NMR Solution Structure and Dynamics of the Peptidyl-prolyl cis–trans Isomerase Domain of the Trigger Factor from *Mycoplasma genitalium* Compared to FK506-binding Protein

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We have solved the solution structure of the peptidyl-prolyl cis–trans isomerase (PPIase) domain of the trigger factor from *Mycoplasma genitalium* by homo- and heteronuclear NMR spectroscopy. Our results lead to a well-defined structure with a backbone rmsd of 0.23 Å. As predicted, the PPIase domain of the trigger factor adopts the FK506 binding protein (FKBP) fold. Furthermore, our NMR relaxation data indicate that the dynamic behavior of the trigger factor PPIase domain and of FKBP are similar. Structural variations when compared to FKBP exist in the flap region and within the bulges of strand 5 of the β sheet. Although the active-site crevice is similar to that of FKBP, subtle steric variations in this region can explain why FK506 does not bind to the trigger factor. Sequence variability (27% identity) between trigger factor and FKBP results in significant differences in surface charge distribution and the absence of the first strand of the central β sheet. Our data indicate, however, that this strand may be partially structured as “nascent” β strand. This makes the trigger factor PPIase domain the most minimal representative of the FKBP like protein family of PPIases.

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Keywords: trigger factor; peptidyl-prolyl cis–trans isomerases; NMR structure determination; protein dynamics; FKBP

Introduction

Peptidyl-prolyl cis–trans isomerases (PPlases) catalyze the isomerization of peptidyl-prolyl bonds,¹ for which significant barriers of 80 kJ mol⁻¹ between their cis and trans conformations exist.² The slow interconversion between these conformations is often the rate-limiting step in protein folding and justifies enzymes catalyzing this process. By contrast, chaperones, which are another class of proteins assisting protein folding, do so by preventing unwanted tertiary interactions, protein aggregation and misfolding.³,⁴

Three classes of PPlases have been found so far; the cyclophilins, the FK506-binding proteins (FKBPs) and the parvulins. Selected members of each of these three PPlase classes have been
characterized thoroughly, both functionally and structurally. Cyclophilins and FKBPs are important targets for the immunosuppressive drugs cyclosporin and FK506, respectively. FKBPs and parvulins were found to adopt a similar fold of their PPIase domains, which has been called the FKBp fold, whereas the cyclophilins are not structurally related to the other two groups of proteins.

Another PPIase is the trigger factor (TF). Originally, this 59 kDa protein was found to be involved in Escherichia coli membrane transport of secretory export proteins by presumably stabilizing their partially unfolded states. Subsequently, TF was found to associate with the 50 S subunit of the ribosome, and hence thought to be involved in initial folding steps of the nascent peptide chain. Its PPIase activity in vitro is very low, but its protein folding activity is the highest among all known PPIases. TF is a modular protein consisting of three domains: an N-terminal ribosome-binding domain, a central PPIase domain, and a C-terminal domain of unknown function. The intact protein has a high affinity toward unfolded polypeptides, even those that do not contain proline residues.

The isolated PPIase domain, however, prefers peptides containing proline. The current view of the role of TF in vivo is that of a multifunctional folding catalyst that possesses both PPIase and chaperone activity. Hence coexpression of TF has been reported to increase the yield of correctly folded overexpressed proteins, which would have otherwise been insoluble. Mutants lacking the TF gene compensate for its function by increased expression of DnaK, while a lack of both genes (TF and DnaK) is reported to be lethal. This may indicate partially overlapping functions of both enzymes. Additionally, TF associates with the chaperone GroEL and enhances its ability to bind other proteins. TF also seems to act as a “cold-shock” protein to enhance bacterial viability at low temperature.

Database searches indicate that proteins homologous to TF are present in all sequenced prokaryotic genomes. In the small Mycoplasma genitalium genome, TF appears to be the only protein associated with PPIase activity. This emphasizes the important role of TF within the PPIase class, since Mycoplasma bacteria are believed to possess the smallest functional genome of a free-living organism. Furthermore, inhibition of TF might be the basis for antibiotics targeted against these pathogenic organisms.

Here, we will focus on the central domain of the TF, TF_{151-251} (denoted TF_{PPIase} in the text), which was found to be responsible for the PPIase activity of the protein. Hydrophobic cluster analysis of the amino acid sequence of TF_{PPIase} revealed a similar pattern of hydrophobic residues for TF_{PPIase} and FKBp. On the basis of the published coordinates of FKBp, several 3D homology models of TF_{PPIase} have been built, all of which feature the typical FKBp fold consisting of a five-stranded β-sheet and an α-helix crossing this sheet. Based on the moderate sequence homology between TF_{PPIase} and FKBp (27% identity) conserved residues were predicted to define the active site of TF_{PPIase}. Important differences of the modeled TF_{PPIase} as compared to FKBp were predicted to exist both in the flap region and in strand β5 of the sheet. These regions show the greatest sequence variability when compared to FKBp. Hence, our primary goal was to determine the solution structure of the TF_{PPIase} at atomic resolution and to compare these findings.
with the structure of FKBP and other known PPIases structures. A second objective was to explain why TF, despite its homology to FKBP, does not bind the immunosuppressant drug FK506.

### Results and Discussion

#### Assignment of resonances

For all NMR studies, the 101 amino acid residue PPIase domain of the TF was used. This domain, TFPPIase, begins at Glu13 (Glu151 in the intact trigger factor) and ends with Lys113 (Lys251). The N terminus of TFPPIase was tagged with 12 amino acid residues containing a His6 tag to enable rapid purification. None of the resonances of the His6 tag, with the exception of the Ser immediately preceding Glu13, could be assigned to resonances in the 1H–15N heteronuclear single quantum coherence (HSQC) spectrum. These residues are all presumed to be unstructured (see below).

We have ruled out interactions between the basic histidine tag and the rest of the protein using a construct lacking the His-tag and instead just having a Gly immediately preceding Glu13. This construct, as compared to the His6-tagged protein, has identical chemical shifts in the 1H–15N HSQC spectrum (see Figure 1 of the Supplementary Material) as well as identical nuclear Overhauser effect spectroscopy (NOESY) patterns in the 15N-edited NOESY spectrum.

Backbone and Cα resonances were assigned using a combination of 3D HNCA(CO)NH26 and 3D CBCA(CO)NH spectra. For the structured region of TFPPIase all residues, except Ala50, give rise to distinct amide cross-peaks. The assignment and the extent of assigned resonances have been published.27

#### Secondary structure of the trigger factor

For the N-terminal stretch (Ser12 to Lys29), only intra-residual and sequential NOEs could be detected. This type of NOE signature is typical of unstructured polypeptide chains.28,29 The well-defined structured region of TFPPIase begins with Leu30. Figure 1 shows local NOEs, HN–Hα coupling constants, H/H amide backbone exchange rates, and the associated secondary structure elements. For consistency, all secondary structure elements of TFPPIase are named analogously to those of FKBP.

