{Y}_{730}^\bullet$ (B). The amino acid subsequent in PCET pathway was exchanged by site directed mutagenesis into one without an H bond donor function as phenylalanine and alanine.
4.3.2 Kinetic Characterization of NH$_2$Y$_{731}$/$Y_{730}$F and NH$_2$Y$_{730}$/C$_{439}$A

The radicals formed in the double mutants NH$_2$Y$_{731}$/Y$_{730}$F and NH$_2$Y$_{730}$/C$_{439}$A were characterized by SF-vis measurements observing the decay of Y$_{122}•$ at 410 nm and the formation of NH$_2$Y• at 320 or 325 nm with wt-β$_2$/CDP/ATP in assay buffer (Figure 4-9). The results are summarized in Table 4-3.

![Figure 4-9: Kinetics of NH$_2$Y• formation in α$_2$-NH$_2$Y$_{731}$/Y$_{730}$F:β$_2$-wt (A) or α$_2$-NH$_2$Y$_{730}$/C$_{439}$A:β$_2$-wt (B) with CDP and ATP by SF Vis spectroscopy. Double exponential fits in A or mono exponential fits in B to the data are shown in black. Residuals for the fit for NH$_2$Y• formation is in magenta for Y• disappearance is in cyan. The results represent the average of 6 to 8 spectra and fits were calculated with OriginPro software to minimize residuals (Table 4-3). This figure is cited from ref. 254.](image)

The yield of NH$_2$Y$_{731}$/Y$_{730}$F with 34±3% is identical within error to the yield of the corresponding single mutant (32±3%). However, the rate constants are both slower, a factor of 6 is found for the fast rate constant and a factor of about 3 for the slow rate constant.

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>$k_1$ (s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$%A_1$</th>
<th>$%A_2$</th>
<th>$%NH_2Y•$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α$_2$-NH$<em>2$Y$</em>{731}•$</td>
<td>9.6 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>27 ± 2</td>
<td>13 ± 1</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>α$_2$-NH$<em>2$Y$</em>{730}•$</td>
<td>12 ± 1</td>
<td>2.4 ± 0.2</td>
<td>20 ± 2</td>
<td>19 ± 2</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>α$<em>2$-NH$<em>2$Y$</em>{731}•$/Y$</em>{730}$F</td>
<td>1.5 ± 0.1</td>
<td>0.3 ± 0.03</td>
<td>14 ± 2</td>
<td>20 ± 1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>α$<em>2$-NH$<em>2$Y$</em>{730}•$/C$</em>{439}$A</td>
<td>0.13 ± 0.01</td>
<td>-</td>
<td>14 ± 1</td>
<td>-</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

a) Rates were obtained from double exponential fits of 6-8 spectra of SF UV-vis spectra of the reaction with 5 μM α$_2$-NH$_2$Y$_{731}$ and 5 μM wt-β$_2$ with CDP/ATP (1 mM/3 mM) in assay buffer. The rate constants for NH$_2$Y$_{730}$ and NH$_2$Y$_{731}$-α$_2$ have been reported previously. 280

The NH$_2$Y$_{730}•$/C$_{439}$A shows a reduction in overall yield by a factor of two and the rate constant of NH$_2$Y$_{731}•$/Y$_{730}$F is 10-fold diminished. Here only one rate constant was sufficient
to fit the kinetic data. The effect of C_{439}A mutation is quite large in terms of radical build up. This implies an effect on the PCET efficiency. Therefore another mutation was tested. Unfortunately α_{2}-NH_{2}Y_{730}/C_{439}S could not be successfully expressed.

4.3.3 Structural Comparison of NH_{2}Y_{731•}/Y_{730}F and NH_{2}Y_{730•}/C_{439}A with Their Single Mutants

In order to exclude larger structural rearrangements in the α_{2}/β_{2} complex of either NH_{2}Y_{731•} or NH_{2}Y_{730•}, the diagonal distance to Y_{122•} was measured for both double mutants. After the PCET reaction 3-amino tyrosine radical and the stable Y_{122•} are located in diagonally opposite monomers of α and β (Figure 1-8, p. 12). Distances between radicals pairs can be measured at X (9 Ghz) and Q-band (34 Ghz) by pulsed electron double resonance (PELDOR/DEER) spectroscopy. The reaction of the αβ complex (final concentration: 130 µM) was performed with the same substrate and effector mixture, additional glycerol was added at 20 s and 25 s, for NH_{2}Y_{731}/Y_{730}F and NH_{2}Y_{730}/C_{439}A, respectively. Glycerol is not only a versatile cryoprotectant, but it prolongs T_{2} relaxation as well as deuterium exchange. For PELDOR spectroscopy long T_{2} relaxation times in the order of several µs are required. The reaction was quenched at 40 s (NH_{2}Y_{731}/Y_{730}F) or 1 min (NH_{2}Y_{730}/C_{439}A) in ice-cold 2-methylbutan (~113 K) to insure a good heat conductivity for the larger Q band tubes.

4.3.3.1 Diagonal Distance from NH_{2}Y_{731•}/Y_{730}F to Y_{122•}

The diagonal distance can be measured directly, if the whole spectrum is excited by the pump and detect pulses as common at X band. However, concentration sensitivity increases with the MW frequency, thus the measurement at Q band (34 GHz) is more sensitive. Isabel Bejenke from our group demonstrated that complete distance distributions at Q band require orientation averaging. With the power of 2W five field points on the NH_{2}Y_{730•}-Y_{122•} radical pair would be necessary. However, the distance did not change at different field points. Thus if the distance is observed and it does not represent a double frequency (the parallel part of a pake pattern, see Figure 2-12), the distance is robust.

In Figure 4-10 the ESE-field sweep spectrum at 34 GHz shows NH_{2}Y_{731•} with a yield of 28%. Three pump and detect frequencies were set up covering the spectral width of the NH_{2}Y_{731•}. A dipolar oscillation was obtained from each detection position after
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procession and analysis of the data (Figure 4-10B). By adding the normalized spectra a dipolar oscillation comparable to earlier X band studies was obtained.

Figure 4-10: 34-GHz distance measurements between NH$_2$Y$_{731}$• and Y$_{122}$• in the α$_2$-NH$_2$Y$_{731}$/Y$_{730}$ F double mutant at 5 K. (A) The ESE spectrum of unreacted Y$_{122}$• (blue) has been subtracted from the observed spectrum (purple), yielding the NH$_2$Y• (28%) spectrum (red). Pump (P, π = 46 ns) and detect (D, π = 56 ns) pulses were separated by 55 MHz and are indicated by arrows and shifted stepwise (ΔB = 11 G) over the EPR line. (B) The three consecutive (1,2,3) four-pulse DEER traces were normalized and background corrected. C) Average trace as sum of the three normalized traces. The red line describes a fit using DeerAnalysis$^{286}$ and Tikhonov regularization$^{287}$ procedure. D) Distance distribution obtained from the analysis in (C). The measured distance distribution shows 3.84±0.15 nm as main distance.$^{254}$
Fitting of the time domain trace including a Tikhonov regularization procedure a distance distribution was obtained as shown in Figure 4-10D (DeerAnalysis 2013). The distance distribution of 3.84±0.15 nm is identical to the distance distribution observed previously for the single mutant with 3.81±0.12 nm.

4.3.3.2 Diagonal Distance from NH$_2$Y$_{730}$•/C$_{439}$A to Y$_{122}$•

In another set of experiments the NH$_2$Y$_{730}$•/C$_{439}$A distance to Y$_{122}$• was tested by the same procedure, as shown in Figure 4-10. The measurement was carried out by I. Bejenke in our group. NH$_2$Y$_{730}$• yielded 17%, compared to Y$_{122}$• (Figure 4-11A) in agreement with the low yield observed in SF-vis measurements (§4.3.2).

Processing procedure of the three measured field points was performed with DeerAnalysis. A distance distribution from an incomplete powder average results (Figure 4-11D). Two components with 3.9 nm and 3.3 nm are prominent, which arise from NH$_2$Y$_{730}$•-Y$_{122}$• and Y$_{122}$•-Y$_{122}$• distances, respectively. The ratio is not as expected from the last dataset (Figure 4-10D). Here the 3.9 nm distant is the minor contribution. Reasons could be the lower yield, unfavorable orientation selection and the first detection (D1, Figure 4-11A) outside the spectral width of NH$_2$Y$_{730}$• the contribution of the longer distance (3.9 nm). However, compared to the single mutant study the 3.9 nm distance probability (cf. Figure 4-10D) decreases from NH$_2$Y$_{731}$• to NH$_2$Y$_{730}$• as well. In order to support the long distance, the effect of suppressing the long distance is shown in B2 and C in green. The RMSD in C (B2) is changing from 0.0025(0.0016) to 0.0036(0.0023) for the blue and green fit, respectively.

The distance measurements performed here provide evidence against global structural distortion introduced into the active enzyme for the observed NH$_2$Y$_{730}$• intermediate by blocking the radical transfer. However, without a resolved dipolar oscillation (Figure 4-10B) with a frequency consistent to the long distance the assignment for NH$_2$Y$_{730}$•/C$_{439}$A is questionable. Overall one can state that the longer distance observed in the single mutants was found as well in the double mutants.

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This includes weighting of the smoothing effect versus the RMSD.
Removal of one H Bond Partner by Double Mutants

Figure 4-11: 34-GHz distance measurements between NH$_2$Y$_{730}$$^•$ and Y$_{122}$$^•$ in the α$_2$-NH$_2$Y$_{730}$/C$_{439}$A double mutant. (A) The composite EPR spectrum at 5 K (purple) followed by subtraction of the Y$_{122}$$^•$ (blue) gives the NH$_2$Y$_{730}$$^•$ spectrum (red) in 13% yield. (B) The DEER traces at 20 K were taken with detection pulses (π/2(π) = 20(40) ns) separated by 50 MHz from the pump pulse (π = 56 ns). (B) Three consecutive traces (1, 2, 3) were measured at D1, D2 and D3 (see A), respectively, with 11 G spacing. (C) The averaged traces were summed and fit by DeerAnalysis$^{286}$ using Tikhonov regularization.\textsuperscript{287} (D) In the distance distribution the distance of 3.29 ± 0.15 nm and 3.9 ± 0.1 nm is shown. B and C) In green the frequency suppressing distances larger than 3.45 nm is shown.

4.3.4 Electrostatic Environment and Conformeric State: Comparison of the Radicals Formed in Double Mutants and Single Mutants

The electronic structure reflected in the g-factor can be studied for NH$_2$Y$_{731}$$^•$/Y$_{730}$F and NH$_2$Y$_{730}$$^•$/C$_{439}$A. To compare the results with the single mutants and estimate also external effects like H bonding high-field EPR spectra were recorded and compared as shown in Figure 4-12. The spectra of the double mutants reveal the same coupling pattern for B$_0$ along g$_y$ and g$_z$. Along g$_x$, all features are broader than observed in the single mutants. This is typical for a distribution of electrostatic environments around the oxygen of the phenyl ring. The
observed $g$ shift is here 0.3-0.4 ppt. NH$_2$Y$_{730}/C_{439}$A shows a slightly smaller $\beta$-methylene coupling at $g_x$ ($\approx$24 MHz) the couplings at $g_y$ are identical with approximately 32 MHz to the value of the single mutant. The coupling obtained from the spectra for $g_z$ is 38 MHz.

The ND$_2$Y$_{731}$ double mutant (orange spectrum) compared to the single mutant (red spectrum) show an increase in dominant C$-\beta$ HF coupling ($A_{iso} \approx$26 MHz, Table 4-1 p.80). In order to disentangle the spectral contributions from $g$ value and HF couplings further 263 GHz spectra were recorded as shown in Figure 4-13. Low signal intensity and yield (15%) of NH$_2$Y$_{730}/C_{439}$A at 70 K prevented an artifact free echo detected spectrum. Another signal is marked as an artifact with an asterisk (*). The stimulated echo sequence was used to compensate the weak signal, thus a shorter $T_m$ filtering has been used. Thus in part the signal could arise due to the Y$_{122}^*$ signal. From 94 GHz spectra $g_x$ was located in the center of the doublet splitting (38 MHz) at the low field side. The obtained parameters are summarized in Table 4-4.

The spectrum of NH$_2$Y$_{731}/Y_{730}$F (gray spectrum, Figure 4-13) displays a distinct triplet at the low field side of the 263 GHz spectrum. Compared to the single mutant (red spectrum), the dominant C$-\beta$ coupling increases ($A_{iso} \approx$ 26 MHz, Table 4-1 p.80). C$-\beta$ couplings are the only larger coupling contributions in these spectra, because the amino protons have been exchanged to deuterons. However, this HF coupling in combination with a weaker coupling to the second C$-\beta$ proton ($A_{iso} < 26$ MHz) is not sufficient to generate the large triplet splitting (peak separation of ~ 1.1 and 1.4 mT). Thus the splitting is assigned to a second component. Two different $g_x$ values, but similar $g_y$ and $g_z$ values are typical for a second component with a different electrostatic environment. The individual parts of these two components are demonstrated via simulation of a larger (red) and smaller (blue) $g_x$ value component. The weighted sum simulation (gray --) supports of the two component interpretation with a weight of 45% to 55% for red and blue, respectively (Table 4-4). These two contributions cannot be resolved at 94 GHz, where the spectral region around $g_x$ shows only broadening (area marked in Figure 4-12). The results indicate that, as expected, removal of the H bond to Y$_{730}$ perturbs the electrostatic environment at NH$_2$Y$_{731}$ and destabilizes the radical. The broadening observed at 94 GHz originates from two distinct environments. Thus no Gaussian distribution of H bond interactions is present, a prerequisite for a further ENDOR investigation. The simulation demonstrated a good fit to the corresponding 94 GHz spectrum, as shown in the Figure 4-12.
Removal of one H Bond Partner by Double Mutants

Figure 4-12: 94-GHz ESE spectra of NH₂Y₇₃₀•/C₄₃₉A and NH₂Y₇₃₁•/Y₇₃₀F compared to the corresponding single mutants. The spectra show NH₂Y₇₃₀•/C₄₃₉A (gray) and NH₂Y₇₃₁•/Y₇₃₀F (orange) together with NH₂Y₇₃₀• (black) and NH₂Y₇₃₁• (red) in D₂O exchanged buffer. The shift of \( g_x \) is marked in black. The simulation (orange, --) uses the parameters of Table 4-4. Exp. details: ESE, \( T=10 \text{ K} \), \( \pi/2 = 18 \text{ ns} \); \( \tau = 260 \text{ ns} \); SRT = 6 ms; SPP = 100; scans = 15-110. To build the derivative spectra a 5 point, second order Savitzky-Golay filter was used.

Table 4-4: Parameters obtained from the double mutant NH₂Y₇₃₀•/C₄₃₉A and NH₂Y₇₃₁•/Y₇₃₀F EPR spectra and simulation parameters of Figure 4-12 & Figure 4-13.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( g_x )</th>
<th>( g_y )</th>
<th>( g_z )</th>
<th>( A \text{ strain} )</th>
<th>( A \text{ [MHz]} )</th>
</tr>
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<td>2.0022</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>A(H₂) -</td>
<td>-</td>
</tr>
<tr>
<td>NH₂Y₇₃₁•/Y₇₃₀F</td>
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<td>2.0042</td>
<td>2.0022</td>
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<td>28</td>
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<td></td>
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<td>A(H₂) 6</td>
<td>4</td>
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<td>2.0042</td>
<td>2.0022</td>
<td>0.15 ppt</td>
<td>34</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A(C-β-H₁) 34</td>
<td>6</td>
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3-Amino Tyrosine Radical Intermediates
4.3.5 Assignment of the H Bond Donors by ENDOR Spectra of the Double Mutants.

The high-field EPR spectra are partially consistent with a loss of a hydrogen bond. Thus it is interesting which \(^2\)H nuclei can be detected with \(^2\)H Mims ENDOR spectroscopy. Therefore, ENDOR spectra were recorded for the samples with the highest yield of 33\% at 20 s and 14\% at 30 s for \(\text{NH}_2\text{Y}_{731}\cdot /\text{Y}_{730}\text{F}\) and \(\text{NH}_2\text{Y}_{730}\cdot /\text{C}_{439}\text{A}\), respectively. The \(^2\)H Mims ENDOR spectra at \(g_y\) are displayed in Figure 4-14, using deuterated assay buffer and a \(\tau\) value of 200 ns. They are directly compared with their single mutant counterparts described in §4.2.1. \(^2\)H ENDOR spectra of both double mutants showed an astonishingly decrease of sharp peaks at ± 0.6-0.8 MHz. No sharp features are observed in \(\text{NH}_2\text{Y}_{731}\cdot /\text{Y}_{730}\text{F}\), whereas a loss of ~ 70\% intensity compared to the single mutant is apparent in \(\text{NH}_2\text{Y}_{730}\cdot /\text{C}_{439}\text{A}\)
Removal of one H Bond Partner by Double Mutants

Notably, this is in agreement with the assignment made before (§4.2 p.81) and previously (§1.5.3, p.21).\textsuperscript{110,254}

Figure 4-14: \textsuperscript{2}H Mims ENDOR spectrum of the double mutants NH\textsubscript{2}Y\textsubscript{730}\textsuperscript{*}/C\textsubscript{439}A and NH\textsubscript{2}Y\textsubscript{731}\textsuperscript{*}/Y\textsubscript{730}F (top) compared to its corresponding single mutant spectrum (bottom). A) The simulated tensor shape assigned to the H bond of the DO-Y\textsubscript{731} is shown in red. The resonance assigned to an H bond of D-C\textsubscript{439} has a blue gradient. B) No sharp feature was detected in the NH\textsubscript{2}Y\textsubscript{731}\textsuperscript{*}/Y\textsubscript{730}F, in agreement with the loss of a perpendicular H bond. Exp. details: T = 70 K, $\pi/2 = 20$ ns, $\tau = 200$ ns, $\pi_{RF} = 40$ $\mu$s, SRT = 5 ms, random RF acquisition\textsuperscript{279} at 1 shot/point, acquisition time is 24 h (green) 50 h (gray).

Thus NH\textsubscript{2}Y\textsubscript{730}\textsuperscript{*} harbors two perpendicular H bonds and NH\textsubscript{2}Y\textsubscript{731}\textsuperscript{*} has one strong to moderate perpendicular H bond donor, Y\textsubscript{730}. The broad range of the amino deuterons shows, furthermore, slight changes for NH\textsubscript{2}Y\textsubscript{730}\textsuperscript{*}, the double mutant has a 10% smaller HF coupling to the ND\textsubscript{2} deuteron, whereas the other coupling stays in the same range as the single mutant. At NH\textsubscript{2}Y\textsubscript{731}\textsuperscript{*} both amino deuterons have a smaller coupling (~20%). This could indicate a slightly changed distribution of spin density over the ring or a change in direct intermolecular interaction at the amino group. Nevertheless, both contributions can be simulated using a simply scaled tensor of the corresponding single mutant (data not shown).

Interestingly, the inner coupling range is also changing for NH\textsubscript{2}Y\textsubscript{731}\textsuperscript{*}, out of the unstructured matrix line a clear pattern emerges. It could be a small axial part of a powder pattern. Then a dipolar tensor with values of $T_1 = -0.3$ MHz and $T_2 = 0.6$ MHz would be present. Otherwise a larger resonance contribution with 1.2 MHz is possibly overlaying with
the amino deuteron tensor resonances. This is in agreement with a low $g_x$ value observed for 55% contribution of the NH$_2$Y$_{731}\cdot$/Y$_{730}$F. In contrast the inner coupling of NH$_2$Y$_{730}\cdot$, which shows a tensor in agreement with distal water, does not change. The NH$_2$Y$_{731}\cdot$ double mutant located near the interface could compensate the loss of an H bond, which could explain good yield and the in part small $g$ shift observed. Therefore it was interesting to observe from which direction this new contribution could arise from.

**4.3.5.1 Orientation Selective ENDOR from NH$_2$Y$_{731}\cdot$/Y$_{730}$F**

A set of $^2$H ENDOR experiments along all canonical orientations of the $g$ tensor was performed. As we have seen before only three deuterium nuclei are present in this system two from the ND$_2$ group and one from a here better resolved matrix line. The point of interest here was the inner coupling range and the orientation of a distant $^2$H coupling. For $B_0$ oriented along $g_x$, the features of the inner line get a single crystal like sharpness. The features get broader at $B_0\parallel g_y$ and at $B_0\parallel g_z$ the inner coupling has an overlap with resonances origin from the two amino deuterons. Section§5.3.4 (p.142) will show that in plane $^2$H HF couplings have quadrupole splitting in the order of $\approx0.15$ MHz. The splitting observed at $g_z$ could be representative for an axial HF ($T_\perp \approx 0.22$ MHz) including a quadrupole splitting of 150 kHz. Due to orientation selection no parallel HF tensor component for $B_0\parallel g_x$ is visible for an H bond collinear ($\pm30^\circ$) to the $g_y$ plane.$^{55, 155}$ A moderate H bond consistent to such a coupling ($2.2\,\text{Å}$, Eq. (2-17)), would be sufficient to explain the difference in $g$ value between the two environments. Without better resolved spectra (Figure 4-15), a simulation of these spectra would be speculative at this point.
Comparing Structural Models from DFT with EPR Parameters

At the beginning of this project, the overall idea was not only to measure EPR and ENDOR spectra but, ambitious as it sounds, to obtain a structural model of the radical state. Based on T. Argirević’s EPR and Riplinger’s DFT work (Introduction §1.5.3, p.21), a suitable model for NH2Y731• in agreement with the EPR results of the previous sections should be found. This time the large models (>200 atoms), used for transition state calculations previously,\(^\text{110}\) could be augmented by an NH2 group on Y731.