Four strands of the central β-sheet of TFPPIase were identified by slow H/H amide exchange and/or large 3JHN–Hα couplings. This sheet is shown in Figure 2 and is defined by 51 characteristic NOEs (arrows) and 27 slowly exchanging H-bonds (bold lines). Strand β4 consists of residues Gly33 through Asn43. It is separated from strand β5 (Tyr57 to Ile61) by a β-turn (Val44 to Lys47) and a stretch of seven amino acid residues (Lys48 to Gln55) that show some β-sheet character (large 3JHN–Hα couplings, strong sequential HN–Hα NOEs and weak sequential HN–HN NOEs) but little H/H exchange protection. Gly62 to Gly69 do not show any characteristic structural features. For residue Phe70 to Ala76, the NOE pattern, slowly exchanging amide protons and small coupling constants define helix α1. Residues Asn80 to Phe89 and Val103 to Lys113 form β-strands β2 and β3, respectively. Amino acid residues Pro90 to Pro102 separating strands β2

![Figure 2. Hydrogen bond and NOE network in the extended β-sheet: from top to bottom, strands 5, 4, 3 and 2 (following the FKBP nomenclature) are indicated. NOEs that define the orientation of the strands are depicted by arrows. Hydrogen bonds identified by slowly exchanging amide protons are shown by bold lines.](image-url)
Table 1. Structure calculation statistics of the 12 lowest-energy structures from an ensemble of 200 calculated structures

<table>
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<tr>
<th>Average energy values (kcal mol⁻¹)</th>
<th>455 ± 3</th>
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<tr>
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<tr>
<td>E improper</td>
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<tr>
<td>E vdW</td>
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<tr>
<td>E NOE</td>
<td>35 ± 3</td>
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<td>E dihedral</td>
<td>0.4 ± 0.05</td>
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<tr>
<td>E J coup</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>E vector-angle</td>
<td>79 ± 2</td>
</tr>
</tbody>
</table>

RMS deviation from idealized covalent geometry

| Bond length (Å)                  | 0.0025 ± 0.00006 |
| Angles (deg)                     | 0.538 ± 0.005 |
| Improper (deg)                   | 0.55 ± 0.008 |
| Dihedral angles (deg)            | 31 ± 0.6 |

RMS deviations from experimental data

| Distance restraints (Å)           | 0.0210 ± 0.0008 |
| Dihedral restraints (deg)        | 0.21 ± 0.01 |
| JHN–Hα (Hz)                      | 1.06 ± 0.02 |

Coordinate rmsd deviation (Å) (residues 30–112)

| Backbone atoms                   | 0.234 |
| All heavy atoms                  | 0.725 |

Analysis of Ramachandran plot of structured region of TF_PPIase (residues 29–113), number of residues in the ensemble of 12 structures

| Residues in most favored regions (%) | 574 (62.9) |
| Residues in additional allowed regions (%) | 296 (32.5) |
| Residues in generously allowed regions (%) | 30 (3.3) |
| Residues in disallowed regions (%)    | 12 (1.3) |

Analysis of residual dipolar couplings

| Q-value                           | 0.25–0.26 |
| Correlation coefficient           | 0.97 |

These 12 structures were submitted to the Protein Data Bank, database entry 1HXV. Statistics were generated from CNS and PROCHECK output.

...and β3 correspond to the “flap” region in FKBP and consist of a loop-like structure followed by a short 3₁₀ helix (α2).

**Tertiary structure**

Using standard molecular dynamics protocols (see Materials and Methods), an ensemble of 200 structures was calculated with the following NMR data: 959 interresidual NOEs, 35 hydrogen bonds, 72 JHN–Hα coupling constants, 68 dihedral angle restraints and 2628 dipole–dipole projection restraints30 derived from 68 amide dipolar couplings (Table 1). Figure 3 shows the distribution of NOEs and the resulting backbone rmsd as a function of sequence position. The backbone rmsd clearly anticorrelates with the number of NOE restraints per amino acid residue, identifying several loops with reduced constraint density. On average, 11 interresidual NOEs per residue could be extracted from the NMR data. These restraints define a tight ensemble of structures, for which the 12 lowest-energy structures (of 200 calculated) display a backbone rmsd of 0.23 Å and an rmsd of 0.72 Å for all heavy atoms. No NOE violations over 0.5 Å and maximally one NOE violation between 0.2 and 0.5 Å were found. Statistics for this ensemble, as calculated by CNS,31 PROCHECK32 and DipoCoup,33 are compiled in Table 1. Figure 4 shows a stereo drawing of these 12 lowest-energy structures, fit for minimal backbone rmsd of the region Lys29-Lys113.

The TF_PPIase structure was additionally restrained using residual dipolar couplings (RDCs). The same data set without RDCs leads to a distinctly higher backbone rmsd of 0.3 Å versus 0.23 Å including RDCs. Concomitantly, the RDC Q value34 drops from 0.5 without RDCs to 0.28 with RDCs, hence the agreement of the structure with the measured couplings is greatly improved. Especially the flap region (residues Pro90 to Pro102) becomes much better defined with the use of RDCs, balancing the relative scarceness of NOEs in this region. It should be stressed that the NOE and the RDC data sets do not contradict each other, which is reflected by unchanged NOE energies when RDC-restraints are used in the simulation.

NMR data indicate a β1 turn between strands β4 and β5, which implies that Asp46 is the only residue that is found in a disfavored region of the Ramachandran plot (Φ = 80°, ψ = −40°). To support the positive Φ angle, we have analyzed the doublet splitting arising from the HN–Hα coupling, which can be resolved by an 1H–15N transverse relaxation optimized spectroscopy (TROSY) experiment.35 The relative intensities in this doublet depend on Φ due to dipole–dipole cross-correlated relaxation.36 For Asp46 the high-field component of the doublet is the stronger, consistent with a positive Φ angle.

The trigger factor adopts the FKBP fold

Figure 5 compares an averaged structure of TF_PPIase derived from the 12 lowest-energy structures to the structure of uncomplexed FKBP.37 Clearly, the overall fold of the TF_PPIase is similar to that of FKBP. This had been predicted by homology modeling.20,24 It is therefore reassuring that the main structural features of this model agree well with our experimental structural findings. To ease comparisons between human FKBP12, the different trigger factor variants discussed (M. genitalium and E. coli), and human Pin1, Figure 6 shows a sequence alignment and structure comparison between these proteins.