The geometry optimization was performed by S. Kossmann in F. Neese’s lab. An unrestricted gradient functional BP86 was employed with dispersion correction and a triple zeta $\zeta$ functional operating in the basis set limit (Methods §3.6, p.71). Three models were studied with one, zero and two water molecules named Model 1, 2 and 3, respectively (Figure 4-16, p.100). The models were originally based on the inactive crystal structures\(^67\) and took all known amino acids within 5 Å around the NH2Y731 into account. In the optimized models the distance to the phenol H bond donor Y730 varies from 2.7 Å in Model 1 to 2.6 Å in Models 2 and 3 (Figure 2-16).
Nonetheless, all optimized models revealed a decreased O\textsubscript{730}-O\textsubscript{732} distance of 0.9 to 0.5 Å compared to the crystal structures (Figure 4-17). In the absence of a water molecule (Model 2) α-R411 approaches the NH\textsubscript{2}Y\textsubscript{731∗}. The guanidinium group comes here within H bond distance R(N\textsubscript{411}-O\textsubscript{731}) ≥ 2.9 Å, where the closest encounter found in crystal structures is 3.6 Å (cf. Figure 2-16). The O\textsubscript{wat2}-O\textsubscript{731} distance, of a second water, considered in Model 3 is with 2.8 Å comparable to the distances (R\textsubscript{O-O} ≈2.6-3.6 Å) observed in the vicinity of some X-ray structures of α-wt and α-NH\textsubscript{2}Y\textsubscript{731/730} (see Figure 2-16). Despite the second H bond having a distance of R\textsubscript{O-H} ≈1.9 Å, the stronger perpendicular H bond to Y\textsubscript{730} remains.

Both interactions described by Model 2 and 3, find precedents in the literature. Common π-cationic interactions has been described for aromatic amino acids like tyrosine to amino acids like arginine.\textsuperscript{288,289} A arginine next to a tyrosine revealed a reduction in redox potential in small peptide model studies.\textsuperscript{290} Strong H bond networks, including two water molecules, have also been proposed in PS II to have a strong effect on g values and an activation role for Y\textsubscript{z}’s high activity.\textsuperscript{156,291}

In our models, the g values and the HF interaction were calculated using B3LYP and EPRII as functional and basis set (§2.3 p.47). The gauge origin of the g tensor had to be laid into the radical (fixed at C4) to reduce gauge-dependent errors. The main two variables in the 3-amino tyrosines extracted from the high-field EPR spectra are the g values and the β-methylene couplings that are tabulated in Table 4-5. For the g values we see that within the uncertainty of the DFT of 0.5 ppt all values are consistent with the experimental g values. Within the models g\textsubscript{z} is not varying significantly and g\textsubscript{x} follows the trend of g\textsubscript{x} between Model 1 and 2 to a smaller extent as expected by g value theory (Theory §2.1.2, p.31).
Comparing Structural Models from DFT with EPR Parameters

Figure 4-16: Central part of the DFT geometry optimized NH$_2$Y$_{731}$• models. The models are based on the models used for the energy barrier calculation (Introduction §1.5.3, p.21) of the Y$_{732}$-Y$_{731}$-C$_{439}$ triad. In gold the residues directly affecting the model are highlighted. Model 1 contains the water molecule wat1, Model 2 has no water molecule, and Model 3 considers an additional water (wat2) close to NH$_2$Y$_{731}$•. The complete models are depicted in Figure A-5 (p.196).

Figure 4-17: X-ray structures including water molecules in a subunit. Crystal structures of: A α-wild-type, PDB ID 2X0X, molecule C, 2.3 Å resolution; (B) α-NH$_2$Y$_{730}$ PDB ID 2X04, molecule B, 2.7 Å resolution; (C) α-NH$_2$Y$_{731}$ PDB ID 2X05, molecule C, 2.5 Å resolution. Only water oxygen atoms (red spheres), which are near to the phenolic oxygen (≤ 5.5 Å) of the tyrosines, are displayed. The distances are given in Å.
Table 4-5: Summary of g values and C-β HF couplings of NH₂Y• at residues 730, 731, 356. The values were obtained from combined simulations of the 263 and 94 GHz spectra and compared with those obtained from DFT calculations. The ¹⁵N hyperfine tensor of the NH₂Y• was not varied in the simulations and kept \( A_x = 2.4 \text{ MHz}, A_y = 1.6-5 \text{ MHz}, A_z = 30.7 \text{ MHz}. {134} \) Uncertainty in g values is about 0.05 ppt for the experiments and 0.5 ppt for DFT calculations. Uncertainty in HF couplings is up to 10% from spectral simulations and up to 20% in DFT calculations.

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<th></th>
<th>( g_x )</th>
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<th>( g_z )</th>
<th>( A_{\text{iso}}(\text{C-β}) ) [MHz]</th>
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<tr>
<td>NH₂Y₇₃₀•</td>
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<td>2.0040</td>
<td>2.0022</td>
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<td>2.0041</td>
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<td>2.00415</td>
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<td>26</td>
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<tr>
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<td></td>
<td></td>
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<td></td>
</tr>
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<td>2.0042</td>
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* Value reported ref. \(^{134}\).* ‡ value from 2-amino-4-methyl-phenol radical.\(^{110}\)

To explain the dependence of the \( g_x \) values for the three models, one has to take a close look at the H bond interactions of the different models. It seems that weak (2.1 Å) to moderate (1.9 Å) H bonds make an effect of 0.4-0.5 ppt if they act together with a moderate H bond perpendicular oriented to the ring system. This is consistent with small model studies (Figure A - 6, p.197). It is worth mentioning that Model 2 and 3 lie closer to the observed experimental values. The β-methylene is best captured by Model 3. This model has a dihedral \( \theta_{\text{Cβ}} \) of -47.2°. As shown in Figure 4-18, this will decrease the second beta methylene coupling (H₁β) to nearly zero with \( a_{\text{iso}}(\text{H}_1\beta) \approx 3-4 \text{ MHz} \) (Model 1 & 3). Within these models \( a_{\text{iso}}(\text{H}_1\beta) \) cannot cause the HF interaction observed as a triplet at \( g_x \). The next largest HF coupling is from the C₆-H with \( A_{\text{C₆-H}} = -9, 1, -13 \text{ MHz} \) (Model 3), therefore we assigned this coupling tentatively to \( A(\text{C₆-H}) \). This is a structural restrain, which could be considered in future studies, defined as in Figure 4-18. Since the \( \theta_{\text{Cβ}} \) angle is connected to the possible coupling sizes, for this angle only one dominant β-methylene ¹H coupling is expected. It should be mentioned, that slight sterical changes from unconsidered interactions to the β subunit could introduce changes in the considered models.
Comparing Structural Models from DFT with EPR Parameters

Figure 4-18: Ring dihedrals $\theta_{C\beta}$ of the crystal structure (green) compared to models 1, 2 (black) and 3 (blue) considered for NH$_2$Y$_{731}^•$ and Y$_{731}^•$ (PDB IDs: 2XOX, 2XO5 and 2XO4). The figure explains the observation, that both C-$\beta$ couplings become smaller, by an increase in $\theta_{C\beta}$.

In order to answer which DFT models are in agreement with the $^3$H ENDOR results Table 4-6 summarizes the acquired values. Direct comparison of Models 1 and 3 are in a good agreement to the experimental ENDOR work. However, all depicted models show the effect of a strong H bond toward Y$_{730}$ nearly perpendicular to the ring plane. Uncertainty can be up to 20% for the DFT values and unique Euler angle sets are hardly found for orientation selective ENDOR, increasing the error to about 10%. Thus within the uncertainty only the coupling sizes of Model 2 disagree with the observed values. Especially, the ND$_2$ tensor is in best agreement with Model 3, were the H bond, as depicted in Figure 4-19, is in best agreement with Model 1. Possibly an intermediate model could fit to both properties even better. The wat2 position could be modified as a more distant binding to the amino group ($\geq 2$ Å) was obtained in an early optimized structure. Missing contacts have rendered the flexibility of a single water molecule on a more distant side too large to lead to an optimized geometry. Here structural information of any contact to the $\beta$ subunit is of essence. The HF values of the wat2 deuterium with its calculated value of $A_{\perp,\parallel} = -0.19, 0.37$ MHz could be unresolved from the discussed narrow matrix line of NH$_2$Y$_{731}^•$ ($\pm 0.2$ MHz).

Addressing the Euler angles and the angular dependence of the H bond to Y$_{730}$ the following Figure 4-19 should visualize the results. In order to compare on the same molecular bases, the HF tensor of the ENDOR simulation was rotated into the individual $g$ tensor system of the model. The angle differences show a stronger disagreement to Model 2 as discussed before based on the coupling size. Although Model 2 agrees well with the $g$ values, the H bond direction does not. The other two models are within the uncertainty of $30^\circ$ identical to the ENDOR simulation. All models are in a good agreement with the
H bond direction to Y_{730}. Even if an additional water molecule is considered as in Model 3 the formed H bond interaction is weaker compared to the perpendicular H bond from Y_{730}.

Figure 4-19: Comparison of DFT models (right) and H bond orientation from ENDOR simulation (left) parameters in their respective molecular frame. The $g$ tensor orientation (blue arrows) is defined in respect with the molecular frame and therefore for each model slightly different (up to $10^\circ$). Row A, B and C are in the molecular frame of Model 1, Model 2 and Model 3, respectively. The hyperfine tensor and $Q_z$ are displayed as mint green and green vectors. In all models $Q_z$ has been found collinear to the O_{Y730}-H bond and has been omitted for clarity.
Comparing Structural Models from DFT with EPR Parameters

Table 4-6: Summary of EPR parameters for the H bond to NH$_2$Y$_{731}$*. Parameters were obtained from simulations of the orientation selective 94-GHz ENDOR spectra and comparison with the DFT models. Uncertainty in the parameters from the DFT and ENDOR simulations is estimated up to about 20%.

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<th>$A_y$ [MHz]</th>
<th>$A_z$ [MHz]</th>
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<th>$\beta$ [$^\circ$]</th>
<th>$\gamma$ [$^\circ$]</th>
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<tr>
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<td>72</td>
<td>-0.05</td>
<td>-0.06</td>
<td>0.11</td>
</tr>
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<td>109</td>
<td>-124</td>
<td>-0.06</td>
<td>-0.07</td>
<td>0.13</td>
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</tbody>
</table>

a) The signs of the couplings from the simulation are only relative to each other within one tensor. The Euler angles ($\alpha$, $\beta$, $\gamma$) are defined from the $A$ or $Q$ to the $g$ tensor based on the $y$ convention (positive sign for a rotation is counter clockwise, second rotation is around the $y$ axis). The $A$- and $Q$- (quadrupole) tensor are chosen such that $|A_x|<|A_y|<|A_z|$. Within this definition, for both the amino deuterons and the H bond deuteron the $A_x$ direction results along the bond direction. Euler angles from DFT (in the ORCA output positive rotations are defined clockwise) were transformed into the magnetic resonance convention, for comparison.
4.5 Discussion of the PCET in the α Subunit with NH₂Ys•

Prior work has demonstrated that unnatural amino acids can be used successfully to obtain information from the αβ₂ active *E. coli* RNR Ia enzyme.⁶⁹ Not only the globular structure but also positioning of NH₂Ys• within the PCET relative to Y₁₂₂• has been accessible (see Figure 1-8, p.12).⁴³ Whereas kinetic studies delivered rates of several PCET steps, the incorporation of NH₂Y offered the opportunity to measure the radical state directly after a reversible oxidation step and reorganization of the protein thereafter.¹¹⁰, ¹³⁴

Especially in α RNR the site selective incorporation⁴⁴ of Y₇₃₀/₇₃₁ with NH₂Ys offered the possibility to study the H bond network and electrostatics of the formed transient radical.⁹², ¹₁⁰ This information can be probed by two EPR accessible parameters: the gₓ value affected by (partial) positive charges and the hyperfine interaction of protons forming H bonds in varying strength. The two NH₂Ys at Y₇₃₀/₇₃₁ showed a strong decrease in gₓ of 0.7-1.0 ppt compared to the value of the free NH₂Y• (Table 4-5, p.101).⁹² This electropositive microenvironment was previously correlated to a DFT calculation (≈140 atoms, model 4) including all assigned H bond contacts assigned in the ²H ENDOR simulations as reported in the introduction (Figure 1-14, p.23).¹¹⁰ In analogy ²H ENDOR spectra of NH₂Y₇₃₁• have been assigned preliminary to two perpendicular H bonds (≈1.8 Å) within T. Argirević’s dissertation.⁹² A spectroscopical evidence, however, for the H bond donor groups assigned in these models has not been presented and a comparison to the transition state calculations (>200 atoms, models 7 and 8) is compromised by the difference in model sizes.⁹², ¹¹⁰ Additionally, highly accurate (<0.1 ppt) and resolved EPR spectra were not presented allowing several interpretations, as the number of conformers observed at NH₂Y₇₃₁•.⁹²

In this chapter, high-field 263-GHz EPR has demonstrated a slight, but significant, increase (0.3 ppt) of electrostatic interaction from the well-structured NH₂Y₇₃₀• to NH₂Y₇₃₁• and has found NH₂Y₇₃₁• to be a single species within the reaction time of seconds and minutes. This thesis revealed that the number, orientation and strength of exchangeable H bonds differ at these two residues. The ENDOR data are consistent with one strong and one moderate perpendicular H bond toward NH₂Y₇₃₀•, but displays only one strong perpendicular H bond toward NH₂Y₇₃₁• (Figure 4-14). The corresponding double mutant ENDOR spectra were consistent with a loss of an H bond. Additionally, they exhibited a decrease in radical build up rate. A connection between the change in H bond network and
Discussion of the PCET in the α Subunit with NH2Ys•

the lower reaction rate might be drawn. This would emphasize that a loss of an H bond leads to a decrease in radical build up rate. A analog argumentation explains the tendency in the PS II, were the stronger H bonded Y731• reacts faster than Y730•. 

Spectroscopic evidence for the assignment of the π-stacking between Y731-Y730 has been obtained by several independent observations. I) The direction of the H bond and the alignment to the axis of the H bond donor describes one perpendicular H bond toward Y730 from NH2Y731• and vice versa for Y731 toward NH2Y730•. II) The H bond dihedral angle (cf. Figure 4-8, p.85) indicates an intervening water molecule as described by a model of Kalia and Hummer to be unlikely. In their structure an H bond, from the water, shows an angle of about 45° between g_z and g_x. In this case the largest quadrupole splitting would be observed along g_x and g_z equally, which is not the case. On the contrary, the ENDOR spectrum at B_0∥g_x shows the smallest quadrupole splitting at this orientation (see Figure 4-8). However, both H bond angles are in agreement with an angle nearly perpendicular toward the aromatic plane (50° to 70°, see §4.2.2, p.83) as modeled to be ideal for this π-stacked interaction. III) The double mutant (NH2Y731•/Y730F) misses the typical perpendicular H bond pattern. Whereas the H bond network at NH2Y731• has a moderate to strong perpendicular H bond. This demonstrates the H bond loss is directly dependent on the phenoxy group at the 730 residue. IV) The DFT models revealed that no matter if zero, one or, two waters are considered in the large models (1-3) the strongest interaction is the H bond formed between the π stacked residues (see Figure 4-16). Thus the results support the current PCET model that Y731• is the proton acceptor in the next forward PCET step to Y730 (Figure 1-10, p.14). Notably, this geometry is present in several, but not all α2 structures. Other conformer states have been observed in wt yeast and human, and in NH2Y RNR α2 structures, as shown in Figure 1-3 (p.5) and Figure 1-12B (p.18). Geometric restraints like the nearly perpendicular H bond (§4.2.2, p.83) and a ring dihedral (§4.4, p.98) have been formulated, and underline the necessary geometry for this adiabatic CPET step. The optimum efficiency of the transfer has been studied by theorists, pointing toward an adiabatic electron and proton transfer in a geometry consistent with our data.

So far we could show that Y731 interacts to Y730 by a strong to moderate perpendicular H bond and can have only a weak H bond interaction toward the interface. DFT studies were performed by C. Riplinger from the Neese group (Mülheim) to explain
the observed g shift in great detail and to include electronic, steric and energetic effects for orientations of the H bonds of Y\textsubscript{730} and Y\textsubscript{731} toward each other (§1.5.3.2, p.26). In this thesis the optimized NH\textsubscript{2}Y\textsubscript{731}•• models 1 and 2 were obtained from the transitions state wt models 1’ and 2’ (models 6 and 7 in ref. \textsuperscript{110}), as illustrated in Figure 4-20 left. Therefore the NH\textsubscript{2}Y\textsubscript{731}•• and Y\textsubscript{731}•• models have the same structural basis and can be compared.

In this thesis Model 1, 2, and 3 were related to the EPR and ENDOR spectra of NH\textsubscript{2}Y\textsubscript{731}•• and had one, zero and two waters in their models, respectively. Models 1 and 3 were within uncertainty in agreement with the \textsuperscript{2}H HF couplings from the amino group and the g values. Model 3, however, could not be considered for a comparison of NH\textsubscript{2}Y\textsubscript{731}•• to Y\textsubscript{731}••, because Y\textsubscript{731} lacks the NH\textsubscript{2}-group necessary to stabilize a water molecule (wat2) at a distant (r\textsubscript{O-H} = 1.9 Å) position (see Figure 4-16, p.100). Based on the assignment one can return to the recent study of the transition states (TS) and energies of Model 1’ and 2’ by Riplinger.\textsuperscript{110} For position NH\textsubscript{2}Y\textsubscript{730}• the ENDOR data showed an H bond consistent with wat 1 in Model 1’. Therefore, a model with wat 1 near Y\textsubscript{730} is supported for Y\textsubscript{731}•• and Y\textsubscript{730}•• equally. The energetic pathway has been predicted and compared to Model 2’ without wat 1 and with a cation-π interaction of R\textsubscript{411} and Y\textsubscript{731}••, as shown in Figure 4-20 left.

In the calculation performed on the wt enzyme the structural effect in terms of energy barriers are now displayed. The lowest energy barrier reported so far between two tyrosines is observed between Y\textsubscript{731}•• and Y\textsubscript{730}•• for Model 1’. Additionally, the barrier heights of other DFT models from the literature are displayed in Figure 4-20.\textsuperscript{107, 112} Siegbahn et al. as well as Kalia and Hummer have modeled their DFT work solely with a di tyrosine peptide model between Y\textsubscript{731} and Y\textsubscript{730} with or without a water molecule in between. Although they pointed out either the importance of (non-equilibrium) electrostatics\textsuperscript{106, 113} or considered H bonds\textsuperscript{107, 232} they could not consider them. Both aspects have now been tested and compared to the EPR work biased by the absence of structural knowledge at the interface. Therefore Model 1’ takes these important interactions into account. Bu et al. states that the protein environment stabilizes the radical intermediates (by up to ~3 kcal/mol) and thus increases their observed barrier height. Comparing Model 1’ and 2’ it is evident that the close electro positive influence of R\textsubscript{411} (with a g\textsubscript{e} shift in NH\textsubscript{2}Y• of 0.5 ppt) in Model 2’ can destabilize the Y\textsubscript{731}•• compared to the TS and diminish the activation barrier.\textsuperscript{110} The geometry with the water (wat1) close to Y\textsubscript{730}, however, stabilizes the C\textsubscript{409}•• and decreases the necessary energy for this endergonic forward PCET step.\textsuperscript{110} Therefore it is difficult to
Discuss the PCET in the α Subunit with NH2Ys•

quantify the effect for all considered interactions, however, compared to Siegbahn et al. the barrier height decreases by 2 kcal/mol for the first step between Y731 and Y730 and is identical within error of 1.2 kcal/mol for the second step to C439•.110 Notably, all DFT models used the same hybrid functional, the work of Kalia and Hummer even by the same dispersion corrected diffuse TZVPP basis set. Furthermore Siegbahn et al. mentioned that the effect of the total energy does not depend strongly on the basis set, thus the comparison is admissible. Generally, it is expected that B3LYP works well for PCET barrier heights, but underestimates them.113, 192, 294 This is an important point before one compare these barriers to kinetic rates.

Figure 4-20: Energy diagram for the PCET in α. Here the calculated energies from C. Riplinger are shown for the preferred Model 1’ (red) and Model 2’ (blue). Additional other DFT energy barriers are shown as HAT energies from Siegbahn et al. (violet),107 the CPET energies of Kalia et al. (green)106 and work form Bu et al. (orange)112. Barrier heights in solid lines are a direct PCET step, were points mark a water assisted transfer. The energies obtained from QM/MM structures of Bu et al. are 2-51 kcal/mol higher and were omitted here. Modified from ref. 110.

Kinetic rates were recently measured for the PCET step from a higher potential 2,3,5-F2-Y356 to Y731-Y730-C439-nucleotide.86 Activated by a photo trigger the 2,3,5-F3-Y356• decayed with a rate of 14000 s⁻¹,86 this is still slower than rates from B3LYP studies between Y731-Y730-C439 with 10⁷ per s.110 The slower rate of 14000 s⁻¹ is in agreement with the proposed rate limiting step for this reaction to be the nucleotide reduction.86, 110 Although this comparison does not enable us to judge the activation energy of single PCET steps, it tells us that no slow structural conversions can take place between our investigated triad of Y731-Y730-C439.