The main structural element of FKBP38–40 as well as of TF_PPIase is the extended multi-stranded anti-parallel β sheet with its distinctive topological connectivity. Surprisingly the first strand (1) of this sheet is absent from our structure. Residues of this N-terminal segment of TF_PPIase appear to be unstructured, on the basis of the absence of tertiary NOEs and the presence of fast-exchanging amide protons. Also, for strand β2, there is no evidence for an additional strand (inter-strand NOEs or slow exchange of the relevant amide protons).
The lack of this β1 strand in TF\textsubscript{PPlase} circumvents the +3, +1, −3, −1 sheet topology\textsuperscript{31} that is distinctive for the FKBP fold and which would involve an unfavorable strand crossing near residue Lys29. Although the N terminus of TF\textsubscript{PPlase} may be structured in the full-length protein due to domain–domain interactions, testing this hypothesis is not within the scope of the current study. All we can say is that this extra strand does not contribute much to the integrity of the central sheet of TF\textsubscript{PPlase}. This is consistent with the structures of the homologous proteins hPar14\textsuperscript{32} or hPin1\textsuperscript{32} showing a similar absence of the first β strand.

To verify that the N-terminal β1 strand is not structurally relevant, a shortened TF\textsubscript{PPlase} fragment containing only the folded part of the TF\textsubscript{PPlase} domain, i.e. residues 168–251 of the full-length TF (denoted TF\textsubscript{168–251}), was expressed. Comparing \textsuperscript{1}H–\textsuperscript{15}N HSQC spectra of TF\textsubscript{168–251} and TF\textsubscript{PPlase} we

Figure 3. NOE data and rmsd statistics of the folded PPlase domain of the trigger factor (residues 29–113). The number of interresidual NOEs per residue is displayed as vertical black bars. On average, more than 11 interresidual NOEs per residue could be observed. Above the NOE statistics the per-residue backbone rmsd of the 12 lowest-energy structures obtained from an ensemble of 200 calculated structures is shown. The overall backbone rmsd in the resulting structure (omitting the N- and C-terminal lysine residues) is 0.23 Å.

Figure 4. Stereo drawing of the superposition of the 12 lowest-energy TF\textsubscript{PPlase} structures. Only the folded parts of the TF\textsubscript{PPlase} structure (residues 29–113) are shown. The orientation of the structure is the same as in Figure 5, with the active-site cleft towards the viewer and the extended β-sheet at the bottom.
found that most of the protein amide resonances are unaffected by the deletion (Figure 2 of the Supplementary Material). Thus, the global fold of the shortened and the full-length variant is identical. Surprisingly, as seen in Figure 7, the only peaks that have shifted slightly, but significantly (0.1 ppm in both dimensions) in the $^1$H–$^{15}$N HSQC spectrum of TF$_{168}$–251 (see Figure 2 of the Supplementary Material) cluster in a well-defined region comprising the strand $\beta_2$ and the solvent-exposed part of helix $\alpha_1$. This is exactly where the missing $\beta_1$ strand would be expected. Hence, we believe that the N terminus, despite being essentially unstructured, does interact with the fully structured strand $\beta_2$ and is best described by a partially structured or “nascent” $\beta_1$ strand.

Figure 5. Structural and dynamic comparison of (a) TF$_{PPlase}$ and (b) human FKBP-12. All secondary structure elements, including the flap and the bulge region are labeled. These proteins are very similar with respect to their overall folding topology. The main differences between the two structures are in the 40s bulge of strand $\beta_5$, in the flap region and in the absence of the $\beta_1$ strand. Both proteins are color-coded to represent the relaxation data. Colored regions mark residues that are flexible, and white areas those that are more rigid.
Structural similarity and diversity across the FKBP fold

The term FKBP fold has been suggested when comparing the fold of four PPIases of known structure (FKBP12, hPin1, hPar4 and GreA tcf). Figure 6 compares TF_PPIase with other members of this family. The conserved moiety of this fold (black) comprises the loop-helix-loop motif, including helix 1 and four strands of the five-stranded β sheet. On the other hand, a high level of diversity among these structures is found within the flap region, within the N-terminal half of strand β5, the “40s bulge” in FKBP, and in the presence or absence of the N-terminal β-strand (β1).

To estimate structural similarities between TF_PPIase and FKBP, we have superimposed the two structures, using the alignment shown in Figure 6. The agreement of the complete structures (aligning analogous amino acid residues in both proteins) is poor, resulting in a backbone rmsd of 3.7 Å. It
drops to 1.5 Å when the “bulge” (residues 50–56) and the flap (residues 88–101) are omitted. Obviously, these two regions account for most of the sequential and structural variations between the two proteins. The backbone rmsd drops further to 1.3 Å when only the amino acid residues marked by an asterisk in the alignment are used for fitting. These residues correspond to the black areas in the secondary structure topology drawing in Figure 6. The resulting superposition of FKBP and TF<sub>PPIase</sub> shown in Figure 8 illustrates the fact that the main deviations between the two backbone geometries are in the flap and the bulge regions.

Figure 7. Mapping of chemical shift differences between the complete TF<sub>PPIase</sub> domain and the deletion mutant TF<sub>168–251</sub> lacking 17 N-terminal residues. Amino acid residues are color-coded depending on the magnitude of the chemical shift differences between both proteins. These shifts indicate weak interactions of the N terminus (corresponding to the β1 strand in FKBP) with the β2 strand and helix α1, forming a nascent β1 strand.

Figure 8. Superposition of the lowest-energy solution structure of TF<sub>PPIase</sub> (blue) and the X-ray structure of FKBP (red). For FKBP the N terminus (residues 1–15) is omitted for clarity. Coordinates for FKBP were taken from the FKBP–FK506 complex, PDB entry 1FKF. The structures were superimposed using residues marked by asterisks in Figure 6, resulting in an rmsd value of 1.3 Å.
The sequence identity (27%) of the structurally aligned sequences of TF_{PPIase} and FKBP is higher than between TF_{PPIase} and other members of the PPIase family (virtually no sequence identity). Pin1 and parvin both lack the flap, and both feature an extended α-helical insert. While TF_{PPIase} and Pin1 can be aligned structurally with a slightly worse rmsd (2.4 Å), using analogous amino acid residues as for the TF_{PPIase}-FKBP superposition, these structural alignments do not correspond to homologies in the sequence (Figure 6).