The interaction of Y731 to C439 was proposed to be direct in previous ENDOR studies. It was also observed in all resting state α2 structures of wt, NH2Y730, and NH2Y731.22,67

3-Amino Tyrosine Radical Intermediates
Bu et al. have strongly challenged this assignment. They claim that their two shell DFT (ONIUM) model based solely on the inactive α2-NH2Y730 structure shows wat1 (H2O138 in their notation) moving into a gap between Y730 and C439 during geometry optimization.\textsuperscript{112} Notably, this has also been observed in some of our DFT models, if no restraints were used. It has been considered as an effect of the imperfect model based on the inactive crystal structure. In this chapter it could be demonstrated that the H bond resonance is missing in the NH2Y730•/C439 ENDOR spectrum, in agreement with the energy models presented by Riplinger (Figure 4-20).\textsuperscript{110} On the contrary, barrier height (Figure 4-20) and geometry of the intervening water of Bu et al. is similar for a water molecule (H2O138) or a cysteine S-H, as illustrated in Figure 4-21 (orange). Nevertheless, it can be stated that in the radical state of NH2Y730• the water they assigned to change its position (wat1/ H2O138) is still present in a distal position (cf. Figure 4-21, blue). Otherwise our methods cannot distinguish between a moderate H bond from and OH or an SH group. Agreement can be reached that assisting water molecules can stabilize the transition state as in the trend observed between Model 2’ and Model 1’ (cf. Figure 4-20). Nonetheless, a large energy barrier (60 kcal/mol) reported by them could not be reproduced even without wat1 in any other Y to Cys study including their own (Figure 4-20).\textsuperscript{112} Though it could point out the strong distance dependence for a PT in a single PCET step.\textsuperscript{101,112}

Figure 4-21: H bond environment of Y730• from DFT Model 1’ (blue) and ONIUM model of Chen et al. (orange).\textsuperscript{110,112} The H bonds to Y730• are marked for Model 1’ in red and for the ONIUM model in blue. The distance of the H bond is given next to the individual proton in Å. The distance between Y730 phenoxy oxygen and H-C439 is marked in gray.
4.6 High-Field EPR Spectra of NH$_2$Y$_{356}$• in the β Subunit

This part of the chapter will describe the results obtained using the 3-amino tyrosine mutation in the β subunit, more specifically at Y$_{356}$ placed in the C terminal domain of the β protein. In β, this part is highly flexible, nevertheless the last 32 amino acids were used to stabilize the α complex in order to crystalize it (Figure 1-4 p. 7). Furthermore, it could be shown by NMR studies§§123 or by the NH$_2$Y$_{730}$• stabilized complex$^6$ that this part becomes ordered in the “active” complex. Focused on position 356, PELDOR studies based on a Dopa mutation have shown a narrow distance distribution (3.1±0.1 nm) at Dopa$_{356}$ (Figure 1-6A p.10). Recently, also a narrow distance distribution of the NH$_2$Y$_{356}$•-Y$_{122}$• radical pair could be reported by PELDOR spectroscopy$^2$ (I. Bejenke, unpublished results). Based on these studies it became evident that a structuring around the flexible region takes place, but what does the local electronic structure look like?

In the following part the protein was prepared as published by E. Minnihan (Methods §3.2), the EPR samples, measurements and analysis were done in this thesis. The NH$_2$Y$_{356}$•-β mutants have the intrinsic problem of the placement of an electronic sink near the “stable” tyrosine Y$_{122}$•, thus only 0.5 radical/β$^2$ compared to 1.2 Y$_{122}$• per wt-β$^2$ could be generated by reconstitution methods.$^2$ A second batch in a 1:1 complex with a His6 tagged α mutant was purified by W. Lee in the Stubbe lab at MIT.

4.6.1 EPR Spectra of NH$_2$Y$_{356}$• for Several Reaction Times

For the investigation of the electronic structure and the H bonding at Y$_{356}$•, the time point for quenching the reaction had to be selected. These time points should be reproducible and should not contain further radical species. Although the SF-vis spectroscopy demonstrated a steady state at 1 s,$^6$ it is also interesting how the spectra develop during the radical decay with time points from s to minutes. As an example, conformational changes in this time scale could report on general local flexibilities of NH$_2$Y$_{356}$•. Therefore EPR experiments were performed at 94 GHz for several time points from 6 s to 2 min, as shown in Figure 4-22. The spectra looked slightly different due to their contributions of glass peaks (high-field asterisk *) and another contribution (low field asterisk *) quite intense at 2 min and quite low in intensity for the 44 s. For both later time points the low field line at $B_0$$_g$ seem to be

$^\S$ This study has been performed with mouse RNR Ia.
broadened, therefore further studies were performed at earlier time points up to ≈20 s, where also the highest radical yield was obtained ≈20%.

Figure 4-22: 94 GHz-Echo detected spectra of NH₂Y₃₅₆• recorded at different freezing time points during the reaction. The spectra are sorted from 6s quench (bottom) to 2 min quench (top). The derivative was obtained from a 3 G pseudo modulation. Exp. details: 70 K; \(\pi(\pi/2)=32(16)\) ns; SRT=5 ms; Number of averages from (6-120 s): 1300, 6200, 450, 1500 and 3800.

4.6.2 Polarity around NH₂Y₃₅₆• from 263 GHz and 94 GHz Spectra

In order to characterize the electrostatic environment and the electronic structure of NH₂Y₃₅₆• spectra were measured and compared at various frequencies, E. Minnihan reported on 9 GHz spectra and T. Argirević on 94 GHz spectra. The 263 GHz spectra should be compared to the \(g\) values already obtained HF parameters necessary for simulating the field dependent 94 GHz EPR spectra. 263 GHz spectroscopy was available to us since the beginning of 2012, but not all functions worked properly at the beginning. For example, the linearization procedure elongates the measurement time to an extent, for which at low radical yields no good S/N can be reached to measure in the accuracy of 50 ppm.
Thus, non-linearized field sweeps with an error of about 90 ppm had to be used. To show, however, that the spectra were consistent with or without linearization the first two spectra are included in Figure 4-23. It is clear that differences can be seen by eye. For instance the spectral width of the non-linearized field sweep is slightly larger. Nevertheless, the same $g$ values are obtained within an error of 90 ppm (Methods §3.5). Another point is the signal contribution marked as artifact with an asterisk (*), which is not present in the reactions quenched later. Hence, it was neglected as a signal arising from the background due to low radical yield of the sample ($\approx$10%). Possibly a deterioration upon freeze and thawing cycles of the prepared enzyme could also explain this contribution.

The $g$ values match already with the other investigated NH$_2$Ys•. They are more clearly resolved in the derivative spectra of the linearized spectrum (Figure 4-24). A comparison with a simulation based on the previous results is shown in Figure 4-24. With a $g_x$ of 2.0049 based on calibration with Y$_{122}$• (Figure A - 7, p. 198), the radical has the highest polarity of all three NH$_2$Ys•. The values are summarized in Table 4-7. The β-methylene HF coupling is within the range of 22 MHz and 29 MHz of the single mutants in α. A consideration beyond the semi empirical McConnel equation is discussed together with single amino acid DFT models in §4.6.4 (p.115). The trend in $g_x$ values is consistent with the decreased spectral width of the ND$_2$Y$_{356}$• spectra. As the $g_x$ values is proportional to the spin density population of the oxygen nucleus, the atom in Y$_{356}$• with the largest spin-orbit coupling, the result can be directly related to an increase of electrostatic environment around the oxygen (Theory §2.1.2). This leads to a trend of decreasing electrostatic interaction from NH$_2$Y$_{356}$ over NH$_2$Y$_{731}$ to NH$_2$Y$_{730}$, the in direction of forward PCET.
Figure 4-23: 263-GHz EPR spectra of ND$_2$Y$_{356}$ measured at three reaction time points. Exp. details (from top to bottom): green: SE, $\pi/2$=90 ns, $\tau$=260 ns, SRT =2 ms, SPP=1000, 150 scans; red: ESE, $\pi/2$=100, $\tau$=240 ns, SRT=3 ms, SPP=100, 250 scans; black: ESE, $\pi/2$=100, $\tau$=240 ns, SRT=3 ms, SPP=100, 160 scans; black and red are not linearized.

Figure 4-24: 94 and 263-GHz EPR spectra (blue) of NH$_2$Y$_{356}$ and simulation (gray, Table 4-7). Exp. details: A) see Figure 4-23 (green); B) ESE at 94 GHz; T=70 K; $\pi/2 = 32$ ns; $\tau = 260$ ns; SRT = 5 ms; SPP = 50; scans = 750. The derivative is obtained by 10 and 3 points second order Savitzky-Golay filter for A and B, respectively.
High-Field EPR Spectra of NH₂Y₃₅₆• in the β Subunit

Table 4-7: Summary of g values and HF couplings observed in the EPR spectrum of NH₂Y₃₅₆•.
The ¹⁴Ν hyperfine tensor of the NH₂Y• was not varied in the simulations and kept \( A_x = 2.4 \text{ MHz} \), \( A_y = 5 \text{ MHz} \), \( A_z = 30.7 \text{ MHz} \).¹³⁴ Uncertainty in g values and HF couplings is about 0.05 ppt and 10%, respectively.

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<th>( g_y )</th>
<th>( g_z )</th>
<th>( A_{iso}(C-\beta) )</th>
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4.6.3 H bond interactions at NH₂Y₃₅₆•

The observation of a polar environment around NH₂Y₃₅₆• has enhanced the interest in the identification of its origin. Up to now, H bonds orthogonal to the ring plane were observed by ENDOR spectroscopy at NH₂Y₇₃₁• and NH₂Y₇₃₀•. These H bond interactions have always sharp features at \( B_0 \parallel g_y \) (cf. Figure 4-7). In ND₂Y₃₅₆• the spectra at 94 GHz at \( B_0 \parallel g_y \) (Figure 4-25) did not show this sharp feature in a range of ±0.6 to ±0.8 MHz. It was quite astonishing to also see in the Q-band spectra (Appendix Figure A - 8, p.199) only contributions from the internal couplings of the amino deuterons and a matrix line with small contributions up to 0.48 MHz. A simulation considering only the matrix line and the amino deuterons is shown below the experimental result. The parameters are based on previous simulations of ND₂Y₇₃₁• and its double mutant (§4.2.1). The difficulty in this case was that the structure and even local structural motifs are unknown. Thus, large DFT models as in the previous case could not be constructed. To evaluate the structural surrounding small models can be notwithstanding helpful. Up to now it can be stated that no strong exchangeable external H bond to NH₂Y₃₅₆• is present, but a high electropositive environment around the oxygen is present.
Figure 4-25: $^3$H ENDOR spectrum of NH$_2$Y$_{356}^•$ quenched at 10 s compared with simulation. The experimental spectrum (gray) taken at 70K and $B_0 \parallel g_y$ was smoothed (blue, adjacent averaging, 10 points). The simulation is shown below the parameters are reported in Table 4-8. Exp. details: Mims $^3$H ENDOR, $\pi/2$=20 ns, $\tau$= 200 ns, 1 SPP random acquisition, SRT=7 ms, acquisition time = 48 h.

Table 4-8: Summary of EPR parameters for the exchangeable protons at NH$_2$Y$_{356}^•$. Parameters were obtained by 94-GHz $^3$H ENDOR spectra. Uncertainty in the parameters is up to 20%. The central line has not been assigned to a coupling, but to a matrix line. The size is given in the first line.

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<th>$A_z$ [MHz]</th>
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4.6.4 Modeling NH$_2$Y$_s^•$ by DFT Calculations Considering One Amino-Acid

Quite unexpected results for NH$_2$Y$_{356}^•$ were found, a high polarity on the one hand and on the other hand no strong resolved H bond interaction as in the other two NH$_2$Y single mutants. Any large DFT models at this residue have no structural foundation, thus DFT
calculations with single amino-acid models were performed on individual observable effects. First the effect of the conformer on the $g$ value was investigated. In the second step the effect of the orientation dependence of a single H bond to NH$_2$Y• was evaluated on g values and $^3$H HF couplings of the H bonding nucleus. Finally the polarity effect should be modeled by two approaches. For all three DFT studies there are examples in the literature$^{144, 154, 175}$ Within these studies tyrosines or semiquinones have been investigated. The outcome cannot be directly transferred to the $g$ values in 3-amino tyrosines. A linear scaling by $g_x$ values or phenoxy oxygen spin density population cannot be assumed.

The error of treating the effects of conformer and H bonding separately is small as will be shown by the calculations. Generally, it should be noted that the uncertainties of DFT calculations are higher than the size of the effects studied here with 0.5 ppt for $g$ values and up to 20% for HF couplings. Therefore the consistency within the models and the trend of the values can only be discussed here. The reason why we still describe these effects will also be highlighted by the experimental results of Chapter 5.

4.6.4.1 The Conformeric State of a NH$_2$Y•

A relaxed surface scan over the ring dihedral $\theta_{C\beta}$ has been performed as a DFT calculation. The B3LYP$^{268, 271, 272}$ hybrid functional and (def2-)TZVPP$^{207}$ have been used to obtain a geometry optimized dihedral within $10^{-9}$ E$_h$ (Methods §3.6.2.2). The EPR parameter were calculated on the same basis including a continuum polarization model (COSMO$^{296}$) adjusted to the polarizability of ethanol to account for a polar environment. The diheadral is defined by the angle between C$_\beta$-C$_\alpha$ and C$_1$-C$_6$ axis, as illustrated in Figure 4-26A. The used tyrosine model is illustrated in Figure 4-26B with typical Löwdin spin density population$^{297}$ of $\rho_O$ 24% and $\rho_C$ about 14%, here $\theta_{C\beta}$=60° is shown. As an additional control of the relaxed surface scan the absolute energies report about a global minimum at $\theta_{C\beta}$=90° (Figure 4-26C), the local maximum arises due to a sterical interaction of the backbone amine with a ring proton. This local maximum is not reported in a larger calculation on a dipeptide radical$^{129}$ so could be a model error of the peptide bond removal.

The $g_x$ value is minimal, when the C$_\alpha$ is in eclipsed conformation to the p$_z$ orbital (Figure 4-26D) due to the hyperconjugative interaction. The effect on the $g$ value was found to be minor and in a range of 0.15 ppt. This change in g value is not significant, therefore only the trend within this model can be considered here. In Figure 4-26E&F a $\sin^2(\theta_{H\beta})$ dependence of the HF coupling to the dihedral angle becomes evident. The $\beta$-methylene
HF coupling is increased if one of the protons is parallel to the π system, i.e., $H_{β1}$ overlaps with $p_z$ of C\(_1\) (Figure 4-26A). This hyperconjugation leads to an increase in spin density population in the nucleus parallel to the aromatic π system, therefore a nearly isotropic and positive HF interaction results. The calculation of the conformational state reproduces the form of the McConnell equation.\(^{295}\) The β-methylene proton behaves similar as the Karplus equation in NMR\(^{299}\) on the overlap of the spin interactions. The lowest HF interaction for both beta methylene couplings is observed at for $C_β$ axial and the highest for $C_β$ parallel to the ring plane, with 2.0061 and 2.0059 respectively.

Although the $g$ value is in the typical range of a free NH\(_2\)Y, the HF couplings for $H_β$ display the full range of observed $C_β$ HF couplings. The maximal coupling of 34 MHz found in α-NH\(_2\)Y\(_{730}\)*/C\(_{439}\)A as well as the smallest coupling of 22 MHz of α-NH\(_2\)Y\(_{731}\)* (cf. Table 4-7) lie on the curve. The $H_β$ HF coupling has been reported in tyrosines to be inversely proportional to the oxygen spin density population.\(^{277}\) The effect is quite small in NH\(_2\)Y’s, due to the second electronegative group in ortho position to the oxyl function. Still the phenoxy spin density population varies within the models in this section from 24% to 19% (p.124). In this case, the 27 MHz of NH\(_2\)Y\(_{356}\)* from $a_{ωω}$ for $H_β$ (Table 4-7) would correspond to a dihedral angle $θ_{Cβ}$ from 10-20° or 50-60°, as directly obtained from Figure 4-26E. Only in the latter case (55±5°) the second β-methylene coupling is smaller than 10 MHz and thereby unresolved, thus $θ_{Cβ}$ is approximately 55°. An uncertainty of 15° can be estimated.
4.6.4.2 Water Dihedral Scan

The same procedure of a dihedral relaxed surface scan was applied to calculate the dihedral of one H bond from a water molecule ($\theta_{H_2O}$, Figure 4-27A) to NH$_2$Y$^\cdot$ (§4.6.4.1). Because no
further restraints were applied, the lowest lying conformer was found in all geometry optimization models for the individual $\theta_{\text{H}_2\text{O}}$. Also the H bonding geometry changes slightly in course of the calculation Figure 4-27B. The $O_{\text{NH}_2\text{Y}}$-$\text{H}_2\text{O}$ distance obtained correlates nicely to the $g_x$ value of the EPR parameter calculation (Figure 4-27C).

The energy variation $10^3$ $E_h$ between the models is approximately an order of magnitude smaller than in the conformeric scan (Figure 4-27C). However a clear minimum is found for the water within the plane (up to ±30°) with the phenoxy ring. This is in contrast to the $\theta_{\text{H}_2\text{O}}$ of single mutant NH$_2$Y$_{\text{•}}$ discussed so far. Here in both cases a perpendicular H bond has been found. An in-plane H bond is in agreement with other tyrosine radical H bonds as found in yeast RNR Y$_{122}$• correspondent or in the Y$_D$ of photosystem II. The H bond angles of tyrosine radicals in α are the exception in the literature, to the best of my knowledge.

The HF tensor of the H bond was therefore a core interest. Although the values obtained from the calculation had a rhombicity increasing up to $\theta_{\text{H}_2\text{O}}$=40°, and only then an absolute axial symmetric tensor forms. For the sake of argument, only the averaged axial component $T_\perp$ of the individual tensors are plotted in Figure 4-27E. Both the orthogonal and the parallel HF interaction increase with dihedral angle $\theta_{\text{H}_2\text{O}}$. This is in absolute contrast to the decrease in distance and increase of $g_x$ value, in C. The orthogonal component of the HF values $T_\perp$ increase faster than the parallel $T_\parallel$ values. The increase is consistent with an increasing isotropic HF interaction $a_{\text{iso}}$, which acts on each individual HF component. It changes $A_x$, $A_y$ and $A_z$ in the principle axis system simultaneously. The isotropic HF $a_{\text{iso}}$ interaction is a direct indication for orbital interaction, because it is only governed by spin density in the 1s orbital of the 'H nucleus (Theory §2.1.3, Eq. (2-13). The increase of interaction, albeit the longer H bond distance, is a consequence of the non-spherical symmetry of the $p_z$ orbital. This is nothing astonishing $per se$, but explains why a tensor can appear a lot weaker by a change in H bond angle. Mostly the orthogonal component of the tensor is observed in the HF spectra, like ENDOR. The broad parallel component can be difficult to observe in these overlaying spectra. The $g$ value variation with H bond dihedral is about 0.2 ppt. As a comparison, this is the difference between α-NH$_2$Y$_{731}$• and β-NH$_2$Y$_{356}$• assuming an in-plane H bond at β-NH$_2$Y$_{356}$•. The C$_1$ spin density population was only minor effected by the change of H bond dihedral with standard deviation of 1.4% of the $\rho_{C1}$ value ($\Delta\rho_{C1,\text{Löwdin}}$=0.2%).
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Figure 4-27: Dihedral scan over the H bond dihedral. A) The ring dihedral $\theta_{\text{H}_2\text{O}}$ is defined as $C_3$-$C_4$-$O$-$\text{H}_2\text{O}$ on a model of the neutral 3-amino tyrosine• (B). C) The $g_z$ value (red) correlates well with the H bond length (green). D) The energy in Hartree against the dihedral $\theta_{\text{H}_2\text{O}}$ is consistent with a global minimum at $\theta_{\text{H}_2\text{O}}=0\text{°}$-30°. E) The HF value (for $^1\text{H}$) depending on the H bond dihedral is plotted. The anisotropic part is listed separately for its averaged axial ($T_{\perp}$, green) and absolute parallel ($T_{\parallel}$, blue) value. Isotropic HF $a_{\text{iso}}$ is shown in red. F) shows the H bond angle ($C_4$-$O$-$\text{H}_2\text{O}$) as a control.
4.6.4.3 Water Network around NH$_2$Y$_{356}$

The last section opened up a new explanation for the $^3$H Mims ENDOR spectrum obtained with NH$_2$Y$_{356}$•. In plane water can have small axial HF tensor contributions of $\approx 0.4$ MHz, but will still affect the $g$ value by $\approx 0.5$ ppt. Despite the high polarity used in the continuum model, this is still not the size of the effect of 1.1 ppt experimentally observed $g_x$ shift (vs. free NH$_2$Y•, Table 4-5), compared to several reported DFT models of the isolated 3-amino tyrosine. The high uncertainty of DFT of about 0.5 ppt$^{110}$ will not deliver quantitative data, i.e., the number of H bonds present at the NH$_2$Y$_{356}$•. Therefore only the trend should be further investigated. Especially steric effects and polarity effects of a free NH$_2$Y• should be considered. The idea of modeling of NH$_2$Y$_{356}$• with water network emerged. Seveant et al. contributed to this idea by demonstrating a water network as a functional PCET system (Theory §2.4.3).$^{250,251}$

The models of the 3-amino tyrosine were built up successively with 1, 2 and 3 water molecules. After each additional water molecule the geometry was optimized and the EPR parameters were calculated by single point calculations. To observe the $g$-shift depending on weak H bonds formed by each water molecule $g$ values and HF couplings were obtained for each H bond. Furthermore, one can observe how water molecules locate toward phenoxy oxygens, because the energy barriers are small enough to be overcome by the geometry optimization cycles. One water molecule is positioned after geometry optimization within the ring plane (Figure 4-28A) the minimum found in Figure 4-27C. After the addition of a second water in hydrogen bond distance of the phenoxy oxygen in the ring plane, the geometry optimization end up in model Figure 4-28B. Here one water is perpendicular (75°) to the ring plane H bonded to the phenoxy oxygen (wat1), the other one is H bonded to the amino proton and is located in the aromatic plane (Figure 4-28B wat2). If wat2 is removed from model B a water geometry comparable to Model A is regained, after geometry optimization. The addition of a third water molecule produced too much flexibility for its small energetic influence to converge in a DFT calculation. Albeit changes in the integration grid and removal of COSMO$^{296}$ polarities. Therefore, a relaxed surface scan for the third water was performed also without COSMO. It ranged from 0° to 60° H bond dihedral $\theta_{wat3}$ with a step size of 10°. Figure 4-28C shows the converged geometry, a water molecule at 40° H bond dihedral $\theta_{wat3}$. 

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Figure 4-28: DFT models of different H bonding situations at a NH$_2$Y$^\bullet$. The $g$ value is compared for a model with one (A, 2.0057), (B, 2.0058) with two and with three (C, 2.0056) water molecules, named wat1 to wat3. The spin densities $\rho$ varied through the models from $\rho_O = 23\%$ over 21% to 21% and for $\rho_{C1}$ from 14% over 13% to 12% (after Löwdin$^{297}$). The H bond length between the oxygen of the NH$_2$Y$^\bullet$ and the wat1 increases from B to C slightly with 1.79 over 1.81 to 1.83 Å. The distances in the figure are given in Å. The dihedrals are given on the left side. Calculation details: B3LYP, def2-TZVPP, COSMO(ethanol), energy converged to 10$^{-6}$ Eh.

From the $g$ values point of view a clear trend of the H bond distance on the $g$ value was found, as reported by C. Riplinger (Introduction, Figure 1-14C, p.23). Two H bonds almost axial to the ring plane have also been shown from his studies to suppress the $g$ values by 0.5 ppt.$^{110}$ As shown on the small model calculations performed previously (Figure 1-14C, p.23), one axial H bond does not shift the $g$ value strongly, only with a second H bond to the oxygen directed perpendicular toward the ring one could reduce the $g$ by 0.9 ppt (Appendix: Figure A - 6, p.197). Model C shows the same tendency. However, the basis set used here gives slightly larger $g$ values, because it is not as flexible as EPR II in the core region (cf. §2.3.3 p.51). Recalculating model C in order to compare it with the large models
with the smaller EPRII basis set reduces the \( g \) values to \( g_x=2.0022, g_y=2.0043 \) and \( g_z=2.0054 \). These values are within 0.5 ppt uncertainty of the DFT calculations consistent to the experimental ones (Table 4-7, p.114). Considering the large distances (≥1.8 Å) of the two H bond partners, especially wat2, the \( g_x \) value is low enough to explain the \( g \) values observed at \( \text{NH}_2\text{Y}_{356}^\bullet \). A protein environment can influence the H bond length for water for instance by polarization and local environment effects.\(^{300, 301}\) Notably, the obtained ring dihedral \( \theta_{C\beta}=68^\circ \) of model C is within the prediction based on the conformeric DFT calculation with \( \theta_{C\beta}=55\pm15^\circ \) (§4.6.4.1) in agreement to the experimental HF coupling (§4.1.2, p.77).

Additionally, one needs to discuss the HF tensors and sizes. Both H bond interactions to the oxygen from wat2 and wat3 in Model C are close to the ring plane and do not overlap with the \( p_z \) orbital of the oxygen. The \( a_{iso} \) values are with -40 and -70 kHz (for \( ^3\text{H} \)) are in the lower range of Figure 4-27E. Although all water containing models would result in a decrease of \( g_x \) value similar to the experiment, they do also show an HF tensor in a size, which would be larger than the observed matrix line. The values are collected in Table 4-9.

Table 4-9: EPR parameters from DFT of \( \text{NH}_2\text{Y}^\bullet \) in water Model C. The HF couplings are within 20% uncertainty and the \( g \) values have an uncertainty of 0.5 ppt. Both HF and \( g \) values are identical within uncertainty for two basis sets used here.

<table>
<thead>
<tr>
<th>Proton HF</th>
<th>( A_x ) [MHz]</th>
<th>( A_y ) [MHz]</th>
<th>( A_z ) [MHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wat1-(^1\text{H})</td>
<td>-2.7</td>
<td>-3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>wat2-(^1\text{H})</td>
<td>-4.0</td>
<td>-4.2</td>
<td>6.8</td>
</tr>
<tr>
<td>wat3-(^1\text{H})</td>
<td>-2.7</td>
<td>-3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>H(_{\beta1})-(^1\text{H})</td>
<td>2.2</td>
<td>-0.9</td>
<td>-1.1</td>
</tr>
<tr>
<td>H(_{\beta1})-(^2\text{H})</td>
<td>23</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Deuteron HF</td>
<td>( A_x ) [MHz]</td>
<td>( A_y ) [MHz]</td>
<td>( A_z ) [MHz]</td>
</tr>
<tr>
<td>wat1-(^2\text{H})</td>
<td>-0.42</td>
<td>-0.46</td>
<td>0.77</td>
</tr>
<tr>
<td>wat2-(^2\text{H})</td>
<td>-0.41</td>
<td>-0.47</td>
<td>0.77</td>
</tr>
<tr>
<td>wat3-(^2\text{H})</td>
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<td>-0.64</td>
<td>1.04</td>
</tr>
<tr>
<td>Basis set</td>
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<td>( g_y )</td>
<td>( g_z )</td>
</tr>
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<td>2.0022</td>
</tr>
<tr>
<td>EPRII</td>
<td>2.0054</td>
<td>2.0043</td>
<td>2.0022</td>
</tr>
</tbody>
</table>

A perpendicular H bond orientation to \( \text{NH}_2\text{Y}_{356}^\bullet \) could be strongly disfavored based on the comparison between \( ^2\text{H} \) ENDOR spectra of \( \text{NH}_2\text{Y}_{356}^\bullet \) in \( \alpha \). A proton bound within the ring
plane, however, with couplings (\(^1\text{H}\)) of -0.5, -0.5 and 1 MHz could be unresolved in the \(\text{NH}_2\text{Y}_{356}\cdot\ ^2\text{H}\) ENDOR spectra. This coupling can be consistent to the experimentally observed matrix line ±0.4 MHz, if the uncertainty of the calculated values and the broadening by quadrupole interaction are considered. The amino deuteron couplings, however, prevent an unambiguous assignment.

4.6.4.4 Positive Charges in the Surrounding of \(\text{NH}_2\text{Y}_{356}\cdot\)

A second hypothesis for the structure between \(\beta\)-W\(_{48}\) and \(\beta\)-Y\(_{356}\) was postulated by Bollinger et al. in 2006. They proposed Mg\(^{2+}\) interaction along the PCET. They observed in a Y\(_{122}\)F mutant upon cofactor assembly a tryptophan radical W\(^+\)\(^\cdot\). The lifetime and the kinetic formation of a Y\(^\cdot\) in \(\beta_2\) was dependent on the presence of Mg\(^{2+}\) and \(\beta\)-Y\(_{356}\). It was proposed that Mg\(^{2+}\) in RNR is not only essential for nucleotide reduction,\(^{302}\) but also for mediating the PCET between W\(_{48}\) and Y\(_{356}\).\(^{74,303}\)

On the other hand earth alkaline metals in their +II oxidation state have been used in the literature to model polarity effects in DFT calculations. These models were used especially if direct treatment of explicit water was not enough to sufficiently reduce the \(g\) value to the experimental values.\(^{175,304,305}\) It has been as well an alternative to “correction factors”\(^{216}\) for treating H bonding effects in EPR/DFT calculations. Magnesium(II) is usually coordinated by six ligands, therefore a small DFT model taking these interactions into account was set up. It includes an essential glutamate residue either \(\beta\)-E\(_{52}\) or \(\beta\)-E\(_{350}\) modeled by acetic acid required for charge balance. The geometry optimized model is shown in Figure 4-29. The \(g\) values reproduced a highly polar environment with 2.0051, 2.0040 and 2.0022 which is in excellent agreement with the experimentally observed spectra (Table 4-7). Although there is no evidence for such an Mg\(^{2+}\) coordination, it demonstrates the effect of positive charges in reducing the \(g\) values significantly. The oxygen spin density population is, after the Löwdin analysis with \(\rho_0=19\%\), 2\% lower than in the water network models.

In a more general picture positive charges are present in proteins also within individual amino acids.\(^{306}\) Especially at the interface salt bridges and interactions with aromatic systems might govern protein-protein interaction.\(^{307}\) Positively charged amino acids are not present in the 32 C-terminal amino acid tail. The difference in their isoelectric point has helped for instance in the separation of truncated and full length \(\beta\) peptides.\(^{308}\) However, positively charged arginine’s are observed in the \(\beta\) subunit and in the \(\alpha\) subunit as essential for activity.
and could have an effect in subunit interaction. The β 32 amino acid tail harbors two glutamates which on the other hand introduce a negative charge. By forming salt bridges these positive charges could be neutralized to enhance a folding of this β C terminal tail. Therefore, positive charges residues should also be considered to lie within the interaction sphere of $Y_{356}^\cdot$.

Figure 4-29: DFT model demonstrating the effect of positive charges (Mg$^{2+}$) in the surrounding of the phenoxy nucleus. In this model an Mg$^{2+}$-Ion (green) was placed and a coordination sphere (yellow dotted line) was modeled. An acid, an alcoholic function and 4 waters are included. Calculation details: B3LYP, def2-TZVPP, COSMO (ethanol), Energy converged to 10$^{-6}$ Eh.

4.7 Discussion of the $\beta$-NH$_2$Y$_{356}^\cdot$ Radical Intermediate

The essential radical intermediate formed at position 356 is of key interest in understanding how the RNR transfers an electron selectively through the α/β subunit interface. There are no detailed structural information on Y$_{356}$. The diagonal distance between Y$_{122}^\cdot$ and NH$_2$Y$_{356}^\cdot$ could be determined in the active enzyme (Figure 1-8, p.12). The comparison between the pK$_a$ values of Y intermediates using NO$_2$Y mutations has demonstrated that residue 356 is more polar than the Ys in α (§1.4.5, p.16). Investigations on NH$_2$Y$^\cdot$s show a similar electropositive environment at position Y$_{731}$, Y$_{730}$ and, Y$_{356}$, (Table 4-5, p.101). So far no conclusions in agreement to the redox potential difference ($\approx$60 mV, Figure 1-11, p.17) between Y$_{731}$, Y$_{730}$ and, Y$_{356}$ could be drawn. Furthermore, there is no information about the H bond network, which is crucial to understand the forward PCET mechanism.

$^3$H ENDOR spectra on NH$_2$Y$_{356}^\cdot$ did not resolve any exchangeable H bond in contrast to the Y intermediates in the α subunit. The $g_z$ value from 263 GHz EPR, as indicator for an electrostatic environment was correlated to number of intermolecular
Discussion of the $\beta$-NH$_2$Y$_{356}\bullet$ Radical Intermediate

H bonds. An apparent contradiction between zero perpendicular H bonds and a $g$ tensor indicating high polarity has been discussed. On the one hand, an H bond network within the aromatic plane, could have unresolved HF interactions. The parallel component of the HF interaction $T_{\parallel}$ is reported to be difficult to detect in Ys• due to their broad, low intense line shape. On the other hand positive charges could be modeled to account for the $g$ tensor and the HF tensor contributions.

Despite these problems, it could be demonstrated that a perpendicular strong or moderate H bond is absent at NH$_2$Y$_{356}\bullet$. Thus it seems to be unlikely that the NH$_2$Y$_{356}\bullet$ can engage in a similar "$\pi$-stacking" geometry as Y$_{731}$ and Y$_{730}$. Additionally, NH$_2$Y$_{731}\bullet$ has not shown any strong or moderate H bond toward the direction of residue 356. From a mechanistic point of view a perpendicular H bond has been pointed out to be a prerequisite for a "HAT geometry", as illustrated in Figure 2-15A (p. 51), allowing an adiabatic proton transfer. This point indicates that a collinear CPET step from Y$_{356}$ to Y$_{731}$ is unlikely. On the other hand drawbacks of the NH$_2$Y mutation became obvious in the investigation of NH$_2$Y$_{356}\bullet$. The introduced amine function can act as an H bond donor and thereby influences the H bond network. Especially for an in-plane H bond as found at residue 356 NH$_2$Ys could be non-innocent reporters for H bonding networks (cf. Figure 4-28, p.122).

The NH$_2$-group also changes the EPR property of Ys investigated. The broad ENDOR absorption of amino proton $^1$H resonances is spectroscopically a resolution problem, which can lead to unsolvable H bond interactions. In the case of $\beta$-NH$_2$Y$_{356}\bullet$ it is aggravated by the intrinsically low S/N due to low Y$_{122}\bullet$/\$\alpha_2\beta_2$ ratios (0.5, see Methods) in $\beta$ mutants.

The introduced H bond donor function (NH$_2$) has been relatively unproblematic in the well-structured surrounding of $\alpha$-NH$_2$Y$_{730}\bullet$ as could be revealed by no additional H bond found compared to Y$_{730}\bullet$ large model. In the $\alpha$-NH$_2$Y$_{731}\bullet$ for the first time water H bonded to the amino group (Model 3, p.98) was considered and reproduced the experimental results better. In contrast, at $\beta$-NH$_2$Y$_{356}\bullet$ no structural information is at hand to separate the NH$_2$ effect from the Y$_{356}\bullet$ H bond network. Thus, further experimental studies are necessary on a natural Y•. Therefore, we decided to simplify our system and searched for an alternative probe, which is described in the next chapter.
5 2,3,5-F₃Y₁₂₂• TO GENERATE Y• INTERMEDIATES IN THE PCET

5.1 Introduction of a New Rate Limiting Step to Generate Y₃₅₆•

The idea of investigating naturally occurring radicals in the RT of RNR is tremendously appealing. While amino tyrosines offered the opportunity to compare all three transient tyrosin based radical intermediates, trapped tyrosines on the pathway have complimentary advantages. Recently, Yokoyama et al. published a new way of by-passing the natural conformational gating in ribonucleotide reductase.⁸⁴,¹¹⁸ Here not the potential of the radical intermediate was lowered; instead they increased the potential of the Y₁₂₂ to prevent a completion of the reverse PCET. The formed intermediate radical is to a large extent (90%) Y₃₅₆•. In this study, they have used β₂-NO₂Y₁₂₂ with a large excess potential of 200 mV (cf. Figure 5-1A). This NO₂Y₁₂₂• not only has a short lifetime in β₂, but also can populate non-pathway side reactions. Additionally, it has to be coupled to cofactor assembly to reduce β₂-NO₂Y in the first place. Thus the individual PCETs to Y₁₂₂ and from Y₁₂₂• to Y₃₅₆• cannot be studied separately.⁸⁴

Nevertheless, the idea to trap a tyrosyl radical in its natural protein environment is appealing. 2,3,5-F₃Y has shown to overcome the conformational gating despite the absence of a large over potential as in NO₂Y.¹¹⁹,¹²⁷ 2,3,5-F₃Y is approximately 30 mV harder to
Introduction of a New Rate Limiting Step to Generate Y₃₅₆•

oxidize than Y to its neutral radical state. In Chapter 4, it was demonstrated that an H bond interaction between α₂-Y₇₃₁ and α₂-Y₃₅₆ is unlikely. If an H bond interaction occurs this interaction would be only consistent with a weak H bond (α₃₅₆≈0). Therefore, it seemed to be possible to investigate with this 2,3,5-F₃Y₁₂₂• the forward (Figure 5-1C) as well as the reverse PCET (Figure 5-1B). For the former case, the pathway is blocked by a phenylalanine at α-Y₇₃₁ after PCET initiation by 2,3,5-F₃Y₁₂₂•. In the latter case, wild type α is used with β-2,3,5-F₃Y₁₂₂. Here it has been postulated that a reverse PCET takes place based on the identical rate constant (20 s⁻¹) of Y₃₅₆• and CDP formation. This argument is analog to the one used for β-2-NO₂Y₁₂₂•. As foundation to future work this thesis will investigate β-2,3,5-F₃Y₁₂₂:α-wt & α-Y₇₃₁F trapped on the second time scale. This time scale is comparable to the 3-amino tyrosine reaction times (Chapter 4). Initial studies of β-2,3,5-F₃Y₁₂₂:α-wt have shown identical 9 GHz spectra of 20 s freeze quench or RFQ samples. However, the relative ratios between Y₃₅₆• and 2,3,5-F₃Y₁₂₂• change from 50% to 30% for Y₃₅₆•.

The focus of this thesis is the identification of intermolecular interactions by HF ENDOR. For this purpose, a natural tyrosine is beneficial, because the absence of ND₂ couplings in the ²H Mims ENDOR spectra in deuterated buffer offers a better resolution for small radical concentrations. The spin density population on the phenoxy nucleus is higher in Ys• than NH₂Ys•. Therefore one should expect an increase in dipolar HF couplings to the phenoxy oxygen [see Eq. (2-16)]. This larger HF coupling can increase the Mims ENDOR efficiency (cf. Figure 2-10B p.43). Up to now the forward radical transfer was discussed using NH₂Ys•. Hence, the focus of this chapter will be the forward transfer.

*** Kanchana Ravichandran and J.A. Stubbe, MIT, unpublished results.
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

Figure 5-1: Reduction redox potentials are shown along the PCET in mV relative to the oxidation of $Y_{122} \rightarrow Y_{122}^\bullet + e^- + H^+$. The potential height is based on the work of recent publications.\textsuperscript{41,110} A) To trap $\beta$-$Y_{356}^\bullet$ in a wild type environment the redox potentials of two mutations $\beta$-2,3,5-F$_3$Y$_{122}$ and $\beta$-NO$_2$Y$_{122}$ are shown. B) By using $\beta$-2,3,5-F$_3$Y$_{122}$ with $\alpha$-wt a radical assigned to $\beta$-$Y_{356}^\bullet$ could be trapped in the $s$ timescale.\textsuperscript{28} Yields of 27% $\beta$-$Y_{356}^\bullet$ together with 3% of $\alpha$-$Y_{731}^\bullet$ and $\alpha$-$Y_{730}^\bullet$ are expected.\textsuperscript{84} The PCET is free to proceed to nucleotide turnover and back, thus in the second time scale an equilibrium radical distribution will be observed. C) To observe a radical species of the forward PCET, most likely $Y_{356}^\bullet$, a PCET blockade at $Y_{731}$ is introduced with $Y_{731}$F.

Prior investigations of the radical intermediates a high-field EPR characterization of $\beta$-2,3,5-F$_3$Y$_{122}$ was conducted. This was necessary due to several reasons. First, this species had already been demonstrated to have changed relaxation behavior in CW EPR spectra as observed by saturation experiments compared to $\beta$-$Y_{122}^\bullet$.\textsuperscript{28} We have observed in pulse EPR spectra that the relaxation filter as introduced for $Y_{122}^\bullet$ does not work as efficiently with 2,3,5-F$_3$Y$_{122}^\bullet$. Thus, possible contributions to the spectra have to be identified. Most importantly, one needs to check, if the newly incorporated mutation induce disturbances in the structure as compared to $\beta$-$Y_{122}$. 

$2,3,5$-F$_3$Y$_{122}^\bullet$ to Generate $Y^\bullet$ Intermediates in the PCET 129
The work of Seyedsayamdost et al. showed that 2,3,5-F₃Ys are deprotonated in solution while Ys are protonated. So it is necessary to understand more of the electronic structure of this mutant, and possibly understand how the conformational gating is circumvented by this UAA.

5.2 Multifrequency EPR Characterization of 2,3,5-F₃Y₁₂₂•

In order to characterize the electronic structure of a radical the HF couplings and the g values have to be determined. Large HF couplings in 2,3,5-F₃Y₁₂₂• are the β-methylene ¹H and the ¹⁹F couplings. To compare the effect of the mutation on the environment, especially the β-methylene coupling can give indications which conformer is present. Large couplings can be observed in the 9 GHz spectrum, whereas the g tensor is observable at high-field/frequency measurements. A simulation with a shared parameter set delivers the best obtainable parameters. In Figure 5-2 the spectra of 3 frequencies are shown with the best simulation below. The 9 GHz spectrum consists of a triplet, quartet, and triplet pattern. This demonstrates that the large β-methylene coupling is roughly the same size as the 2-¹⁹F HF interaction (Table 5-1). From a doublet of doublets a distorted triplet arises, as observed on the low and high-field side of this spectrum. The last triplet in the center overlaps with the rest of the spectrum consistent with a doublet observed in the higher field spectra at B₀∥gₓ or gᵧ. At 94 GHz, the strong orientation dependence of the ¹⁹F HF tensors becomes evident, here B₀∥gₓ and gᵧ show only a doublet contribution. The triplet, quartet, and triplet pattern are only observed at B₀∥gₓ. The triplet at the high-field side seems to be strongly distorted and only merely visible within the S/N. One explanation could be a not completely integrated echo signal, diminishing weak intensities at the edges of the spectrum from these large HF couplings. The low field side overlaps with the gₓ doublet, thus only at the edges weak peaks are resolved. The 263 GHz spectrum continues the trend and delivers stronger constrains for the gₓ and gᵧ values. As an internal standard the strong contribution of Mn²⁺ ions can be used. These sharp peaks are clearly visible in the spectrum and distort the high-field line shape. For the simulation the β-methylene coupling and the g values were taken from the HF spectra were the large HF couplings present at B₀∥gₓ, whereas taken from the 9 GHz spectrum. This resulted in a reasonable agreement of the simulation to the experimental spectrum in a small number of iterations if a full matrix diagonalization for the electron spin was used (EasySpin; cf. §2.1.6, p.36). This is necessary in this case due to the
large $^{19}$F HF couplings observed here. To improve the values of the $\beta$-methylene couplings a Davies ENDOR improved variant$^{310}$ was recorded by R. Rizzato of our group (unpublished data).