Assuming that conserved structural features indicate common function and structural variability encodes differing function, it is intriguing to postulate different functional roles for conserved and variable regions. For PPIases, the common function is peptidyl-prolyl cis-trans isomerase activity. Most of the residues that comprise the proline binding pocket (excluding only Tyr82 for FKBP or Tyr93 for TF_{PPIase}) are located within the structure-conserved parts of these proteins.\(^{38,40}\) Non-conserved structural features may encode peptide substrate specificity\(^{43}\) or may facilitate specific interactions with other domains (TF) or even proteins (FKBP).

The flap and the 40s bulge region are different from FKBP

As seen in Figure 8, the major differences between FKBP and TF_{PPIase} are found within the flap region connecting strands β2 and β3 and in the 40s bulge that interrupts strand β5. In TF_{PPIase}, the flap (Thr88 to Pro102) is shortened by six amino acid residues when compared to FKBP (Thr76 to Ser96). In FKBP, this region has no amino acid residues when compared to TF_{PPIase} or Glu180 in E. coli TF, which correspond to Asp37 of FKBP, will be unlikely to affect the PPlase activity, as has been observed for E. coli TF mutants.\(^{46}\)

Both the 40s bulge and the flap region modulate the protein surface around the PPlase active site. These regions show a high degree of variability across the whole family of PPlases of the FKBP superfold.\(^{40,42}\) On the other hand, the variations between FKBPs originating from different species are rather low.\(^{37}\) Hence, these regions can be regarded as functional fingerprints. They may modulate specificity towards prolyl bonds in various sequence contexts or enable interactions with other proteins.

In addition to shape, the surface charge distribution may determine substrate specificity. Hence, we have calculated the electrostatic surface potential of both proteins, TF_{PPIase} and FKBP. Both proteins feature a hydrophobic active-site cleft, framed by the flap region, which is dominated by basic histidine side-chains and the 40s bulge, which differs in charge distribution between the two proteins (see Figure 2 of the Supplementary Material). For FKBP, a basic patch of Arg42, Lys44, and Lys47 dominates the 40s bulge. The same region in TF_{PPIase}, a regular β-sheet region, is distinctly acidic in character due to the presence of Glu58 and Asp34.

Peptide substrates can interact with the flap region and the 40s bulge. If peptide substrate recognition is driven by electrostatic interactions with the acidic patch, then basic sequences would be preferred over acidic sequences. This effect has been observed experimentally.\(^{43}\)

The importance of shape and charge complementarities in protein–protein interactions is well documented for the FKBP–FK506 complex, which forms a tertiary complex with calcineurin.\(^{48}\) Indeed, a lot of critical interactions of the A and B chain of calcineurin involve binding to the 40s bulge and to the flap region. Furthermore, the loop of calcineurin A that packs tightly against the 40s bulge of FKBP is rich in acidic side-chains, being complementary to the basic 40s bulge. For TF_{PPlase} probably the most relevant protein–protein (or domain–domain) interaction is the tertiary organization of the complete three-domain TF protein, for which few if any data are available. It is only known that the full-length TF interacts with GroEL,\(^{17}\) but it is not clear which domain is responsible for this interaction.

The trigger factor does not bind FK506 due to steric hindrance

One of the first differences observed between FKBP and TF_{PPlase} is that FK506 is a potent inhibitor of FKBP’s PPlase activity, while TF_{PPlase} remains unaffected by FK506.\(^{8}\) We have therefore interpreted this difference from a structural point of view.
In the past, the question of whether FK506 affects TF PPIase activity has been somewhat controversial. Although convincing data exist that *E. coli* TF does not bind FK506, weak inhibition of PPIase activity by FK506 has been observed for *Bacillus subtilis* TF. We have reinvestigated the binding of FK506 to *M. genitalium* TF PPIase using NMR techniques that provide a generic binding assay independent of any biochemical assay and can detect even weak binding. Saturation transfer difference spectra of TF PPIase plus FK506 show no signals. Also, upon addition of FK506, no amide resonances were shifted in the $^1$H–$^{15}$N HSQC spectrum of TF PPIase. Furthermore, FK506 did not compete with dimethyl sulfoxide (DMSO) which binds weakly ($K_D = 200$ mM) to the active site of TF PPIase. Hence, there is strong evidence that FK506 does not bind the TF PPIase domain of *M. genitalium*. As described below, our structural data strongly support these findings.

The crystal structure of the FKBP–FK506 complex identifies residues involved in the interaction of FKBP with FK506. Many of these residues are conserved if not identical in FKBP and TF PPIase (Table 2 and Figure 9). Two aromatic side-chains are swapped (Tyr26/Phe46 in FKBP versus Phe40/Tyr57 in TF PPIase) and the conserved Trp59 is replaced by a phenylalanine residue both in *M. genitalium* and *E. coli* TF.

Whether the conserved Trp59 in FKBP is an essential mediator for FK506-binding has been tested for *E. coli* TF by creating a more “FKBP-like” TF mutant W59F. Unfortunately, this mutation led to a protein that was highly unstable.

Table 2. Equivalent residues or functional groups that are involved in binding of FK506 in FKBP and of the substrate in both proteins

<table>
<thead>
<tr>
<th>FKBP</th>
<th>TF_PPIase</th>
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<td><strong>A. Hydrophobic contacts</strong></td>
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</tr>
<tr>
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<td>CO</td>
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</table>

Figure 9. Ribbon representations of the putative active sites of TF_PPIase (left) and FKBP (right). Conserved side-chains and functional groups that were found to be important for FK506 binding to FKBP and their equivalents in TF_PPIase are displayed as stick representations in both structures.
Trp59 in FKBP has hydrophobic contacts with two valine residues (Val24 and Val63), whereas their bulkier counterparts in *E. coli* or *M. genitalium* TFPPIase, leucine and isoleucine, leave insufficient space for a Phe-Trp replacement. Likewise a F70H mutant was not stable, since a polar side-chain is not compatible with the hydrophobic environment of Phe70.

Another difference is Asp37, which does not have a structural analog, since the backbone structure is altered significantly in this region. However, the H-bond acceptor O$_g$ of Asp37 is replaced adequately by the backbone oxygen atom of Ser51, as discussed above. Since the 3D arrangement of all other critical residues is essentially the same for both proteins, these subtle differences alone can hardly explain why FK506 does not bind to TFPPIase.