A conformeric change can be observed from the obtained $a_{iso}$ of $C_{\beta}$-$H$ with 45 MHz. A semi-empirical formula \[ [\text{McConnell Eq. (5-1)}] \] connects this $a_{iso}$ value with the rotation angle around $C_{\beta}$-$C_1$ axis, the ring dihedral $\theta_{pz\beta}$. In this equation the spin density population at $C_1$ ($\rho_{C_1}$) is a linear proportional to $a_{iso}(C_{\beta}$-$H)$ and $B_1$ is an empirical constant. Wt-$Y_{122}$ has an $a_{iso}$ value of 55 MHz, which is significantly larger. The spin density population is not \textit{a priori} known for the new UAA; it can be derived either experimentally by stepwise isotopic labeling or theoretically by a DFT calculation. The DFT can give an estimate of the typical $C_1$ spin density population $\rho_{C_1}$ to determine the $C_{\beta}$-$H$ hyperfine couplings \[ [\text{see Eq. (5-1)}].^{178, 295} \] The $C_1$ spin density population $\rho_{C_1}$ is experimentally obtained for $Y_{122}$ with 38%. DFT structures of $Y^•$ under similar conditions obtain with 37% similar Mulliken$^{312, 313}$ spin density populations. The spin density populations calculated for a 2,3,5-$F_3Y_{122}$ (Figure 5-3A) are with 34% roughly 10% smaller and would lead to an $a_{iso}(C_{\beta}$-$H)$ = 49 MHz for the same conformer. This indicates a slight but significant conformeric change of the 2,3,5-$F_3Y_{122}$. Using the McConnell Eq. (5-1) the angle $\theta_{pz\beta}$ between the projection of $p_z(C_1)$ and $C_{\beta}$-$H_{\beta}$ is estimated to be $16$-$25^\circ$. For $Y_{122}$ a $\theta_{pz\beta}$ was obtained by single crystal EPR data to be $12$-$16^\circ$, thus the value is $0$-$9^\circ$ larger. The $sp^3$ geometry at $C_{\beta}$ has angle of $117.8^\circ\pm 0.6^\circ$, thus a $\theta_{\beta\beta}$ value between $44$-$53^\circ$ is obtained (Figure 5-3B). X-ray diffraction has shown a dihedral $\theta_{\beta\beta}$ of $46.2^\circ$, as seen in Figure 5-3C. This gives a tendency of the occurred change in ring dihedral.

\[
\begin{align*}
  a_{iso}(C_{\beta}-H) &\approx B_1 \cdot \rho_{C_1} \cdot \cos^2 \left( \theta_{pz\beta} \right) ; B_1 = 147-162 \text{ MHz} \\
  \Leftrightarrow \theta_{pz\beta/11\beta} &\approx \left( \arccos \left[ a_{iso}(C_{\beta}-H) \cdot (B_1 \cdot \rho_{C_1})^{-1} \right] \right)^{1/2} 
\end{align*}
\]  
\text{(5-1)}
Multifrequency EPR Characterization of 2,3,5-F₃Y₁₂₂•

Figure 5-2: EPR spectra of 2,3,5-F₃Y₁₂₂• at different observing frequencies (9, 94 and 263 GHz). The experimental trace (red) and the simulation (black) are compared based on the same simulation parameters (Table 5-1). The black dashed lines show the HF coupling pattern. The canonical g values are marked as blue dashed lines in the 94 and 263 GHz ESE spectra. Right: The 2,3,5-F₃Y• and the HF coupling pattern for B₀||g₁ is shown. Exp. details: 9 GHz: CW-EPR spectra of 2,3,5-F₃-Y₁₂₂• in H₂O at 80 K, MA=1 G, conversion time=80 ms, power attenuation = 17 dB, scans=16; 94 GHz: ESE spectrum of 2,3,5-F₃-Y₁₂₂• in D₂O (50 µM) at 10 K, π/2(π)=30(60) ns, τ =240 ns, SRT=200 ms, number of averages = 16; 263 GHz: ESE spectrum of 2,3,5-F₃-Y₁₂₂• in D₂O (50 µM) at 10 K, π/2(π)=60(120) ns, τ =290 ns, SRT=20 ms, number of averages =1950. The 263 GHz spectra show a 3% Mn²⁺ contribution (g_{eff}= 2.00110) with an HF coupling of A_{Mn} ~ 94 G marked with an asterisk (*). The derivative of the pulse spectra was built using a 10 points second order Savitzky-Golay filter.
The g-values obtained by simulation are a source of information about the local electrostatics. In this case, the environment around Y_{122} is reported to be apolar and influenced by van der Waals interactions. To separate the effect from the UAA 2,3,5-F₃Y and the local polarity of the environment around 2,3,5-F₃Y_{122} a DFT calculation delivered g values. The environment around Y_{122} has a lower polarity as other radicals discussed up to now. A continuum polarization of chloroform (ε=4) was used in agreement with previous DFT calculations. The g tensor shown in Figure 5-3 and its values are summarized in Table 5-1. The g tensor is identical within error to the simulation. The β-methylene in the final model was chosen similar to the McConnell estimate θ_{Cβ}=55°. In this case an a_{iso} of 47 MHz is within uncertainty identical to the experimentally obtained β-methylene HF coupling (a_{iso} = 45 MHz). However, other HF couplings, like 3-F and 2-F, are generally too large and too less dipolar. In order to discriminate the error between neglected contributions of the protein and DFT more sophisticated calculations would be necessary. This is not within the scope of this thesis. Here one could clearly show that a difference in conformer change and the already adjusted polarity around Y_{122} is in reasonable agreement with the experiment.

Table 5-1: Summary of obtained g values, C-β HF couplings, and ^19F HF couplings. The results from the simulation are compared to results reported earlier from 9 GHz EPR spectra and with a small DFT Calculation (Figure 5-3).
2,3,5-F₃Y₁₂₂• has a similar environment as Y₁₂₂• including a small change in ring dihedral (<10°). This could be obtained from the multi-frequency EPR work supported by a small DFT model. The small structural changes might shed light on how 2,3,5-F₃Y₁₂₂ is able to omit the conformational gating. On the one hand, this UAA has a lower pKₐ with 6.4 compared to 9.9 and could be deprotonated. On the other hand the protein has shown no deprotonation of NO₂Y₁₂₂ mutant at this position, even if the pH of the protein buffer was altered up to 2.5 units above Acyl-NO₂Ys solution pKₐ. A conformeric change could be another plausible explanation. Thus, the reason for an omitted conformational gating is still open to further studies, which should consider the small change in backbone dihedral. Ideally the diagonal distances of the two β₂-Y₁₂₂• should be compared with two β₂-2,3,5-F₃Y₁₂₂• in the resting β₂ and in the “active” β₂:α₂ complex. The former is an experiment that can be directly done; for the latter one, a rigorous filter of the individual contributions has to be developed.

Figure 5-3: DFT model of 2,3,5-F₃Y₁₂₂•. A) The principal axes of the g and one exemplified prolate ¹⁹F HF tensor are illustrated by green arrows on C₄ and F₅, respectively. In this model the oxygen has 35% and the C 34% spin density population (Mullikan). DFT Calculation details: UB3LYP, def2-TZVPP; COSMO(ethanol); converged to 10⁻⁹ Eh. B) Dihedral angles θₐ and θₚz discussed here are depicted along Cβ-C₁. C) Y₁₂₂ and its dihedral angle θ₂β from the crystal structure is shown.⁶³
5.3 Characterization by Forward PCET Y• Formed with 2,3,5-F₃Y₁₂₂•

5.3.1 Electrostatic Environment

This section starts with the forward PCET transfer, because many results indicated that the intermediate NH₂Y₃₅₆• accumulates during forward PCET.⁶⁷, ⁸³, ⁹¹ The relative reaction potential to the Y• intermediates in α was identified for a Y₃₅₆• previously.⁸⁴ Derived by the analogy to NO₂Y₁₂₂•: α₂-Y₇₃₁F and 2,3,5-F₃Y₁₂₂•:α₂-wt is was expected to find Y₃₅₆• here.²⁸, ⁸⁴

The reaction was performed as described previously for the NH₂Y's, i.e., it was quenched in liquid nitrogen at two time points 10 and ≈20 s.

The spectra of the obtained pathway radical are shown in Figure 5-4. Both spectra show a typical tyrosine line shape with a pseudo quintet (doublet of triplets) at B₀∥gₓ, a doublet at gᵧ and large doublet with a doublet from doublets at gₑ. These features arise from the isotropic β-methylene HF coupling and an anisotropic 3/5−¹H HF coupling. Where the 3/5−¹H coupling is unresolved at B₀∥gₓ, it is only half the size of the β-methylene HF coupling collinear to gₓ. The factor between the isotropic coupling and the 3/5−¹H coupling is about 2.5 at B₀∥gₓ. Taking this into account the spectra could be simulated as shown in Figure 5-4 (gray). The simulation parameters are identical within error to the ones observed for a putatively forward Y₃₅₆• at 140 GHz, as shown in Table 5-2.⁸⁴ It should be noted that the error in HF couplings in this thesis is smaller than reported previously.⁸⁴ A β-methylene aiso of 47 MHz was obtained here. Typical spin densities ρ_C₁ of tyrosines in polar environments are reported for Y_D•₃¹₁₆-,₃¹₇-, Y_Z•₃¹₈- and Y•₃¹₉-,₃₂₀ in water values with 0.37₃¹₁₆-,₃¹₇-, 0.34₃¹₉-,₃₂₀. Using Eq. (5-1) a β-methylene angle θ_pzβ of 14-28° (θ_Cβ=42-56°) is obtained. A geometry illustrated in Figure 5-3B by θ_pzβ = 25°. To summarize, we can report to have trapped a radical species with a very similar g and HF tensor as observed previously within ≈15 s with the β-NO₂Y₁₂₂ mutant.⁸⁴ A different value of aiso(β−¹H ) could arise from the simulation of the broad lines observed by Yokoyama et al.⁸⁴ The reaction in this thesis uses 2,3,5-F₃Y₁₂₂•, which is uncoupled from the oxidation of the Y₁₂₂ mutant. The radical yield of 10 and 23 s was between 35 and 40%.
The later time point seems to have an additional contribution to the line shape compared to the radical quenched at 10 s. This will be discussed later after introducing the radical formed with wild type α (§ 5.4).

Figure 5-4: 94-GHz EPR spectrum of PCET radical formed with β2-2,3,5-F₃Y₁₂₂:α2-Y731F in deuterated buffer. Three reaction time points 10 s (blue), 20 s (red) and 23 s (green) are compared to the simulation (gray, Table 5-2). The inset on the right side shows a Y•. Exp. details: 100 K ESE; π(π/2)=28/32/48(14/18/24) ns; τ =267/227/272 ns; SRT = 5 ms; 6000/600/600; blue & green: 3 G pseudo modulation, red was gained by a 15p second order Savitzky-Golay filter, green trace was additionally smoothed by 10 points.

In order to obtain high restraints for the simulation parameters the parameter set was concomitantly simulated with a Q-band spectrum (Figure 5-5). The Q-band spectrum shows an overlap of the spectral components; for instance part of the pseudo quintet is now observed at B₀∥gₒ. This helps to weight effects from the g values and the coupling size. The simulation aligns well with the spectrum, only at the high-field side (1199.8-1200.7 mT) the spectrum is distorted by the quartz peak from the EPR sample tube. Here the simulation shows discrepancy to the experimental EPR line-shape.
Figure 5-5: 34-GHz EPR spectrum of the PCET radical formed with $\beta_2$-$2,3,5$-$F_3Y_{122}\alpha_2$-$Y_{731}F$ in deuterated buffer. The derivative (blue) is compared to the simulation (cf. Table 5-2). Exp. details: Reaction time 18 s, then 20% glycerol-d$_3$, quenched at 41 s in ice cold isopentane; ESE at 80 K, SRT = 1 ms, $\pi = 40$ ns, $\tau = 210$ ns, number of averages = 600, Savitzky-Golay (8 points, second order) filtered.

Table 5-2: Pathway radical observed with $\beta_2$-$2,3,5$-$F_3Y_{122}\alpha_2$-$Y_{731}F$ simulation parameters and comparison to Yokoyama et al.$^{84}$ The error is estimated with 10% for the HF couplings (>15 MHz) and is given in parenthesis for the last displayed digit for the g values.

<table>
<thead>
<tr>
<th>$\beta_2$-$2,3,5$-$F_3Y_{122}\alpha_2$-$Y_{731}F$ [this thesis]</th>
<th>$A_x$ [MHz]</th>
<th>$A_y$ [MHz]</th>
<th>$A_z$ [MHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_x$</td>
<td>$g_y$</td>
<td>$g_z$</td>
<td>$\beta$-$^1$H</td>
</tr>
<tr>
<td>$g$ values</td>
<td>2.0072(1)</td>
<td>2.00446(5)</td>
<td>2.0022</td>
</tr>
</tbody>
</table>

| $\beta_2$-NO$_2$Y$_{122}$ $\alpha_2$-$Y_{731}$F$^{84}$ |
|------------------|-------------|-------------|-------------|
| $g_x$ | $g_y$ | $g_z$ | $\beta$-$^1$H | 54 | 52 | 54 |
| $g$ values | 2.0072(5) | 2.0044(1) | 2.0022(4) | $3/5$-$^1$H$^+$ | 4 | 18 | 26 |

a) The Euler angles are $\alpha,\beta,\gamma = 90, 90, \pm 20$, for the definition $|A_x|<|A_y|<|A_z|$.

For tyrosine radicals, it has been reported that the g shift is in most cases influenced from H bond interactions as already discussed in the previous chapter. A DFT study of $p$-methylpenoxy radical on its dependence of a single H bonding distance has led to an empirical formula, based on a least square fit of the obtained data.$^{143}$ This formula (5-2) is displayed below and should govern our expectations.
Here the $g_x$ value is directly dependent on the H bond distance $r$ in Å. Thus the expected value would be with 1.72 Å, which is rather short. However, this formula assumes a single H bond as the sole origin of electrostatic effects.

$$ g_x = 2.0094 - \frac{0.0033}{(r_{O-H} - 0.5)^2} \quad (5.2) $$

Up to now, the EPR spectra recorded in deuterated buffer were discussed, because they facilitate the $g$ value determination by sharpening the EPR spectrum. Based on the expectations one would expect similar results for the reaction performed in H$_2$O buffer but modified by a coupling of exchangeable protons. As discussed earlier, the gyromagnetic ratio is 6.5 higher thus the coupling size increases directly proportional [cf. Eq. (2.14) p.34]. The spectra were recorded under the same reaction conditions only in an H$_2$O buffer. For further tests 15 mM MgSO$_4$ required for nucleotide reduction was exchanged stoichiometrically by $^{25}$MgCl$_2$. The results in Figure 5-6 show only minor changes in the line shape. Couplings up to the size of ~0.3 mT are normally not resolved, but still contribute to line broadening as observed here.

![Figure 5-6](image.png)

Figure 5-6 Comparison between radical formed from β2-2,3,5-F$_3$Y$_{122}$:α2-Y$_{731}$F in H$_2$O (blue) and D$_2$O (green). Exp. details: H$_2$O ESE spectrum at 80 K; quenched at 15 s, $\pi(\pi/2)$=56(28) ns, $\tau$=240 ns, SRT= 6 ms, number of averages = 6300. For parameters to the D$_2$O spectrum, see Figure 5-4 with 10 s reaction time.
5.3.2 Assignment of the Pathway Radical by the Diagonal Distance to 2,3,5-F₃Y₁₂₂•

One way to characterize this pathway radical is to measure its location along the PCET. This has been done for several radical intermediates along the pathway. Thus, we can easily compare these results, as illustrated in Figure 1-8 (p.12).

The differences of the two simulations parameters between this pathway radical and previous reported Y₃₅₆• require additional confirmation of the assignment to residue β-356.⁸⁴

Thus, Q-band distance measurements (DEER/PELDOR) were performed to obtain experimental evidence for the assignment of the observed radical species to β-Y₃₅₆•. In order to maximize the S/N Q-band was used instead of X-band PELDOR spectroscopy.²⁸²-²⁸⁴ To compensate the effect of an incomplete spectral excitation, three experiments at 3 different field points (1-3) for pump (P) and detect (D) were performed (Figure 5-7A). At all field points a pronounced oscillation frequency was observed (Figure 5-7B). The averaged dipolar oscillations can be Fourier transformed; a pake pattern results with a perpendicular frequency, resulting in a distance of 3.06±0.03 nm (Figure 5-7C, Inset).

The averaged time traces can also be fitted (Eq. (2-18), Figure 5-7C) under a Tikhonov regularization²⁸⁷ procedure a distance distribution as a probability function of distances is obtained (Figure 5-7D, DeerAnalysis).²⁸⁶ Here a full pake pattern is assumed, but the value is still within the error of the pake pattern distance. The distance for this system was obtained with 3.04±0.06 nm (Figure 5-7C), identical within error to value obtained for Y₁₂₂• to the Dopa₃₅₆• (3.05±0.06 nm) or NH₂Y₃₅₆• (3.02±0.16 nm)²⁸, p.177. And also identical to the value reported by Yokojama et al. for NO₂Y₁₂₂• to Y₃₅₆• (3.01±0.04 nm).⁸⁴

In summary, the same forward radical distance is observed as reported in the millisecond time scale by Yokojama et al.⁸⁴ This distance has already been assigned to several UAA at the 356 position and can be assigned to β²-2,3,5-F₃Y₁₂₂• -- β¹-Y₃₅₆•. ²⁸, p.177, ⁴³ Noteworthy, also the β²-NO₂Y₁₂₂• -- β¹-Y₃₅₆• distance is stable up to the minute time scale.⁸⁴ Thus, despite the decay of roughly 10% of radical content in the second timescale the radical position is in respect to the diagonal distance to residue 122 stable.²⁸ Additionally, the PCET disruption at position 731 by phenylalanine does not change the distance compared to other reports of diagonal distances between the 122 and 356 position in β (cf. Figure 1-8, p.12). If Y₇₃₁ is H bonded to Y₃₅₆ a difference between α₂-wt and α₂-Y₇₃₁F should be observable in the ²H ENDOR spectra.
Characterization by Forward PCET Y• Formed with 2,3,5-F₃Y₁₂₂•

Figure 5-7: 34-GHz diagonal distance measurement with the pathway radical produced by β₂-2,3,5-F₃Y₁₂₂: α₂-Y₇₃₁F. A) ESE spectrum at 40K (violet) and spectrum of pathway radical alone at 70 K (red) are shown together with the pump and detect positions in the DEER traces. Three consecutive measurements are spaced by 11 G. Pump (P1, P2 and P3; π = 56 ns) and detect (D1, D2 and D3; π = 46 ns) are separated by 54 MHz. The dipolar oscillations (green = 1, blue = 2 and red = 3) in B are illustrated. The dipolar oscillations are normalized and averaged to form C. From Fourier transformation a dipolar coupling pattern is obtained the perpendicular component (red, •) of the powder pattern has a frequency of 1.82±0.05 MHz (C, inset). This frequency results to an interspin distance of 3.06±0.03 nm using Eq. (2-18). This trace was fitted to obtain a distance distribution D. The main observed distance is 3.04 ±0.06 nm. The distances >3 nm (frequencies < 0.8 MHz) are in respect to the recorded dipolar oscillation not in the reliable distance range. Therefore they are regarded as unresolved in the pake pattern.

5.3.3 Mims ENDOR of Forward Radical Transfer to Y₃₅₆•

The $g_x$ value of β₂-Y₃₅₆•:α₂-Y₇₃₁F is comparable with the $g_x$ value of Y₀• in the photosystem II, for which a value of 2.0076 has been reported.³²¹ It has been stated that Y₀• has only one H bond,³²² also the wide doublet tyrosine of prostaglandin-H₂-synthase harbor a single H bond ($g_x=2.0075$).¹⁶⁰ Thus one stronger H bond or a contribution of a second ¹H HF coupling would be expected in these Y₃₅₆• ENDOR measurements. The ENDOR spectra in Chapter 4 show that the large couplings can be measured with an inter pulse delay $\tau$ of

2,3,5-F₃Y₁₂₂• to Generate Y• Intermediates in the PCET
200 ns equivalent to 5 MHz modulation through the Mims whole conditions (Theory §2.2.3 p.40). Thus a range of strong couplings from ±0.5 to ±2 MHz is unsuppressed. The recorded spectra are shown in Figure 5-8. The spectra show a complete dipolar tensor \( A_\parallel = -2A_\perp; A \approx T \) shape as typical for H bonds not interacting with nodal plane of the \( \pi \) system (see Figure 2-12 p.46). The values of the resolved turning points in the pake pattern of the HF interaction are ±0.6 MHz and ±0.3 MHz. It should be mentioned, that they are astonishingly similar to the values observed for yeast RNR with a 0.5 ppt larger \( g_x \) value.\(^{55}\) In the comparison to the \(^2\)H MIMS ENDOR spectra of yeast RNR and \( Y^\bullet \) with a comparable \( g_x \) values (2.0076±0.0001) as yeast RNR, it becomes evident that their central region is occupied by a Mims hole (cf. Table 1-1, p.24).\(^{55,155}\) In contrast the central line in the region of ±0.2 MHz has an intensity twice the size compared to the ±0.3 MHz peaks. This could indicate another not completely resolved weaker H bond. The assignment of the direction of the here detected proton, could specify the direction of the H bond acceptor during forward PCET between \( Y_{122} \) and \( Y_{356} \).

Figure 5-8: \(^2\)H ENDOR of \( Y_{356}^\bullet \) with \( a(Y_{731}F) \). Mims ENDOR spectrum was recorded for three samples with 10-23 s reaction time. All samples at \( B_\parallel g \) show an HF coupling consistent with a weak to moderate H bond and a smaller unresolved central line contribution. Exp. details: \( T=80\text{-}100 \text{ K } \pi/2=20 \text{ ns}, \tau=200 \text{ ns}, \tau_{RF}=40 \mu \text{ s} \), one SPP random RF acquisition; gray: 100 K, SRT=10 ms; red: 100 K, SRT=5 ms; green: 80 K, SRT=10 ms; acquisition time = 42, 23 and 25 h for blue, red and green, respectively. The blue curve is 10 points adjacent averaged.
5.3.4 Direction of the H Bond

The $^2$H Mims ENDOR spectra along each canonical orientation of the $g$ tensor are shown in Figure 5-9. The interpulse delay $\tau$ was adjusted to maximize the Mims ENDOR signal for the small coupling size (cf. Figure 2-10B p.43). Beside the central Mims hole, no other Mims holes are expected in the RF range investigated. The three orientations show three distinct spectra with an axial $T_\perp$ tensor component with resonances of $\pm 0.3$ MHz visible in all spectra, most sharply along $B_0 \parallel g_z$. In this spectrum, the parallel component $T_\parallel$ is absent with resonances of $\pm 0.6$ MHz. For $B_0 \parallel g_x$ the parallel component is shallow but visible, the spectrum. $B_0 \parallel g_y$ was shown before measured with a shorter $\tau$ value (200 ns) suppressing this small coupling range a bit stronger than for $B_0 \parallel g_x$. In this $B_0 \parallel g_y$ spectrum the parallel component is clearly visible and a complete powder pattern can be observed (see Figure 2-12 p.46). The spectra were first simulated with the starting parameters of yeast-RNR, due to the similarity in the size of the HF coupling. The simulation parameters, however, did not fit the obtained spectra. In yeast, an H bond approximately parallel to the C-O bond has been found, but the $B_0 \parallel g_x$ orientation (Figure 5-9) has only a weak parallel component. In a purely dipolar tensor, as assumed here, the parallel component is along the dipolar axis, thus the O--H vector should be along $g_y$, possibly with a minor contribution along $g_x$. Therefore an angle dependent simulation approach was used. It considers each orientation of the HF tensor with respect to the $g$ tensor within a resolution of $10^\circ$ for one octant of the principle axis $g$ tensor system (inset Figure 5-9). The quadrupole tensor is aligned along the H bond donor atom X-H bond (cf. Figure 4-19 p.103), thus it can be assumed to be parallel to the H bond direction and HF tensor for negligible protein structure restraints on the H bond donor. The best simulation has been selected and is shown in Figure 5-9. Simulations in which the parallel HF coupling component is approximately collinear to the $g_x, g_z$ plane could be considered. Therefore the simulated $^2$H HF tensor is from its orientation similar to the one reported in for Y$_D$•, by Kessen et al.\textsuperscript{155} (see Table 1-1, p.24).

To rationalize Euler angles between the $g$ and HF tensor principle axis systems (see Table 5-3) one can relate them to the H bond dihedral angle $\theta_{H2O}$ discussed before (§4.6.4.2 p.118). Then the $\gamma$ is the H bond angle (Hx--O--C$_4$), because the C$_4$-O bond also defines the $g_x$ axis in the PAS. $\beta$ is the H bond dihedral angle $+90^\circ$, as shown in Figure 4-10. The angle $\alpha$ has nearly no effect in the simulation and was set to $0^\circ$. The discrepancy of the simulation and the experimental data arises most probably from another smaller coupling in the range...
of ±0.25 MHz. This small coupling is not resolved from the matrix line and is therefore not assigned.

A single H bond geometry consistent with the obtained angles resembles a 130±15° H bond angle. In agreement to the H bond angle found with nearly no scalar (\(a_{iso}\)) contribution in previous H bond dihedral scans (§4.6.4.2 p.118). The H bond dihedral can range from -35-35° without a strong deviation in the fit. To visualize the effects a small DFT model with a \(\theta_{H2O}=30°\) (Figure 5-10) has been calculated. Here the HF coupling values are overestimated by a factor of 1.5, but the angles are within the error of the experiment.

Figure 5-9: Orientation selective \(^2\)H ENDOR of \(Y_{356}\)• with a-Y731F. The Mims ENDOR spectrum was recorded with \(B_0\) along \(g_x\), \(g_y\) and \(g_z\) (black). The simulation is shown beneath each trace in red. Exp. details: \(g_x\) and \(g_z\): \(T= 40\) K, \(\pi/2=36\) ns, \(\tau=400\) ns, \(\pi_{RF}= 40\) µs, one SPP random acquisition, SRT =10 ms, acquisition time = 24 & 32 h; \(g_y\): see Figure 5-8 (green). A line broadening of 45 kHz was used for the simulation. Further parameters are shown in Table 5-3.

Structurally, this has some implication compared to the work discussed in the NH\(_3\)Y’s (§4.2.2, p.83). This is the first spectroscopic evidence for an in-plane H bond at \(Y_{356}\)•. It has an essentially zero \(a_{iso}\) contribution. This excludes spin polarization through the \(p_z\) oxygen orbital, because the H bond is in the \(p_z\) orbitals nodal plane (cf. Figure 5-10). In \(Y_{356}\)• the stabilization of perpendicular H bond is absent, thus an energetically favored H bond is formed along the filled non-bonding \(\pi\) orbitals (see Figure 4-27D p.120).
Characterization by Forward PCET $Y\cdot$ Formed with $2,3,5$-$F_3Y_{122}\cdot$

Table 5-3: Simulation parameters of the unknown (X) H bond donor to $Y_{356}\cdot$. The Euler angle uncertainties are described in the text. The HF and quadrupole tensor has about 10% error. A DFT calculation with a water in the assumed $\theta_{H2O}=30^\circ$ is compared to the simulation. The DFT calculation has $g$ values of $(2.0077, 2.0045, 2.0022)$.

<table>
<thead>
<tr>
<th>$Y_{356}\cdot$</th>
<th>$X\cdot-D$</th>
<th>$A_x$ [MHz]</th>
<th>$A_y$ [MHz]</th>
<th>$A_z$ [MHz]</th>
<th>$\alpha$ [°]</th>
<th>$\beta$ [°]</th>
<th>$\gamma$ [°]</th>
<th>$Q_x$ [kHz]</th>
<th>$Q_y$ [kHz]</th>
<th>$Q_z$ [kHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulation</td>
<td></td>
<td>-0.44</td>
<td>-0.46</td>
<td>0.91</td>
<td>0</td>
<td>120</td>
<td>130</td>
<td>-54</td>
<td>-72</td>
<td>126</td>
</tr>
<tr>
<td>$\theta_{H2O}=30^\circ$</td>
<td></td>
<td>-0.77</td>
<td>-0.78</td>
<td>1.34</td>
<td>-10</td>
<td>104</td>
<td>138</td>
<td>-54</td>
<td>-74</td>
<td>128</td>
</tr>
</tbody>
</table>

a) Calculation details: B3LYP; basis set def2-tzvpp/EPRII. The polarizability of ethanol was used and dispersion correction. Both basis sets gave within 0.1 ppt or 5% identical HF and $g$ values. b) Obtained with point-dipole approximation (§5.3.5).

Figure 5-10: DFT model of a $Y\cdot$ with one water molecule. A restrained geometry optimization was performed with H bond dihedral $\theta_{H2O}=30^\circ$. An H bond angle (C-O-H$_{wat1}$) of 130° was obtained.

5.3.5 $Y_{356}\cdot$ H bond Length

In the presence of a purely dipolar HF interaction a distance from the electron spin dipole to the nearest point charge can be approximated. Here oxygen is the nearest spin density bearing nucleus and has a $\rho_O$ reported for $Y_D\cdot$,$^{316,317,323}$ $Y\cdot$,$^{319,320}$ in water and $Y_Z\cdot$,$^{315,318}$ to be between 0.28 and 0.25. One obtains a distance of 1.87-1.95 Å for the H bond by Eq. (2-17) (p.35). The nucleus is approximated as a point charge. Hence, the obtained distance is in tendency estimated as too large (≈5%).$^{153,295}$ Nevertheless, this is a moderate to weak H bond. It could be shown before that the H bond distances increase with the number of H bonds (§4.6.4.3 p.121).$^{110}$ In the similar case of $Y_D\cdot$ with an stronger H bond, but the same angle dependence, no matrix line or inner coupling was observed. Also the $g$ value is
0.4 ppt higher than observed for β-Y_{356}•:α-Y_{730}F. The ²H coupling connected to the central line is therefore a most likely more distant.

This has been qualitatively supported by a small DFT model. It considers two water molecules (wat 1&2) in the π-plane and a Y•. The wat2 is scanned for its distance from 2.4 to 2.2 Å. To insure a fast convergence the ring dihedral (θ_{Cβ}=55°) and the dihedral to the first water (θ_{wat1}=20°) was kept constant. The values are summarized in Table 5-4 below. To reproduce the g_x value exact a ²H within a distance of 2.2 Å is necessary. However, all distances have g values within the uncertainty of the DFT. For wat2 with an H bond distance of 2.2 Å, an HF tensor of A_{xyyz}=-0.3, -0.4, 0.8 MHz was found. A perpendicular component of 0.4 MHz could be still unresolved between the observed matrix line width and the larger HF coupling. Wat2 can occupy every orientation toward a Y•, except for orientations exceeding the matrix line width. For instance the line width at g∥B_0 is smaller than 0.4 MHz, therefore A_z and Q_z parallel to g, can be excluded. The HF tensors of wat1 are in agreement with the overestimation of the H bond HF couplings by DFT calculations. This has been observed previously.¹¹⁰, ²⁵⁴

Table 5-4: Small DFT model using a Y• and two waters scanning the distance of a distant of the second water (wat2). ρ_O was 0.34 after Muliken population analysis.¹¹², ³¹³. The H bond dihedrals were kept constant with 20° (wat1) and 167° (wat2). The H_{wat-O-C_4} angle stayed constant with 117° (wat1) and 123° (wat2). The Q_z value was either 130 kHz for wat1 or 142 kHz for wat2.a

<table>
<thead>
<tr>
<th>No.</th>
<th>O--H Distance [Å]</th>
<th>A_x [MHz]</th>
<th>A_y [MHz]</th>
<th>A_z [MHz]</th>
<th>Q_z [kHz]</th>
<th>g_x</th>
<th>g_y</th>
<th>g_z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H_{wat1}</td>
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<td>-0.63</td>
<td>-0.69</td>
<td>1.24</td>
<td>126</td>
<td>2.0072</td>
<td>2.0046</td>
</tr>
<tr>
<td></td>
<td>H_{wat2}</td>
<td>2.20</td>
<td>-0.33</td>
<td>-0.38</td>
<td>0.78</td>
<td>142</td>
<td>2.0073</td>
<td>2.0046</td>
</tr>
<tr>
<td>2</td>
<td>H_{wat1}</td>
<td>1.82</td>
<td>-0.63</td>
<td>-0.69</td>
<td>1.24</td>
<td>126</td>
<td>2.0072</td>
<td>2.0046</td>
</tr>
<tr>
<td></td>
<td>H_{wat2}</td>
<td>2.30</td>
<td>-0.30</td>
<td>-0.34</td>
<td>0.70</td>
<td>142</td>
<td>2.0073</td>
<td>2.0046</td>
</tr>
<tr>
<td>3</td>
<td>H_{wat1}</td>
<td>1.81</td>
<td>-0.64</td>
<td>-0.70</td>
<td>1.25</td>
<td>126</td>
<td>2.0074</td>
<td>2.0046</td>
</tr>
<tr>
<td></td>
<td>H_{wat2}</td>
<td>2.40</td>
<td>-0.27</td>
<td>-0.31</td>
<td>0.64</td>
<td>146</td>
<td>2.0074</td>
<td>2.0046</td>
</tr>
</tbody>
</table>

a) Calculation details: UB3LYP, def2-TZVPP, COSMO(Ethanol), RICOSX, Grimme dispersion corrected. The uncertainty of the coupling values is estimated with 20%. C_{β-1H} HF coupling stayed constant with d_{norm}=55 MHz.
5.4 Equilibrium PCET Radical $Y_{356}^\cdot$ Formed during Reverse PCET

5.4.1 Multi-frequency Characterization

The pathway radical formed in the reaction of $\beta_2$-2,3,5-F$_3$-Y$_{122}$ and $\alpha_2$-wt with substrate (CDP) and effector (ATP) has been quenched in the second timescale. EPR spectra of the putative forward radical transfer and that in PCET equilibrium can then be compared directly. The radical formed in PCET equilibrium can be observed without the disturbance of the mutation ($Y_{731}F$) in $\alpha$, as in the previous section. Therefore the formed $Y_{356}^\cdot$ is also relevant to the discussion of the forward radical transfer. The 94 GHz EPR spectrum Figure 5-11A has the same pattern at $B_0\parallel g_x$ and $g_y$, only at $B_0\parallel g_z$ shows only a dominant $\beta$-methylene HF coupling. To constrain the HF couplings further a 34 GHz spectrum (Figure 5-11B) was recorded, then the two frequencies were simulated simultaneously. Simulating the spectra with one parameter set (Table 5-5) gave a reasonable fit of the experimental trace (Figure 5-11, gray). In addition to the simulation set for a $Y_{356}^\cdot$, a 10% additional contribution of $Y_{731}^\cdot$ and $Y_{730}^\cdot$ was taken into account. This contribution is only observable at the low field side of the 94 GHz and 9 GHz spectrum. Based on the knowledge from the 3-amino tyrosine the $g_z$ value it was assumed to be larger than in the $Y_{356}^\cdot$ case. A value of 2.0078 has been used, other values were not resolved therefore the suggested parameters of Yokojama et al. were used as parameters. Also the parameters of $Y_{356}^\cdot$ along the reverse PCET are identical within error to the published results (Table 5-5). Thus only a single Q-band DEER trace was recorded, to verify the diagonal distance reported for radicals at $Y_{356}$ (Appendix: Figure A - 9, p.200). This is an additional evidence for assigning the observed main pathway radical species to $Y_{356}^\cdot$. 

2,3,5-F$_3$Y$_{122}^\cdot$ to Generate Y• Intermediates in the PCET
Figure 5-11: EPR spectra (blue) and simulation (gray) of the pathway radicals using β2-2,3,5-F₃Y₁₂₂:α₂-wt at two frequencies. The 94 GHz and 34 GHz EPR spectra in D₂O buffer at 80 K are shown left and right, respectively. The simulation parameters are shown in Table 5-5. Exp. details: ESE, 94 GHz: π=40 ns, τ= 270 ns, SRT= 6 ms, the reaction was quenched at 11 s in liquid nitrogen; 34 GHz: ESE, π=40 ns, τ= 220 ns, SRT= 5 ms. The reaction was quenched in ice cold isopentane after 41 s, at 10 s 20% glycerol-D₃ was added. The derivative was obtained by 3 G pseudo modulation or by a Savitzky-Golay (5 points, second order) filter for 94 GHz and 34 GHz, respectively.

Table 5-5: Simulation parameters of the pathway radical observed with β2-2,3,5F₃-Y₁₂₂ and wild type α2 in comparison to Y₃₅₆• formed with β2-NO₂-Y₁₂₂:α2-wt. The errors for the g values were estimated and are given in parenthesis for the last shown digit. The error for the HF values >15 MHz are 2 MHz, the errors for the smaller couplings are estimated to be approximately 3 MHz.

<table>
<thead>
<tr>
<th></th>
<th>gₓ</th>
<th>gᵧ</th>
<th>gᶻ</th>
<th>β⁻¹H</th>
<th>Aₓ [MHz]</th>
<th>Aᵧ [MHz]</th>
<th>Aᶻ [MHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₂-2,3,5F₃Y₁₂₂:α₂-wt [this thesis]</td>
<td>2.0063(1)</td>
<td>2.0045(1)</td>
<td>2.0022(1)</td>
<td>61</td>
<td>52</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>g values</td>
<td>2.0063(1)</td>
<td>2.0045(1)</td>
<td>2.0022(1)</td>
<td>3/5-¹H⁺</td>
<td>-5</td>
<td>-21</td>
<td>-24</td>
</tr>
<tr>
<td>β₂-NO₂Y₁₂₂:α₂-wt³⁴</td>
<td>2.0064(2)</td>
<td>2.0044(2)</td>
<td>2.0022(3)</td>
<td>61</td>
<td>52</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>g values</td>
<td>2.0063(3)</td>
<td>2.0044(2)</td>
<td>2.0022(3)</td>
<td>3,5-¹H⁺</td>
<td>4</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>

a) The Euler angles are α,β,γ = 90, 90, ±20, for the definition |Aₓ|<|Aᵧ|<|Aᶻ|.

2,3,5-F₃Y₁₂₂• to Generate Y• Intermediates in the PCET 147
Interestingly, although $Y_{356}^\bullet$ is formed with α-wt and α-$Y_{731}F$ they have a significantly different $g$ values and β-methylene HF couplings with $a_{iso} = 47$ MHz to $a_{iso} = 56$ MHz, they have the same diagonal distance to $Y_{122}^\bullet$. Hence, either the spin density changes, the ring dihedral angle $\theta_{C\beta}$, or a combination thereof. Studying the semi-empirical McConnel Eq. (5-1) Svistunenko et al. demonstrated that the oxygen spin density population is inversely proportional to McConnel estimate of $C_1$ spin density population. An increase of 20% in spin density population could explain the difference in β-methylene HF coupling. A value of $\rho_{C1}$ between 0.41 and 0.45 would be obtained from $\rho_{C1} = 0.34$-0.38 used for the forward PCET $Y_{356}^\bullet$. The larger value is comparable to the spin density population $\rho_{C1} = 0.44$-0.45 observed in a recent $Y_{2}\bullet$ DFT study with $g_x$ between 2.0055 and 2.0063. A change in dihedral angle $\theta_{C\beta}$ would correspond to 12-22° change for an oxygen spin density population in the range of 34%-38%.

The spectrum recorded for the reaction in H$_2$O buffer shows the same line shape as the spectrum in deuterated buffer (Figure 5-12). The broadening due to D/H exchange is significantly stronger than in the forward PCET $Y_{356}^\bullet$. This can partially be explained by the more intense shoulder at the high-field side of the spectra previously assigned to a contribution of $Y_{731}^\bullet$ and $Y_{730}^\bullet$. The $A_\beta$ coupling is decreasing slightly as observed at $g_z$. And the $g_x$ and $g_y$ values seem to be slightly shifted between the two spectra below. Overall the concomitant change of time point and medium contribute to this spectrum. Here a rigorous investigation of different time points, preferentially by rapid freeze quench would bring more insight. Furthermore we will consider the single spectrum at 11 s. This 11 s sample is further used to record ENDOR spectra, which can be used to further investigate the different spectral line shapes observed here.
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

Figure 5-12: Comparison between an H₂O and a D₂O spectra of the Y₃₅₆• formed β₂-2,3,5F₃-Y₁₂₂• α₂-wt. W-Band ESE spectrum of the reaction of 2,3,5-F₃-Y₁₂₂•(β) and wild type(α) with CDP/ATP. ¹Hβ HF coupling is marked at g with Aβ. Exp. details: Blue: see Figure 5-11; Green: T= 80 K, π(π/2)=56(28) ns , τ= 614 ns, number of averages = 3150. The derivative was built with a Satitzky-Golay filter (20 points, second order).

5.4.2 Mims ENDOR of Y₃₅₆• Using Wild Type α

In order to understand the g-shift of 0.9 ppt (compared to Y₃₅₆•:α₂-Y₇₃₀F) Mims ENDOR spectroscopy was performed for the radical transfer in equilibrium. A larger coupling is expected than observed for the radical in §5.3.3. Therefore the measurement shown in Figure 5-13 was recorded with a larger window for couplings of up to ±2 MHz. With the τ setting of 200 ns we create certain blind spots, thus a second τ value was measured with shifted blind spot criteria (3.3 MHz modulation, cf. Theory §2.2.3, p. 40). Nevertheless, identical spectra are obtained (5.13). The same coupling strength as before (§5.3.3) with ±0.6 MHz for the parallel component is observed. Hence, the strength of the coupling apparently does not correlate with the g value shift. The number of H bonds, however, could correlate to the g value shift. In the amino tyrosine, the ¹H ENDOR intensities relative to each other for inter- and intra-molecular (NH₂) nuclear couplings observed. For tyrosines in D₂O buffer spectra only the anisotropic coupling and the matrix line can be observed. It can
Equilibrium PCET Radical Y\textsubscript{356}\textsuperscript{•} Formed during Reverse PCET

be stated, however, that compared to the Y\textsubscript{356}\textsuperscript{•}:\alpha\textsubscript{2}-Y\textsubscript{730}F spectra with the blocked pathway the parallel component of the hyperfine is stronger and both turning points (± 0.6 and 0.3 MHz) are slightly sharper. This could be an indication of a second anisotropic deuteron coupling.