Our conclusion that TFPPIase–FK506 binding is prohibited originates from the analysis of a hypothetical TFPPIase–FK506 complex, which shows that binding of FK506 is sterically highly unfavorable. This hypothetical complex is modeled utilizing the superposition of TFPPIase and the FKBP–FK506 complex (see Figure 8). Figure 10 shows a detailed view of this hypothetical TFPPIase–FK506 complex compared to the experimental FKBP–FK506 complex. Due to differences in backbone geometry of the flap region, the conserved Tyr93 is located at a position that is shifted by 1–2 Å into the cleft when compared to its position in FKBP, leading to steric overlap with the FK506 pyranose ring (color-coded green in Figure 10 and Scheme 1). Furthermore, Val55 of FKBP, which forms part of the hydrophobic pipecolyyl binding pocket, is replaced by the much bulkier Phe66 in TFPPIase. In TFPPIase Phe66 interferes with binding of the FK506 pipecolyyl ring (color-coded red in Figure 10 and Scheme 1).

Since these arguments are based on a hypothetical model, they cannot account for conformational flexibility of the aromatic side-chains, which could give way to accommodate FK506. They also cannot account for motions of Phe66 and Tyr93 relative to each other, as might be expected from our relaxation analysis (see below). Nevertheless our view is that two main structural factors prevent FK506 Bind.

![Scheme 1](image-url)

**Figure 10.** Model of the hypothetical TFPPIase–FK506 complex (left) and comparison with the actual FKBP–FK506 complex (right). The hypothetical TFPPIase–FK506 complex is based upon the same superposition of TFPPIase and FKBP as in Figure 8. Regions of FK506 and of TFPPIase experiencing steric clashes are indicated by their dotted van der Waals surfaces. Blue, residues Phe66 and Tyr93 (TFPPIase) that interfere with the binding in TFPPIase and the corresponding Val55 and Tyr82 of FKBP. Regions of FK506 in conflict with the binding to TFPPIase are displayed in red and green, respectively, and are colored according to Scheme 1.
binding to TF<sub>PPIase</sub>: (1) the altered backbone geometry of the highly variable flap region; and (2) the side-chain replacement Phe to Val in a conserved loop. Taken together, these two aspects lead to a much narrower hydrophobic binding cleft and make binding of the large FK506 molecule highly unfavorable.

The structure of the active site is conserved between TF and FKBP

The fold of the extended sheets and the loop-helix α1-loop motif is strikingly similar in TF<sub>PPIase</sub> and FKBP. Since this structural homology is found in other PPlases, it appears to be linked to the PPlase activity directly.

Currently, the definition of the PPlase active site remains speculative, since a structure of a protein–substrate complex of TF<sub>PPIase</sub> or FKBP has not been published. Crystallization of such a complex is difficult due to the low (millimolar) affinities of TF and FKBP towards their target peptides. For the interaction of TF<sub>PPIase</sub> with a standard peptide, Suc-Ala-Ala-Pro-Phe-pNO<sub>2</sub>, NMR titration studies yield a <i>K<sub>d</sub></i> of 0.1 mM (data not shown), which compares well with the <i>K<sub>d</sub></i> of 0.52 mM of the same peptide binding to FKBP.<sup>50</sup> Cyclophilins and parvulins have a much higher affinity towards their substrates and thus have beencocystralized with a minimal substrate, Ala-Pro.

The only structural basis of FKBP's PPlase activity can be inferred by using the FK506–FKBP complex as a model, where the pipercolyl moiety of FK506 is assumed to mimic the peptidyl-prolyl bond.<sup>38,40</sup> With this approach, two enzyme–substrate complexes for FKBP were modeled to rationalize enzyme–substrate recognition.<sup>44,45</sup> These studies have identified several residues that appear to be involved in protein–substrate interactions. These residues and their TF<sub>PPIase</sub> counterparts are shown in Figure 9 and listed in Table 2.

FKBP's active-site cleft is defined by the aromatic side-chains of Tyr<sub>26</sub>, Phe<sub>46</sub>, Phe<sub>99</sub>, and Trp<sub>59</sub>. This cleft can accommodate the hydrophobic pipicoline ring of FK506, which is assumed to mimic the proline ring. FK506 binding to FKBP changes its conformation around the pipicoline ring from cis in the free state to trans in the complex. The carbonyl oxygen atom is hydrogen bonded to the amide group of the conserved Ile<sub>56</sub> of FKBP (Ile<sub>67</sub> for TF<sub>PPIase</sub>). In FKBP, this amide has a greatly enhanced hydrogen bond donor potential, leading to the formation of specific hydrogen bonds even to small molecules like DMSO.<sup>37,51</sup> For TF<sub>PPIase</sub>, we find large shifts for the corresponding Ile<sub>67</sub> amide resonances upon addition of the peptide substrate or DMSO. The mode of binding to the peptide substrate can therefore be assumed to be similar for both proteins.

Other hydrogen bonds involving the backbone of Glu<sub>54</sub> and side-chains of Tyr<sub>82</sub> and Asp<sub>37</sub> were considered important for enzyme–substrate recognition (Table 2). In TF<sub>PPIase</sub>, the relative 3D arrangement of these groups is essentially the same (see Figure 9). The only differences are the narrowing of the hydrophobic cleft by the aromatic side-chain of Phe<sub>66</sub>, and a repositioning of one hydrogen bond donor, the tyrosine hydroxy group, by 1–2 Å (see above).

Since the TF<sub>PPIase</sub> substrate-binding site is narrower and thus more restrictive than that in FKBP, the TF<sub>PPIase</sub> domain may be more selective with respect to its substrates. This is confirmed by experimental data concerning the stereoselectivity of both proteins.<sup>52</sup> While FKBP also catalyzes isomerization in peptides that contain a D-proline residue at the critical position, this is not the case for TF<sub>PPIase</sub>. For these D-Pro-containing peptides, the kinetic data yield a much larger Michaelis–Menten constant, indicating that these substrates are bound by TF<sub>PPIase</sub> less tightly than by FKBP.

Peptide substrate variability has been addressed in several additional studies, which have explored the nature of the residue preceding the prolyl bond (the P1 site), both for FKBP and for TF<sub>PPIase</sub> (<sup>8</sup>9,23,46,53) Both proteins show the same preference for bulky, hydrophobic side-chains and no tolerance for negatively charged side-chains. The preference for a bulky side-chain (as judged by relative catalytic efficiencies) is higher for TF<sub>PPIase</sub> than for FKBP, which apparently contradicts the narrower binding-cleft hypothesis. However, the P1 side-chain is located at some distance from the proline-binding site where the narrowing occurs. Nevertheless, detailed knowledge of the peptide’s binding mode will be necessary for a more extensive characterization of substrate binding.