![Graph: B_0 \parallel g_y vs ENDOR Frequency]

Figure 5-13: \textsuperscript{2}H ENDOR of Y\textsubscript{356}\textsuperscript{•} with wild type \alpha. The pathway radical formed after 10 s was measured at 70 K with two different \tau\ values 200 ns (red) and 300 ns (blue) to ensure a broad coverage of possible resonances. The spectra show the B_0 \parallel g_y only a weak to moderate H bond and a matrix line. Exp. details: Mims ENDOR, T= 70 K \pi/2=20 ns, \tau=200-300 ns, one SPP random acquisition, SRT=11 ms, acquisition time = 24 and 48 h. The traces have been smoothed by 5 (red) and 6 (blue) points adjacent averaging.

The form of the tensor indicates that both H bonds should be within the plane of the tyrosine (±30°, g_x, g_y plane), otherwise a stronger non-dipolar contribution would be expected from the spectral analysis performed for the 3-amino tyrosines (Chapter 4). Orientation selective data can give spectroscopical evidence for an H bond along g_x or g_y as shown here (§5.3.4) or previously for instance in yeast RNR.\textsuperscript{55}

5.4.2.1 \textsuperscript{1}H ELDOR Detected NMR of Y\textsubscript{356}\textsuperscript{•} Formed during Equilibrium PCET

The unprecedented low \textit{g}_x value of 2.0063 has shown only distant H bonding protons (≈1.9 Å) for the Y\textsubscript{356}\textsuperscript{•} formed during PCET equilibrium. This seems contradictory at first. Although the effect can be rationalized a control was necessary to check if any larger couplings were not recorded due to line broadening or fast exchange conditions within the

2,3,5-F\textsubscript{3}Y\textsubscript{122}\textsuperscript{•} to Generate Y\textsuperscript{•} Intermediates in the PCET
S/N of the ENDOR measurement. Additionally, we only excited a part of the orientations at 94 GHz, thus couplings larger at other orientations could have been missed. At Q band, we were able to reach a nearly full pake pattern with one measurement, as shown by studies on amino tyrosines.\textsuperscript{92}

Here, the experiment was performed at 80 K to suppress any contribution of $\beta_2$-$2,3,5$-$F_3$-$Y_{122}$ in the spectra. The spin-lattice relaxation ($T_1$) at these temperatures is quite short compared to the long RF pulse. An HF detecting sequence, ELDOR detected NMR, has been often used in metallo proteins with intrinsically short relaxation times.\textsuperscript{186} It has been reported to be less affected from short spin-lattice relaxation times ($T_1$) as ENDOR (cf. Theory §2.2.4 p.43).\textsuperscript{135} Indeed, first ENDOR spectra showed the need for long accumulation to gain sufficient S/N.\textsuperscript{92}

The spectrum in Figure 5-14 was recorded with ELDOR detected NMR. It should be mentioned that the resolutions are not as high as in a comparable Davies $^1$H ENDOR spectrum, but ELDOR detected NMR is more sensitive.\textsuperscript{188,191} Thus measurement times up to 72 h could be omitted.\textsuperscript{92} Additionally, all internal tyrosine couplings contribute to this $^1$H HF spectrum, making an assignment to individual couplings speculative. However, in comparison between the reactions performed in D$_2$O and H$_2$O the spectrum is a valuable control for the estimated couplings sizes (blue, Figure 5-14). Both spectra are completely identical to each other within S/N. Only around the central line the deuterated sample has a sharper central line (5.1 MHz vs. 7.8 MHz). The line width increase is consistent with the coupling observed in the Mims ENDOR spectra of 1.2 MHz ($6.5 \times 1.2$ MHz $= 7.8$ MHz).

Thus, no stronger coupling was missed in the Mims ENDOR spectra, due to blind spots. The broad lines observed in the range of $\pm$(26-31) MHz are identified as beta methylene couplings, here the background subtraction leaded to a higher frequency uncertainty than in the central region of the spectrum. This is especially true at the edge of the experimental resonance conditions for the MW irradiation (DIP).
Equilibrium PCET Radical $Y_{356}$• Formed during Reverse PCET

Figure 5-14: Comparison of $Y_{356}$• with wild type α in protonated and deuterated buffer. The EDNMR centered at the $^1H$ Larmor frequency shows the resonances in $D_2O$ (blue) and $H_2O$ (red) spectrum. The same HF couplings are observed independently of the buffer, beside the $\beta$-methylene coupling (orange) and the broadened central line as discussed in the text. Exp. details: $\tau(\pi/2)=$200(100) ns; $\tau = 500$ ns; HTAELDOR pulse = 2 $\mu$s, SRT =2 ms, acquisition time = 14 h (red) 11 h (blue). The recorded spectra were baseline corrected by a spline function.

5.4.2.2 Correlating of the Number of H bonds to the g Value

The $g_x$ value is an indication for an electrostatic environment mainly around the phenoxy oxygen nucleus. H bonding is a major factor for electrostatic effects beside positive charges in the surrounding. Hence, one might correlate observed $g_x$ value with the number of H bonds. Depending on the number and the distance several H bond networks have been reported, for instance two studies in a highly polar electrostatic environment come close to the $g$ values observed for $Y_{356}$•:α2-wt (Table 5-6). Two H bonds have been reported previously in Y crystals formed in hydrochloric acid solution.161 And three H bonds have even been modeled for YZ• in PSII (Introduction §1.5.2, p.20).156,161

To reduce the number of parameters one can use the point dipole approximation from $Y_{356}$•:α2-Y731F. The distance derived in §5.3.5 (p. 144) can be applied, because even for lower $g_x$ values the oxygen spin density population was found identical to the values used for the distance calculation with $\rho_O = 0.25$.156 Hence, the H bond distance should be approximately $1.87 \pm 0.10$ Å.
As Table 5-6 illustrates, examples in the literature of two H bonds include at least one strong H bond (≈1.6 Å), but this is not in agreement with the derived distance for Y₃₅₆•:α₂-wt (1.87±0.10 Å). Two DFT models were set up to calculate the value of the HF coupling to the external proton in a mean distance of 1.84 Å (model 4) and 1.7 Å (model 5). The former report an HF coupling of $A_z = 1.2$ MHz the latter $A_z = 1.4$ MHz. The shorter distance has a significantly larger value than experimentally observed (0.91 MHz). The value calculated for the longer distance with 1.84 Å is within uncertainty (30%) still in agreement to the $^2$H ENDOR simulation. Both models are within uncertainty to the obtained $g$ values of Y₃₅₆• formed with α₂-wt. The $g_x$ value of both models is 0.2 ppt (model 5) and 0.5 ppt (model 4) larger than experimentally observed. Notably, the H bond environment described in Model 4 is similar to Model C designed for discussing polarity effects around NH₂Y₃₅₆• (§4.6.4.4, p.124).
Table 5-6: Comparison between experimental (white) and calculated (highlighted gray) $g$ values and, number and distances of H bonds.

<table>
<thead>
<tr>
<th>$g_x$ value</th>
<th>$g_y$ value</th>
<th>$g_z$ value</th>
<th>No. of H bonds</th>
<th>$r_{O-H}$ [Å]</th>
<th>Structure description</th>
<th>$T_1$'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0072(1)</td>
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<td>2.0022</td>
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<td>1.9(1)$^d$</td>
<td>$Y_{356}•:Y_{731}F$</td>
<td>-0.45</td>
<td>EPR: This thesis</td>
</tr>
<tr>
<td>2.0063(1)</td>
<td>2.0045(1)</td>
<td>2.0022(1)</td>
<td>≥two</td>
<td>1.9(1)$^d$</td>
<td>$Y_{356}•:wt$</td>
<td>-0.45</td>
<td>EPR: This thesis</td>
</tr>
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<td>2.00673</td>
<td>2.00453</td>
<td>2.00232</td>
<td>one</td>
<td>1.45$^c$</td>
<td>$Y_6•$ (tensed) His-H</td>
<td>-0.80</td>
<td>EPR$^{153}$</td>
</tr>
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<td>-</td>
<td>EPR on crystal$^{161}$</td>
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<td>2.0042</td>
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<td>1.60$^d$</td>
<td>Modeled: COOH function and a polarized HCl molecule</td>
<td>-</td>
<td>EPR on crystal$^{161}$</td>
</tr>
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<td>2.0044</td>
<td>2.0023</td>
<td>two</td>
<td>1.59</td>
<td>Polarized Hs and water molecules</td>
<td>-</td>
<td>DFT calculation$^{156}$</td>
</tr>
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<td>2.0023</td>
<td>three</td>
<td>1.59</td>
<td>Polarized Hs and 2 water molecules</td>
<td>-</td>
<td>DFT calculation$^{156}$</td>
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<td>2.0068(5)</td>
<td>2.0046(5)</td>
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<td>two</td>
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<td>2.0065(5)</td>
<td>2.0045(5)</td>
<td>2.0022(2)</td>
<td>two</td>
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<td>Model 5: 2 water molecules</td>
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a) The error or uncertainty of the values is given for the last digit in parentheses. b) H bond distance between phenoxy oxygen and proton. c) Averaged value in MHz; d) Derived by the point dipole approximation the error of 10% of the HF coupling is not considered. e) Estimated by a relaxed scan over H bond distances in a DFT model. f) Derived by McConnel Eq. $\rho_0=0.28$ g) from the reduced crystal structure. h) $^1$H HF couplings are $A_{yz}= -3.9$, -4.3, 7.8 MHz and $A_{xy}= -3.3$, -3.5, 7.4 MHz. i) Calculation details: UB3LYP, def2-TZVPP, COSMO(Ethanol), RICOSX, D3BJ Grimme dispersion corrected. The H bond distances 1.7 Å were restrained. The uncertainty of the coupling values is estimated with 20% and the uncertainty of the g values is estimated with 0.5 ppt. j) $^1$H HF couplings are $A_{yz}= -4.0$, -4.0, 8.7 MHz and $A_{xy}= -5.1$, -5.1, 9.2 MHz.
5.5 Discussion

5.5.1 Removing the Conformational Gating by 2,3,5-F₃Y

Initial studies demonstrated that β₂-2,3,5-F₃Y₁₂₂ is able to overcome conformational gating with potential difference of about 10 mV to Y₁₂₂. The potential difference is low enough that reverse PCET from β₂-Y₃₅₆• to β₂-2,3,5-F₃Y₁₂₂ could be observed for the first time. At first, it has been discussed that 2,3,5-F₃Y is deprotonated at the 122 position due to its pKₐ of 6.4. Then it was concluded that 2,3,5-F₃Y is protonated based on the inability to observe β₂-NO₂Y₁₂₂• within a pH dependent study (cf. §1.4.5, p.16). In this study, however, the reported solvent inaccessibility of Y₁₂₂ has not been considered. Thus it cannot be excluded that the protonation state of the buried 122 residue is unaffected by the buffer pH.

The EPR parameters of β₂-2,3,5-F₃Y₁₂₂• were compared to β₂-wt(Y₁₂₂•). EPR spectra were recorded at three different frequencies (9, 94 and 263 GHz) to determine g values and the tyrosine ring dihedral angle θ_{Cβ}. One set of simulation parameters has been found for all spectra. The 263 GHz EPR spectra reveal a gₓ value of 2.00832 (±0.00005), which is slightly shifted from the value of 2.00912 (±0.00005) found in wt Y₁₂₂•. A small model DFT calculation could reproduce the g value considering a polarity of ε=4 described previously for Y₁₂₂•. Notably, no H bond interaction has been considered in the model. In a combination of the empirical McConnel equation and DFT calculation the ring dihedral was determined to be θ_{Cβ}=55±5°. The reduced state in the crystal structure reports about θ_{Cβ}=46°, thus a ≈10° larger value is observed. On the other hand, the oxidized Y₁₂₂• displays a 1-5° smaller value than observed for reduced Y₁₂₂. How this structural change could influence the accessibility of residue 122 to the interface requires still a large DFT or QM/MM model of different conformational states taking the Cα-Cβ bond into account.

5.5.2 Comparison between Y₃₅₆• and NH₂Y₃₅₆• Forward PCET Radical Intermediates

Information on the radical transfer along the subunit interface is important to understand the PCET between the subunits and the activity control of RNR Ia. In E. coli RNR Ia several pieces of information have been gathered for the “stepping stone” of the PCET, residue 356. In the reduced state, pKₐ measurements indicated a solvent like environment for NO₂Y₃₅₆.
Discussion

independently from the formation of the αβ₂ “resting state” complex. The transient radical state reports about how the protein has reorganized directly after a single PCET step in the “active” αβ₂ complex. In the “active” αβ₂ complex the radical pair distance between residues β₁-356 and β₂-122 (PELDOR distance) of 3.0±0.1 nm is independent of mutation method and reaction time ranging from 8 ms up to a minute. EPR high-field data has reported that two different polar environments can be obtained at Y₃₅₆•. A combined examination of polarity and H bond network, however, has not been presented before.

As shown in the previous (§4.6 p.110) and in the current chapter, two different approaches have been pursued to characterize radical intermediates at residue β-Y₃₅₆. In the first approach the radical was competitively trapped using an NH₂Y mutant at residue 356 with 3-10 % residual activity. In the second approach a PCET blockade was used with an α₂-Y₇₃₁F mutant, which is inactive. Despite their differences, both radical intermediates formed at residue 356 inform us about H bonding and electrostatic environment. Also in the reaction of the β₂-2,3,5-F₃Y₁₂₂:α₂-Y₇₃₁F RNR the PELDOR distance is reproduced (cf. §5.3.2, p.139). Additionally, Y₃₅₆• and NH₂Y₃₅₆• have no perpendicular strong or moderate exchangeable H bonds. Perpendicular H bonds have been observed in the studies of the PCET transfer in the RNR α subunit (Chapter 4). The in-plane H bond characterized in Y₃₅₆• might be hidden under the wide spectral feature of the amino deuterons for NH₂Y₃₅₆•. The hyperfine tensor is proportional to the nearby phenoxy oxygen spin density population (ρₒ) due to the scaling of its dipolar part. Therefore an H bond observed in an Y₃₅₆• with (T₁=0.45 MHz) has a dipolar strength related to ρₒ. A value around 25-28% is reported for ρₒ. In contrast, the spin density population for NH₂Y₇₃₁• in the α subunit (Chapter 4). The NH₂Y₃₅₆• has a gₓ value (2.0049) slightly lower than the strongly H bonded NH₂Y₇₃₁• with (gₓ=0.29-0.38 MHz for NH₂Y₃₅₆• (see Eq. 2-17). The most prominent axial component T₁ in the spectra would be with ±0.2 MHz not resolved from the matrix line of NH₂Y₃₅₆•, which could explain the absence of clearly resolved H bond features in the 𝟑H ENDOR spectrum of NH₂Y₃₅₆•.

Furthermore, high frequency EPR delivers γ values for the forward radical transfer of NH₂Y₃₅₆• and β-Y₃₅₆•:α-Y₇₃₁F. The NH₂Y₃₅₆• has a γ value (2.0049) slightly lower than the strongly H bonded NH₂Y₇₃₁• in the α subunit (Chapter 4). The β-Y₃₅₆• with α₂-Y₇₃₁F has a γ value of 2.0072. Thus Y₃₅₆• is by 1.9 ppt more affected by electropositive charges than Y₁₂₂• and even 0.4 ppt more than yeast or mouse RNR Y₁₂₂• equivalent with one H bond.
Hydrogen Bonds and Electrostatic Environment of Radical Intermediates in RNR Ia

(1.8 Å). The value is not as low as the metastable “tensed” Y₃• state with 2.0064 with an H bond of approximately 1.5 Å (see §1.5.2, p.20). The electrostatic effect of NH₂Y₃₅₆• should correspond to a moderate to strong H bond (rFH 1.6 Å and 1.8 Å) and at least one moderate H bond (rFO 1.9 Å) found in NH₂Y₇₃₀ and NH₂Y₇₃₁ (see §4.4). This would imply for Y₃₅₆• another partially positive charge in <2 Å surrounding of the oxygen nucleus. However, this could not be observed for Y₃₅₆• with α₂-Y₇₃₀F. Here the g value is consistent with a weak H bond (1.8 Å) and another distant proton (≥2.2 Å, Table 5-4, p.145). A similar environment has been modeled for NH₂Y₃₅₆• (model C §4.6.4.3, p.121). For NH₂Y₃₅₆• the g value could not be reproduced by one moderate and one weak H bond, but was within uncertainty of 0.5 ppt consistent to the DFT model C (§4.6.4.3, p.121). The lack of additional structural information, however, allows various interpretations. Noteworthy, Y₃₅₆• formed during reverse PCET with α₂-wt is more consistent to the g value observed at NH₂Y₃₅₆• and with three weak to moderate H bonds or two H bonds and a positive charge.

Additionally, two conformers were recently proposed for NH₂Y₃₅₆• by their different kinetic phases. Further time-dependent measurements at high frequency EPR are necessary to decide if the NH₂Y changes the equilibrium between the two states observed with Y₃₅₆•. One can point out that the conformer observed for the forward PCET at NH₂Y₃₅₆• (θCβ = 55°±15°) and Y₃₅₆• (θCβ=42-56°) is identical within uncertainty of the measurement and the small DFT models.

5.5.3 Y₃₅₆• in Forward and Reverse PCET

This thesis covers the forward PCET transfer in RNR Ia, which requires an additional mutation to observe the forward Y₃₅₆• species. Therefore the preliminary results of Y₃₅₆•;α₂-wt, which is initially formed during reverse PCET, should be discussed. It could be shown that the reverse Y₃₅₆• shares a common distance with the forward Y₃₅₆• species. This is in agreement with study focusing on the equilibrium radicals formed using β₂-NO₂Y₁₂₂: α₂-wt. Additionally, all Y₃₅₆• using 2,3,5-F₃⁻Y₁₂₂• properties observed here agree well with the properties found using the β₂-NO₂Y₁₂₂•, such as polarity and ring dihedral. However, using 2,3,5-F₃⁻Y₁₂₂• one could separate the investigation of Y₃₅₆• from the cofactor assembly step, necessary to produce β₂-NO₂Y₁₂₂•.

Hence, Y₃₅₆• residue could be observed during putatively reverse PCET with a gₓ value of 2.0063±0.0001 in an unusual polar environment. It has the same ²H HF coupling...
size as observed for $Y_{356}^\cdot$ during forward PCET. The electrostatic environment is within uncertainty of our models in agreement with at least two in-plane H bonds in the range of $1.9\pm 0.1 \text{ Å}$. For longer distances a third weak H bond might be considered as the second weak H bond in the forward $Y_{356}^\cdot$. This implies a structured H bond network, because unstructured $Y^\cdot$ in aqueous solution need an acidic environment to produce similar low $g_x$ values (Table 5-6).$^{161, 319}$ Furthermore a rapid exchange would broaden the EPR line shape $B_0\parallel g_x$ and the $^2$H ENDOR spectra, in contrary the lines are sharper than observed with $Y_{356}^\cdot:\alpha_2-Y_{731}F$. However, other sources of structured polar environments as cations or charged groups are equally possible to explain the $g_x$ shift. As has been pointed out for $\text{NH}_2Y_{356}^\cdot$, positive charges from cations as $\text{Mg}^{2+}$ or guadinium groups can in principle introduce a strong polarity around $Y^\cdot$. Binding of $\text{Mg}^{2+}$, has long been known to play an important, but still poorly defined role in α/α, α/β and β structure/chemistry.$^7$ Though a complex of $Y_{356}^\cdot$ and $\text{Mg}^{2+}$ additional to the two H bonds observed is unlikely considering the strong effect on the $g$ value of a divalent cation.$^{174, 175}$ Orientation selective measurement can further limit the directions of the H bond(s) observed $Y_{356}^\cdot:\alpha_2$-wt, if an additional parallel tensor component ($T_\parallel$) can be observed either along $B_0\parallel g_x$ and $B_0\parallel g_z$ a second $1.9\pm 0.1 \text{ Å}$ H bond would be present. A second H bond in this range would imply a similar geometry as observed for $\text{NH}_2Y_{730}^\cdot$ with three H bonds 1.8, 1.8 and 2.2 Å.$^{110}$

The H bond network found at the $Y_{356}^\cdot$ could be used to obtain the reversible redox potentials. Peptide models have been applied to measure reversible redox potentials of the radical intermediates without dimerization of the $Y^\cdot$ in solution.$^{62, 327, 121, 122}$ The H bond network observed in this thesis at the RNR intermediates might aid the design of adequate peptide models, which are able to measure realistic redox potentials. Generally, it has been found that an additional H bond can reduce the redox potential by typically 60-120 mV.$^{292}$ An approximately 60 mV lower potential of $Y_{356}^\cdot$ has been postulated based on kinetic data compared to the Y PCET intermediates $Y_{731}^\cdot$ and $Y_{730}^\cdot$ in the α subunit (see Figure 1-11, p.17).$^{28, 41, 84}$

Based solely on the Mims ENDOR spectra presented herein, it cannot be evaluated if the two $Y_{356}^\cdot$ resemble two energetic minimum states or if the α- Y$_{731}$F mutation influences the environment. It cannot be ruled out that an H bond bound to $Y_{356}^\cdot$ is lost due to α$_2$- Y$_{731}$F mutation, because a water molecule is also bound to α$_2$- Y$_{731}$. However, in the last
chapter it could be shown that at least NH$_2$Y$_{731}$\textbullet\textit{ interacts} only weakly with the subunit interface.

Additionally, a change in the isotropic $\beta$-methylene HF couplings from 47 to 56 MHz could be observed for forward to reverse Y$_{356}$\textbullet\textit{, respectively. This might be an indication of two conformer-cific states of Y$_{356}$\textbullet\textit{. A large change in C$_p$-H HF coupling is not a prerequisite for a conformational change. Here a significant change of 20\% could be observed between forward and reverse PCET Y$_{356}$\textbullet\textit{. If a conformational change occurs it could change the ET distance in the PCET by more than 6 Å, although the diagonal distance between $\beta^1$Y$_{356}$\textbullet\textit{ and $\alpha^2$-2,3,5-F$_3$Y$_{122}$\textbullet\textit{ might be unaffected (Introduction §1.4.6, p.17). In this thesis, an unchanged diagonal distance has been found between forward and reverse PCET Y$_{356}$\textbullet\textit{. The same was reported using NO$_2$Y$_{122}$\textbullet\textit{ to form forward and reverse Y$_{356}$\textbullet\textit{.84

This could be further tested by rapid freeze quench EPR. In the EPR spectra a change in $g_x$ value from the millisecond time to the second time scale could be investigated. With Y$_{356}$\textbullet\textit{:$\alpha_2$-wt no difference were reported between RFQ and manual freeze quench at 9 GHz.28 This could be an indication that the different H bond networks, observed with Y$_{731}$F and with wt $\alpha$, are indeed formed first during forward then during reverse PCET. A change in the H bond network might affect the PT distances.

5.5.4 Mechanistic Implications

Mechanistically the presence of a bidirectional PCET at Y$_{356}$ has been suggested since the pH dependent measurements with various fluorinated Y$_{356}$\textbullet\textit{.82, 119 In combination with conserved glutamates (i.e., E$_{350}$, see §1.4.1.2, p.7), a geometry similar to tyrosine analogs (1 or 2) of Hammerström et al. was proposed, as shown in Figure S-15.328, 329 In these models, Hammerström et al. could show that the phenol reaction was pH independent in the presence of an intramolecular acid.328 This has been observed during various F$_n$Y$_{356}$ incorporations.82, 119 However, there is not any spectroscopic evidence for a glutamate (E$_{350}$) near residue 356. Additionally, no PT acceptor was found.
This thesis was able demonstrate a polar environment that stabilizes two different H bond environments of Y$_{356}^\bullet$. In both cases forward or reverse PCET only in-plane and moderate H bonds are observed. The H bonds found here might be from the proton acceptor function during forward PCET. Thus a proton acceptor function (X) would be within ≈2.9±1 Å (distance O$_{phenoxy}$ to X) within the ring plane. Small PCET model studies have shown similar geometries.$^{101,102}$ Experimental studies point out that the kinetic rate in a bidirectional CPET by elongation of the ET distance from 12 to 21 Å is within error identical to the rate dependence of a pure electron transfer, for a short proton transport (PT) distance.$^{331}$ On the other hand, PT rates have been investigated for several donor acceptor distances within the ring plane (see 3-6, Figure 5-15), indicating that PT can be rate limiting.$^{101,252}$ Due to the fast decay with PT distance, the H bond ($\approx1.9\pm1$ Å) found here could be a rate limiting with a donor acceptor distance d of 2.9±0.1 Å, as illustrated by Figure 5-16.

However, the rate is strongly dependent on the distance and a change of ~0.2 Å changes the rate constant nearly 3 orders of magnitude. Currently, advanced EPR techniques combined with semi-empirical equations or HF parameter from DFT calculations employed here, are not precise enough to evaluate H bond distance changes of 0.1 Å. Isotopical labeling with $^2$H could increase the resolution of $^1$H ENDOR spectroscopy in comparison to $^3$H ENDOR spectroscopy studied here.
Figure 5-16: Dependence of the estimated PCET rate constant on the proton donor-acceptor distance $d_{O-N}$ for a reaction without driving force ($\Delta G^0=0$). The red line is a fit for $\ln k_{\text{PCET}} = -\beta d_{O-N} + \text{constant}$, with $\beta \approx 27 \text{ Å}^{-1}$. Data plot from ref. 101.
6 Conclusion

The intersubunit radical transfer from the diiron cofactor to the nucleotide reduction side in RNRRs Ia has major unresolved issues. Although three Y• intermediates have been assigned, it is uncertain how these Ys transfer their protons to form Ys•. Structural information of the “active” state is still missing. In this thesis site specific incorporated 3-amino tyrosin forming NH₂Ys• offered the unique opportunity to compare these three Y intermediates in high-field EPR investigations and resolve hydrogen (H) bonds networks and electrostatic environments at these radicals sites. At the Y intermediates β-356 another mutation strategy offered the possibility to characterize H bonds to Y356•.

6.1 NH₂Y• Intermediates Investigated in the α Subunit

Pulsed-263-GHz EPR spectroscopy has been applied for the first time on NH₂Y• to unravel small changes in the electrostatic environment of NH₂Y356•, NH₂Y730• and NH₂Y731•. The $g_x$ value is most indicative for electrostatic changes, which arise due to positive and negative charges and H bond interactions. All three investigated intermediates showed low $g_x$ values (> 0.6 ppt lower as the free NH₂Y•) symptomatic for H bonded polar environments. NH₂Y730• has been assigned before to harbor one moderate H bond (1.8 Å) and two weak H bonds (≥ 2.0 Å),110 in this thesis it had the highest $g_x$ value of the three NH₂Ys• intermediates. The $g_x$ value from 263-GHz EPR spectra of NH₂Y731• is with 0.3 ppt lower that found at NH₂Y730• (see Table 4-7, p.114).
$^3$H ENDOR spectroscopy revealed a strong H bond (1.6-1.7 Å) at NH$_2$Y$_{731}$$^\bullet$ (see Figure 4-7, p.83). A DFT calculation, consistent with the experimentally obtained g values, considered an additional weak in-plane H bond (≥1.9 Å) from a nearby water molecule (Model 3 in Figure 4-16, p.100).

Thorough investigations lead from the initial hypothesis of π stacking between Y$_{731}$ and Y$_{730}$ to the first spectroscopic evidence for their interaction. First, two additional NH$_2$Y$^\bullet$ from double mutants were investigated, and thus underlying amino deuteron resonances could be separated from resonances of intermolecular H bonds. Two perpendicular H bonds at NH$_2$Y$_{730}$$^\bullet$ and one stronger H bond at NH$_2$Y$_{731}$$^\bullet$ were found consistent with the double mutants removing in each case one perpendicular H bond contribution. Second, to support the tensor shape indicating a contact interaction with the spin bearing p$_z$ orbital, orientation selective ENDOR spectra at NH$_2$Y$_{731}$$^\bullet$ have been recorded. The simulation revealed an H bond and its H bond donor bond (H-XDonor) aligned ≈70° perpendicular to the ring plane (see Figure 4-7, p.83). Third, the interaction has been calculated by large DFT structures capturing the essential EPR parameters from 263-GHz EPR and 94-GHz ENDOR spectra. These calculations demonstrated a π- stacked geometry between NH$_2$Y$_{731}$$^\bullet$ and Y$_{730}$ independent of the number of water molecules present in the models (1,2 & 3, Figure 4-16, p.100).

The combination of results from DFT models, EPR and orientation selective ENDOR spectra lead to the assignment of H-O-Y$_{730}$ as H bond partner of NH$_2$Y$_{731}$$^\bullet$. In the same DFT models without the NH$_2$ group the π stacked conformation between Y$_{730}$ and Y$_{731}$ could be found in the oxidized and reduced state. Together with the H bonds interaction at NH$_2$Y$_{730}$$^\bullet$ the mutual H bonding between position Y$_{730}$ and Y$_{731}$ could be demonstrated. This geometry is typical for an adiabatic proton transfer between Y$_{731}$ and Y$_{730}$, formulated as HAT by Siegbahn et al. or as CPET by Kalia and Hummer.

The DFT calculations of the large models could be closely correlated to the transition state calculations done prior to this thesis. A model including a distal water molecule (wat 1), H bonded to Y$_{730}$, is preferred based on the current data for the radical intermediate at 730 and 731 (Figure 1-14C, p.23). The structural bias of investigating α RNR Ia with NH$_2$Y$^\bullet$ has been discussed.

RNR employs a highly selective PCET transfer. Seminal studies on the PCET showed that the removal of an OH group by mutation of a tyrosine to a phenylalanine is able...
to shut down the whole pathway. H bond knock out double mutants (NH₂Y731•/Y730F and
NH₂Y730•/C439A) highlighted this point again. They are intrinsically inactive due to the loss
of an essential amino acid. However it was not expected that the radical build up rate would
be directly affected, as the radical is generated - in PCET direction - before the mutation
position. The double mutants form NH₂Y• by a factor of 2 and 10 slower for
NH₂Y731•/Y730F and NH₂Y730•/C439A, respectively (see Table 4-3, p.88). This showed once
more how delicate this PCET pathway is in terms of activity. This is also in agreement with
the recent slight over potential study of Olshansky et al., even here the radical decay of 2,3,5-
F₃Y₃56-β, was affected by a Y₇30F mutation in α, pointing out how important the PCET is for
reducing the individually formed transient radical.86

Quite intriguing was the finding that the interaction from the α to the β subunit is
not governed by a perpendicular moderate H bond as suggested previously.92 However, a
weak H bond (≥2 Å) from the interface to NH₂Y731• could not be excluded. Nevertheless,
the data indicates that a close encounter as π stacking of Y₃56 and Y₇31 and a collinear PCET
is unlikely.

6.2 NH₂Y• and Y• Intermediates Investigated in the β Subunit

The investigation of radical intermediates in β was performed by the study of tyrosine
intermediates at β2-Y356, in Chapter 4 with Y356 mutated to NH2Y356.

263 GHz EPR spectroscopy revealed the highest polarity found in all NH₂Ys• at
NH₂Y356• with a gx value of 2.0049 which is by 0.2-0.5 ppt significantly lower than in
NH₂Y730/731• (see Table 4-7, p.114). The ENDOR spectrum of NH₂Y356•, however, was only
in agreement with in-plane H bonds. In contrast to perpendicular H bonds, in-plane H
bonds could be affected by the incorporated in-plane amino group. Here the innocent
reporter role of the NH₂Y• could not be anticipated anymore, therefore another mutation
approach was necessary at residue 356. Here one could not find any evidence of a
perpendicular or strong H bond at NH₂Y•.

Y₃56• could be trapped using 2,3,5-F₃Y₁₂₂• with a ~10 mV higher reduction
potential. Why 2,3,5-F₃Y₁₂₂• circumvents conformational gating and forms Y₃56• has not
been understood.28 A characterization of this mutant showed only a minor shift (9°) in ring
dihedral compared to Y₁22 (see Figure S-3, p.134). If a difference in PT or indeed a general
deprotonated 2,3,5-F₃Y₁₂₂⁻ (pKₐ of N-acetyl-2,3,5-F₃Y 6.3) triggers the unhindered PCET is still discussed.⁴¹

It has been discussed that a strong to moderate H bond is not expected between Y₃₅₆ and Y₇₃₁ based on NH₂Y• studies. Therefore a PCET blockade as α₂-Y₇₃₁F should be a reasonable small perturbation to trap Y₃₅₆• on the forward PCET. And indeed the yield was comparable to Y₃₅₆• using α₂-wt with about 30% for the manually quenched samples (s timescale).

The forward PCET Y₃₅₆• was investigated by multi-frequency EPR at Q band and at 94 GHz. The spectra revealed a 0.4 ppt lower gₛ value of Y₃₅₆• than observed for Y₅• with one moderate H bond (1.8 Å), which is an indication for a higher electrostatic interaction. The ²H Mims ENDOR spectra in combination with DFT calculations estimated an H bond distance of 1.9±0.1 Å to the phenoxy nucleus. The weak to moderate H bond was in-plane to the phenyl ring. Orientation selective ENDOR spectra could demonstrate an orientation perpendicular to the C-O bond axis similar to Y₄• in PS II.¹⁵⁵ By comparison to other orientation selective measurements an additional smaller coupling was proposed.⁵⁵ In order to explain a small ³H coupling pattern as well as the lower gₛ value (0.4 ppt compared to Y₄•) a DFT Y• model was created. A moderate and a weak H bond with a distance of 1.8 and ≥2.2 Å could reproduce both EPR parameters.

The control study with β₂-2,3,5-F₃Y₁₂₂•: α₂-wt, reverse PCET Y₃₅₆• showed a ring dihedral (θ₁C₉=55±10°) and polarity similar to the forward NH₂Y₃₅₆• PCET case. The ²H Mims ENDOR spectra, however, reported the same ENDOR pake pattern in size as Y₃₅₆• formed during forward PCET. This apparent contradiction could be explained with a second moderate in-plane H bond (1.8 Å). Additionally, electrostatic influences have been discussed for NH₂Y₃₅₆• (during forward PCET) and Y₃₅₆• (during reverse PCET). Higher resolution is necessary to further resolve the H bond environment during reverse PCET.

The absence of any strong or perpendicular H bond is an indication for a mechanistically different PCET in the β subunit in perspective to the observed π stacking in the α subunit.

6.3 Outlook

For the investigated tyrosyl radicals and their analogs, high frequency up to 263 GHz is necessary to measure resolved and accurate EPR spectra. In future studies the experimental
limit of resolvable nuclear distances can be expanded by the use of deuterated enzymes (Ys) in H$_2$O buffer media to detect $^1$H intermolecular ENDOR resonances. Theoretically, the broadening of the line and concomitant decrease in absorption is partially compensated by a higher detection efficiency for larger couplings (§2.2.3, p.40). Additionally, the splitting of the quadrupole coupling will be removed from the spectra, if intermolecular $^1$H HF couplings are detected. It should be noted, that the quadrupole information on the electric field gradient will be lost. This information was valuable within this thesis. More recently developed high sensitive $^1$H ENDOR schemes can be applied in isotope labeled samples.$^{310, 332}$

Additional investigations on the reverse PCET have to be performed in shorter time scales within RFQ high-field EPR. A change in the $g$ value over the reaction time might demonstrate the exchange between forward and reverse Y$_{356}$• state. Additionally, the KIE should be measured between the PCET of 2,3,5-F$_3$Y$_{122}$• and Y$_{356}$, because for the first time a putatively single PCET step can be investigated between Y intermediates in RNR.

Orientation selective PELDOR studies concomitantly with solid state NMR studies might investigate the structure of the “active” homodimeric complex using NH$_2$Y$_s$ and an isotopically labeled $\beta$ terminal tail.$^{333-336}$ The information from solid state NMR studies would be complementary, i.e., the reduced conformation of $\beta_2$-Y$_{356}$ could be obtained if the reaction is trapped with NH$_2$Y$_{730}$•. New advances in cryo electron microscopy might also lead to structural insight with nearly atomic resolution (<3.0 Å).$^{70, 337, 338}$

Recently, several papers have used QM/MM calculations to investigate PCET reactions.$^{339-341}$ To observe if conformational changes occurring below the ms time scale QM/MM calculations of the subunit interface could contribute valuable insight in the ns timescale.
7 References


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8 Appendices
**APPENDIX 1: PREVIOUS EPR RESULTS FOR NH₂Y**

<table>
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<th>Nucleus/ [MHz]</th>
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<td>β</td>
<td>γ</td>
<td>α</td>
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<tr>
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Table A.1: Summary of EPR parameters from NH₂Y• by simulation of 94 GHz EPR spectra in D₂O and H₂O buffer at position β-356, α-731 and α-730 on the PCET of *E. coli* RNR. T. Argirević presented these hyperfine couplings, Euler angles and g values in his thesis (2011). The large couplings were additionally in agreement with 34 GHz ¹H Davis ENDOR spectra.
APPENDIX 2: APPENDIX TO CHAPTER 4 (NH$_2$Y$_{730/731}$•)

The 263 GHz spectrum in H$_2$O buffer was recorded at a different reaction time point of 2 min, but still shows the g values as demonstrated by the simulations. However, one should note, that due to the absence of sharp features beside g, the accuracy of the g value determination is lower.

Figure A - 1: 263 GHz ESE Spectrum of Y$_{730}$NH$_{2}$• 2 min at 70 K. Blue shows derivative (obtained by 5 points 2nd order Savitzky-Golay filter) and red shows the simulation. Exp. details: ESE, $\pi/2$=64 ns, $\tau$= 270 ns, SRT= 6 ms, SPP=500, 200 scans.
Figure A-2: 94-GHz EPR spectra at various reaction times (10 s- 2 min) of ND$_2$Y$_{71}$. Exp. details: ESE, T = 70 K, $\tau$= 240 ns, SRT = 5 ms, SPP= 50-100, scans=100-200. The derivative was built by a 5–10 points second order Savitzky-Golay filter.
Figure A - 3: ENDOR spectra of ND$_2$Y$_{331}$ at $g_y$ from three preparations and from reaction time points ranging from 10-35 s. All spectra show within S/N the same features. Exp. details: $^2$H Mims ENDOR, T = 10 K, $\tau$=200 ns; $\pi$/2=20 ns, SRT= 150-200 ms; number of averages from top to down: 450 (2 h), 2800 (11 h), 150 (0.6 h), 300 (1.2 h), 50 (12 min), 3000 (12 h); Exp. details of the 30 s trace can be found in Figure 4-7 (p.83).
Figure A - 4: Orientation selective $^2$H Mims ENDOR spectrum of the central line of NH$_2$Y$_{76}$$. Spectral range between -0.3 to 0.3 MHz shows weak couplings. Exp. details: $T$ =70 K, $\pi/2=20$ ns, $\tau=360$ ns, SRT=10 ms, acquisition time 5 h (3000 scans).
Figure A - 5: Large DFT models for NH$_2$Y$_{731}$*. The 210 to 216 atoms large Model 1 (wine red), Model 2 (green) and Model 3 (purple) are depicted. The GDP is modeled by an (3R, 4S)-tetrahydrofuran-3,4-diol.
Figure A - 6: Small DFT models for NH$_2$Y$_{731}$$. Small models were constructed from DFT Model 2 in order to check for the effect of individual amino acid residues on the $g$ values of NH$_2$Y$_{731}$$^\bullet$. For geometry optimization the functional (B3LYP) and the Aldrich’s’ TZVPP basis set of triple-$\zeta$ quality were used. For deriving the EPR parameters, the EPR II basis set was used (Online Methods). Solvent effects were taken into account by a conductor like screening model (COSMO) with the polarizability of ethanol. A) This first small model is built from Model 2 by considering residues only in the first interaction sphere. No further geometry optimization was carried out. The $g$ tensor with R$_{411}$ is $g_{xyz} = [2.0050, 2.0041, 2.0022]$. If R$_{411}$ is removed the $g$ tensor becomes $g_{xyz} = [2.0055, 2.0044, 2.0021]$, i.e., the $g_x$ value shifts by $\Delta g_x = + 0.5$ ppt. B) Small model A after additional geometry optimization, $g_{xyz} = [2.0052, 2.0042, 2.0022]$. C) Small model which mimics the double mutant NH$_2$Y$_{731}$/Y$_{730}$F. The $g$ tensor results to $g_{xyz} = [2.0060, 2.0046, 2.0023]$. D) Small model C after geometry optimization. The $g$ tensor is $g_{xyz} = [2.0052, 2.0043, 2.0023]$, i.e., the computed $g_x$ lowers by 0.8 ppt.
APPENDIX 3: APPENDIX TO CHAPTER 4 (NH₂Y₃₅₆•)

Figure A - 7: 94 GHz field calibration of β-ND₂Y₃₅₆• (blue line) by internal β-Y₁₂₂• (black line). The $g$ values were aligned on Y₁₂₂• with $g_x=2.00912$.\textsuperscript{157} Exp. details (blue/black): ESE, 13 s reaction time, T = 70 K /10 K, $\tau=30$ ns, $\tau=275$ ns, SRT = 5 ms/ 50 ms, SPP = 100/10, scan = 62/1. The derivative was built by a 10 point second order Savitzky-Golay filter. NH₂Y₃₅₆• yield was 13%, estimated by the integral with and without subtraction resting state of β₂-Y₁₂₂•.
Figure A.8: $^3$H Mims ENDOR spectrum of ND$_2$Y$_{356}^\cdot$ compared to ND$_2$Y$_{731}^\cdot$ at 35 GHz. Exp. details (blue/red): T= 10 K, $\pi/2= 20$ ns, $\tau=200$ ns, $\pi_{RF}=30/40$ µs, SRT=30/150 ms, SPP=1, averaging time = 11 h. 20 point Savitzky-Golay second order filter was used to obtain blue and red from the corresponding gray spectrum. Data courtesy belongs to Bejenke and Argirević for blue and red, respectively.
Figure A - 9: Diagonal distance measurement of pathway radical produced with α₂;2,3,5-
F₃Y₁₁₂;β₂-wt. ESE spectrum at 40 K (violet) and spectrum of pathway radical alone at 70 K (red)
are shown together with the pump and detect positions of the PELDOR measurements. From
pump (π = 56 ns) and detect (π = 46 ns) separated by 54 MHz the dipolar oscillation (B) is
obtained after background subtraction and normalization. DEER Analysis using Tikonov
regularization gives a fit (black line) to the individual (B) and summed (C) dipolar oscillation.
This oscillation can be Fourier transformed to a Dipolar pake pattern (D). The perpendicular
component with ±1.86 MHz can be read out. From Eq. (2-19) the dipolar distance is obtained
with 3.0±0.1 nm. The inner part of the pake pattern also show another contribution with ±0.6
MHz, but the length of the recorded time trace is too short to resolve this contribution.