TF<sub>PPIase</sub> and FKBP show similar dynamic behavior

The dynamic properties of TF<sub>PPIase</sub> were probed by measuring <sup>15</sup>N relaxation parameters. Figure 11 shows longitudinal (<i>R</i><sub>L</sub>) and transversal (<i>R</i><sub>T</sub>) relaxation rates as well as <sup>1</sup>H–<sup>15</sup>N heteronuclear NOE values for a total of 91 backbone amide protons (89% of all possible). No data are presented for residues for which assignments are missing (residues 14, 15, 17, 19, 20, 50, and 51) or for those with significant overlap in the <sup>1</sup>H–<sup>15</sup>N-HSQC spectrum (residues 25 and 81). Excluding the N-terminal 18 residues, the average <sup>1</sup>H–<sup>15</sup>N NOE value of 0.80 (±0.03) indicates that most regions of TF<sub>PPIase</sub> are relatively rigid, which is consistent with the narrow distribution of conformers in the calculated ensemble. Within the structured region, only Ile<sub>67</sub> exhibits internal motion on the nanosecond to picosecond time-scale, which may be of importance for its role as hydrogen bond donor. Some residues (residues 33, 34, 37, 63, 64, 70, and 110) show <sup>1</sup>H–<sup>15</sup>N NOE values above the theoretical maximum of 0.834, which may be due to chemical exchange with the solvent, provided the errors of these values are not underestimated.<sup>54</sup> The N-terminal 18 residues are flexible in solution, as evidenced by <sup>1</sup>H–<sup>15</sup>N NOE values
In this region we find large errors of the $R_2$ values due to multiexponential decay of the experimental data. Hence, the flexible tail may not adopt a random-coil conformation. On the basis of our studies on TF168-251 (see above and Figure 7), this observation supports our postulation, that the N terminus forms a “nascent” β strand interacting with the edge of the β sheet.

The considerable variation of relaxation rates over the entire protein ranging from 1.21 s$^{-1}$ to 2.24 s$^{-1}$ for $R_1$ and from 9.57 s$^{-1}$ to 22.5 s$^{-1}$ for $R_2$ indicates that TFPPase is subjected to significant intramolecular motions on the nanosecond time-scale as well as conformational motion on the microsecond to millisecond time-scale.

Reduced spectral density mapping is a convenient method to characterize the motion of each N–H bond at 0, $\omega_N$, and 0.87$\omega_H$ frequency.$^{55,56}$ Values obtained for TFPPase are depicted in Figure 12(a)–(c). In general, the spectral density function $J(\omega)$ of flexible residues decays slowly towards higher values of $\omega$, resulting in an increase of $J(0.87\omega_H)$ (Figure 12(c)) and a decrease of $J(0)$ (Figure 12(a)). On the other hand, exchange broadening gives rise to an increase of $J(0)$. These micro- to millisecond motions, which are most relevant for biological function, have no effect on $J(\omega_N)$ or $J(0.87\omega_H)$. Thus, residues undergoing slow motion can be extracted by analyzing $J(0)$ values (Figure 12(c)).

In the case of anisotropic tumbling, $J(0)$ depends on the dynamics and on the orientation of each N–H vector. Since TFPPase is expected to tumble anisotropically, as judged from the ratio of the eigenvalues of the inertial tensor (1.00:0.84:0.52), we calculated scaled $J^{\text{eff}}(0)$ values that depend only on the dynamics of a given residue.$^{57}$ The averaged $J^{\text{eff}}(0)$ was determined from rigid residues in secondary structure elements and amounts to 3.08(±0.32) ns (Figure 12(d)). Hence, an average

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**Figure 11.** Plots of (a) $^{15}$N longitudinal relaxation rate ($R_1$), (b) $^{15}$N transverse relaxation rates ($R_2$) and (c) $^{1H}$–$^{15}$N NOE (from top to bottom) as a function of residue number for TFPPase at 298 K and 600 MHz. Residues for which no results are shown correspond either to proline residues, to unassigned, or to overlapped residues. The location of the β-strands and the α-helices within the structure is sketched above the panels.
overall correlation time of \( \tau_{m}^{\text{eff}} = 2.5 \). \( J_{eff}(0) \) = 7.7(±0.7) ns can be defined, which is a reasonable value for a monomeric protein of this size. This value cannot be compared to the overall correlation time of FKBP (9.2 ns) directly, because the measurements were carried out at significantly different temperature (303 K) and protein concentration (8.6 mM). The authors attribute the large \( \tau_{m}^{\text{eff}} \) value of FKBP to possible solution viscosity effects arising from the high protein concentration.

Considering only the structured part of TF{PPIase}, we define residues with \( J_{eff}(0) > 3.4 \) ns indicating significant exchange broadening are marked with open circles.

**Figure 12.** Plots of the reduced spectral density (a) \( J(0) \), (b) \( J(\omega_{N}) \), (c) \( J(0.87\omega_{H}) \) and (d) the scaled spectral density \( J_{eff}(0) \) as a function of residue number for TF{PPIase}. The location of secondary structure elements within the structure is sketched above the panels. Values for the reduced spectral density \( J(0) \), \( J(\omega_{N}) \), \( J(0.87\omega_{H}) \) (a)–(c) were calculated using method 2 from Konradi et al.\(^{56} \) (d) Values for the scaled spectral density \( J_{eff}(0) \) were calculated as described.\(^{57} \) The average \( J_{eff}(0) \) is indicated by a horizontal full line and the standard deviation of \( J_{eff}(0) \) by broken lines. For the structured part, values with \( J_{eff}(0) > 3.4 \) ns indicating significant exchange broadening are marked with open circles.
that are hydrogen bonded in well-defined secondary structure elements appear to exhibit conformational motions. Notable are the α1-helix, the end of the strand β5 (Thr60, Ile61), the beginning of the strand β4 (Gly33 to Ile38), as well as the residues Val107 and Thr84 from the central part of the β sheet.

The TF_PPIase structure can be described as a bent hand with the palm being the β-sheet, the thumb forming the flap region, and the fingers comprising the ends of the β-strands plus helix α1. Using this picture, the conformational motion of TF_PPIase describing our relaxation data can be visualized as an opening and closing of this hand. Residues Thr84, Val107, Thr41, and Glu55 (located across all strands of the central β sheet) would act as a hinge. Such breathing motions were observed for FKBP. When all amide protons with chemical exchange line-widths larger than 2 Hz are mapped on the structure of FKBP, the corresponding regions are affected (Figure 5(b)). Thus, from our relaxation data we conclude that TF_PPIase and FKBP exert similar motions.

In both proteins, the residues with increased flexibility include the protein–substrate interface discussed above, most notably the loop including helix α1, residues within the 40s bulge in FKBP (50s bulge in TF_PPIase) and the flap region. It is reasonable to assume that motions within the molecular interface are relevant for the binding process. Indeed, the available data on FKBP support this view. In the FK506–FKBP complex, residues within the flap region (residue 82–87) were found to be more rigid than in free FKBP, as indicated by damped internal motions at these sites. It is therefore probable that a picture similar to that of FK506 bound to FKBP emerges when proline-containing peptides are bound to TF_PPIase. We are currently investigating the effect of the standard substrate peptide Suc-Ala-Ala-Pro-Phe-pNO2 on the dynamics of TF_PPIase.

Conclusions

The NMR structure of TF_PPIase agrees well with previously published structural models that are based on sequence homology with FKBP. TF_PPIase adopts a four-stranded β-sheet structure found also in FKBP and other PPIases. The N-terminal β1 strand of FKBP was not structured in TF_PPIase. However, our data suggest that this N terminus may form a nascent strand by partially adhering to the edge of the central β-sheet. In addition to structural similarity, we observe dynamic similarity of the two proteins. Characteristic differences from the predicted structure were found in those regions that differ in sequence most strongly from that of FKBP, in particular the flap and the 40s bulge regions. These regions may be responsible for substrate recognition and specific interactions with other proteins.

Despite the high degree of conservation within the active site of TF_PPIase, two distinct structural differences can explain the inability of TF_PPIase to bind FK506: (1) a replacement of a non-conserved Val by Phe; and (2) backbone structure variations involving a conserved tyrosine residue. Both effectively narrow the active site, thereby causing steric clashes that prohibit binding of FK506.

Finally, our structure can explain all currently published mutational data on TF. Most of these mutations unsuccessfully attempted to re-establish FK506 binding by designing a more FKBP-like TF_PPIase. From the structure, it is now evident that this could not be expected, since mutations of the most critical residues that narrow the active site were not undertaken. Our structural data suggest that mutating Phe66 and Tyr93 to less bulky hydrophobic residues may enable FK506 binding.

Materials and Methods

Overexpression and purification of the trigger factor

The PPIase domain TF151–251 (denoted TF_PPIase in the text) including an N-terminal His-tag (MRGSHHHHH-HHGS) was cloned and overexpressed in E. coli and purified as described.

The TF_PPIase and the deletion mutant TF168–251 both devoid of the His6 tag were amplified with the N-terminal NcoI-site primers (5′-ggggaggggcctgctggaaagctgg-3′) for TF_PPIase and (5′-ggggaggggcctgctggccaaaactagctaatggtg-3′) for TF168–251, and the C-terminal BamHI-site primer (5′-ggggtttgcctgctggcagctcag-3′) and cloned into the vector pKM263, an expression vector selected in E. coli BL21DEIII pLysS (Novagen). Expression in M9 minimal medium, purification and cleavage of the GST fusion protein were done as described with minor modifications.

NMR spectroscopy

All NMR experiments were recorded at 298 K on Bruker DRX600 and DRX800 spectrometers at 600 and 800 MHz proton resonance frequencies, respectively. For assignment purposes, samples with 2 mM protein concentration were used. The following samples were prepared: (1) U–15N; (2) U–13C/U–15N; (3) unlabeled protein. All spectra were recorded on 50 mM sodium phosphate buffer (pH 6.5). For the HCCH-total correlated spectroscopy (TOCSY), the 80 ms 13C-NOESY-HMQC's and 80 ms 2D-NOESY, the samples were dissolved in 99% H2O/1% 2H2O.

The following multidimensional experiments were recorded and evaluated: (a) backbone assignment: HNCO, HNCA, CBCA(CO)NH, HNCACB; (b) sidechain assignment: (H)CC(CO)NH-TOCSY, HCC-TOCSY, 80 ms 15N-NOESY-HMQC's, 60 ms 15N-TOCSY-HSOCQ, 80 ms 13C-NOESY-HMQC (aliphatic region), 80 ms 13C-NOESY-HMQC (aromatic region); (c) additional spectra for structure elucidation: HNHA, 80 ms 2D-NOESY. NOEs involving aromatic protons were extracted from the latter spectrum. 5% 2H2O and 5% dodecyl (ethylene glycol)hexanol 96:4 w/w were recorded for evaluation of residual dipolar couplings.
All 3D spectra were processed by Bruker xwinmr1.3 software including linear prediction and apodization by shifted sinebell functions. Backbone and side-chain resonances were assigned using Felix (MSI) and xeasy.63 13N relaxation data were obtained on a 1.5 mM concentrated (1-15N)-labeled TFPPIase sample. Backbone amide [1H]-15N NOE, 15N R1, 15N R2 (CPMG) values were measured at 298 K and 600 MHz with conventional pulse sequences.64 The 15N R1 and the 15N R2 relaxation decays were sampled at eight different time-points each (T1 delays = 0.005, 0.079, 0.159, 0.239, 0.359, 0.519, 0.759, and 1.119 seconds; T2 delays = 0.009, 0.017, 0.034, 0.060, 0.086, 0.112, 0.148, 0.190 seconds) with duplicate spectra for all time-points. All R1 and R2 spectra were acquired with 128 x 1024 complex points and with spectral widths of 2.067 x 8.503 kHz in the t1 x t2 dimensions. A recycle delay of three seconds and eight transients were applied. For the unsaturated [1H]-15N NOE measurement, a recycle delay of five seconds was used. This recycle delay was substituted in the presaturated [1H]-15N NOE experiment with a 0.5 second delay followed by a 4.5 second long series of non-selective 120° H pulses separated by 5 ms delay. The NOE-spectra were recorded in an interleaved manner with 64 transients each.

Distance, dihedral angle and orientation restraints

Evaluation and assignment of NOESY spectra was accomplished with xeasy.63 NOEs from the 15N-edited 3D-NOESY-HSQC and from the 2D homonuclear NOESY were classified either as very weak, weak, medium, or strong with an upper distance restraint of 5.6, 4.6, 3.0 and 2.9 Å, respectively. NOEs from 13C-edited NOESY-HSQC spectra were classified as strong to medium (4.5 Å) or weak (5 Å), reflecting the reduced sensitivity and resolution of these 13C-edited spectra. In all cases, no lower distance limit was applied.

Hydrogen bonds were introduced for amide protons where the exchange against deuterium was not complete after 20 hours. One additional hydrogen bond (Ala76(NH)–Thr72(O)) emerged from the resulting structures and was introduced at a later stage of the calculation. Hydrogen bonds were defined as double hydrogen bonds from the carbonyl oxygen atom to the amide nitrogen and nitrogen atoms, using standard lengths for hydrogen bonds.

\[ J_{HN-15N(\psi)} \] coupling constants were extracted from a 3D HNHA experiment.68 In addition to J coupling restraints (see below), dihedral angle restraints for \( \psi \) were applied in an iterative way, i.e. after the corresponding amino acid residues began to cluster within the Ramachandran plot. Generally, values of 120(±40°) for \( \beta \)-sheet regions and 65(±20°) for \( \alpha \)-helices were applied. For residues that emerged in the positive \( \psi \) range, a value of 60(±40°) was applied.

Dipolar splittings were evaluated from the resonance positions derived from two spin-state selective, \( \omega_1 \)-coupled [1H]-15N HSQC spectra.69 Splitting were subsequently converted into vector projection restraints using the DipolCoup program.70

Structure calculation and analysis

Structures were calculated using the CHARMM force field with the CNS program package.71 CNS was extended to incorporate residual dipolar coupling restraints70 (program files available online from ftp://

ftp.EMBL-Heidelberg.DE/nmr/nlges/arianew/cns_1.0_10042000). A four-stage molecular dynamics protocol was used: (a) 500 steps/7.5 ps torsion angle molecular dynamics at 10,000 K; (b) 1000 steps/15 ps cooling to 0 K; (c) 5000 steps/25 ps Cartesian molecular dynamics cooling from 2000 K to 0 K; (d) extensive minimization. Prochiral valine and leucine methyl groups were treated with float at 298 K.

The resulting NMR structures were evaluated with MOLMOL66 and PROCHECK.32 Figures were prepared and electrostatic surface potentials were calculated with MOLMOL.

Structural data for comparison with the FKBP structure were taken from PDB entries 1FKF for the FKBP–FK506 complex,75 1D6O for uncomplexed FKBP and 1D7H for the FKBP–DMSO complex,75 1PIN for human Pin176 and 1EQ5 for parvulin.37

Relaxation analysis

Relaxation NMR spectra were processed and analyzed with Felix 2000 software (Biosym Technologies, Inc.). Backbone amide [15N] R1 and R2 relaxation rates were determined by fitting of peak heights as functions of relaxation decay times to single-exponential decay functions. Steady-state NOE values were calculated from the ratios of the peak intensities with and without proton saturation. Uncertainties in measurements of peak height were estimated from baseline noise. Spectral densities were calculated at \( \omega = 0, \omega_0, \text{and } 0.8\omega_0 \) from \( R_1 \) and \( R_2 \) spectra of [1H]-15N NOE data using the approximation \( J(\omega) = \text{const} or J(\omega) \propto 1/\omega^2 \) for \( \omega = 0 \pm 0.8\omega_0 \). Since the eigenvalues of the inertial tensor are in the ratio of 1:0.008:0.5 for the best calculated structure, TFPPIase is expected to tumble anisotropically. As a consequence, \( J(\omega) \) depends on the orientation of the individual N–H bonds. Therefore, \( J(\omega) \) was scaled up to residue-independent \( J^{\text{eff}}(0) \) values using the procedure described.77 Based on our structure, the anisotropy factor \( \sigma \) was calculated as \( \sigma = D_\parallel/D_\perp = 1.6 \) with the shape of TFPPIase approximated to a symmetrical ellipsoid. The angle \( \alpha \) between the N–H bond vector and the long axis was extracted from the 3D structure. This led to scaling factors between 0.86 and 1.10. Anisotropic effects for \( J^{\text{eff}}(0) \) can be neglected in most real cases. \( J^{\text{eff}}(0) \) then depends on only the dynamics: internal motions give rise to lower than the average \( J^{\text{eff}}(0) \), whereas exchange broadening causes higher values. The average of \( J^{\text{eff}}(0) \) was taken from 19 residues in secondary structure elements that show little flexibility (residues 39, 40, 42–44, 47, 48, 85–89, 97–99, 103–106). It amounts to 3.1(±0.3) ns.

Binding studies

TFPPIase–FK506 binding studies were conducted by recording saturation transfer difference (STD) spectra66 and by monitoring chemical shift changes in 1H–15N HSQC spectra. Spectra with the same amount of DMSO were used as a reference. FK506 (Calbiochem, San Diego) was dissolved in DMSO and added as stock solution to yield a 10% DMSO/90% buffer solution. Under these conditions, ca 0.5 mM FK506 is soluble as judged from relative signal intensities with a standard concentration (0.2 mM 3,3,3-trimethylsilyl-propionate (TSP)). For STD spectra, a tenfold excess of ligands (DMSO or peptide) were added stepwise. In STD spectra, saturation was achieved by a series of equally

1.00:0.84:0.52 for the best calculated structure, TFPPIase is approximated to a symmetrical ellipsoid. The angle \( \alpha \) between the N–H bond vector and the long axis was extracted from the 3D structure. This led to scaling factors between 0.86 and 1.10. Anisotropic effects for \( J^{\text{eff}}(0) \) can be neglected in most real cases. \( J^{\text{eff}}(0) \) then depends on only the dynamics: internal motions give rise to lower than the average \( J^{\text{eff}}(0) \), whereas exchange broadening causes higher values. The average of \( J^{\text{eff}}(0) \) was taken from 19 residues in secondary structure elements that show little flexibility (residues 39, 40, 42–44, 47, 48, 85–89, 97–99, 103–106). It amounts to 3.1(±0.3) ns.
spaced 50 ms Gaussian-shaped pulses, with a peak amplitude of 10 Hz, 10 ms delay between the pulses, and a total saturation time of about two seconds.

**Data Bank accession codes**

The structures shown in Figure 4 have been submitted to the RCSB Protein Data Bank, with ID code 1HXV. Backbone and Cα resonances were assigned using a combination of 3D HNCACB and 3D CBCA(CO)NH spectra. For the structured region of TPerwsc all residues, except Ala50, give rise to distinct amide cross-peaks. The assignment and the extent of assigned resonances have been submitted to the BioMagResBank with accession number 4953.

**Acknowledgments**

We thank Susanne Grimme and Barbara Pescatore (Frankfurt) for overexpression and purification of the trigger factor, Wolfgang Peti and Jens Meiler (Frankfurt) for assistance with measurement and evaluation of residual dipolar coupling data, and Peter Bayer (Dortmund) and Ulf Reimer (Berlin) for many helpful discussions concerning the trigger factor.

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Edited by J. Karn

(Received 29 August 2001; received in revised form 26 December 2001; accepted 12 February 2002)

http://www.academicpress.com/jmb

Supplementary Material comprising two Figures of the $^1$H–$^{15}$N HSQC spectra of TF$_{PPIase}$ and of the deletion mutants, and one Figure showing the electric surface potential and van der Waals surface of TF$_{PPIase}$ is available at IDEAL.