Thermal coefficients of the methyl groups within ubiquitin

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Abstract: Physiological processes such as protein folding and molecular recognition are intricately linked to their dynamic signature, which is reflected in their thermal coefficient. In addition, the local conformational entropy is directly related to the degrees of freedom, which each residue possesses within its conformational space. Therefore, the temperature dependence of the local conformational entropy may provide insight into understanding how local dynamics may affect the stability of proteins. Here, we analyze the temperature dependence of internal methyl group dynamics derived from the cross-correlated relaxation between dipolar couplings of two CH bonds within ubiquitin. Spanning a temperature range from 275 to 308 K, internal methyl group dynamics tend to increase with increasing temperature, which translates to a general increase in local conformational entropy. With this data measured over multiple temperatures, the thermal coefficient of the methyl group order parameter, the characteristic thermal coefficient, and the local heat capacity were obtained. By analyzing the distribution of methyl group thermal coefficients within ubiquitin, we found that the N-terminal region has relatively high thermostability. These results indicate that methyl groups contribute quite appreciably to the total heat capacity of ubiquitin through the regulation of local conformational entropy.

Keywords: NMR; methyl group dynamics; cross-correlated relaxation; thermal coefficients; protein stability; ubiquitin

Introduction
The intricate relationship between structure, function, and dynamics strongly influences such fundamental physiological processes as protein folding, molecular recognition, and thermal stability. All of these processes occur with concomitant changes in the thermodynamic parameters of the system, specifically the enthalpy ($H$) and the entropy ($S$). The linkage between the temperature dependence of $H$ and $S$ is expressed by the heat capacity ($C_p$).1,2 Contributions to the $C_p$ of a protein include hydration of solvent exposed surface area, covalent bonds, electrostatic interactions, and hydrogen bonds. In addition, the local conformational entropy ($S_{conf}$) of residues within a protein must also be considered. The term $S_{conf}$ is directly related to the degrees of freedom each residue possesses within the three-dimensional protein structure. Therefore, an understanding of the dependence of $S_{conf}$ on temperature, encapsulated by $C_p$, yields insight into the role local dynamics play in controlling the stability of proteins.
Nuclear magnetic resonance (NMR) spectroscopy provides a potent tool for characterizing such local dynamics with atomic resolution on multiple time-scales. Analysis of spin-lattice relaxation ($T_1$), spin-spin relaxation ($T_2$), and steady-state nuclear Overhauser enhancements with the “model free” formalism introduced by Lipari and Szabo allows the extraction of a general order parameter ($S^2$) that describes the amplitude of the individual bond vector motions on the ps to ns time-scale.\(^4,5\) The relationship between $S^2$ and $S_{\text{corr}}$ results from the dependence of both parameters on the population distribution of bond vector orientations.\(^6,8\) By measuring relaxation data at multiple temperatures, the temperature dependence of $S^2$ ultimately provides the local $C_p$ for each bond vector.\(^9–13\)

An important probe ideally suited for providing fundamental insight into protein folding, stability, and recognition are methyl groups. Located primarily within the hydrophobic core of proteins, methyl groups are typically quite numerous, well dispersed throughout the core, and possess a wide variety of motional amplitudes.\(^9,14–16\) For these reasons, determining the methyl group order parameters ($S^2_{\text{axis}}$) provides an avenue for understanding how methyl dynamics on the ps to ns time scale are related to protein stability. To obtain $S^2_{\text{axis}}$, typically the methyl groups in a protein are deuterated (–CH$_3$D or –CHD$_2$) for performing deuteration relaxation measurements.\(^17–19\) An alternative to this approach is to utilize cross-correlated relaxation (CCR) between dipolar couplings of two CH bonds ($\sigma$) in the methyl group for extracting $S^2_{\text{axis}}$.\(^20,21\)

An inherent advantage to determining $S^2_{\text{axis}}$ from $\sigma$ versus deuteration relaxation is the savings in measurement time, especially important for measuring $S^2_{\text{axis}}$ at many different temperatures. In the past, $S^2_{\text{axis}}$ calculated at one temperature point with deuteration relaxation studies could take up to 1 week due to the requirement of data being recorded at two fields.\(^17\) Recent advances in determining $S^2_{\text{axis}}$ from the measurement of five relaxation rates for –CH$_3$D\(^19\) and four relaxation rates for –CHD$_2$\(^18\) has effectively reduced the amount of time necessary to obtain the same information to ~1 day. Yet, $S^2_{\text{axis}}$ derived from $\sigma$ can require as little as 1 h of measurement time and, thus, is ideally suited for studying the temperature dependence of $S^2_{\text{axis}}$.

Here, we report the temperature dependence of the methyl group order parameters derived from the CCR between dipolar couplings of two CH bonds in ubiquitin. From these measurements, we calculate the thermal coefficients characterizing this temperature dependence, specifically the characteristic thermal coefficient $\Lambda$ and the local heat capacity $C_p$. Furthermore, we analyze the distribution of methyl group thermal coefficients within ubiquitin, illustrating the relatively high thermostability of the N-terminal region of this protein.

### Results and Discussion

#### Methyl group CCR

Two dimensional constant time $^{13}$C, $^1$H HSQC measurements without decoupling of $^1$H during $^{13}$C-chemical shift evolution leads to splitting of the methyl group $^{13}$C signal into a quartet.\(^22,23\) The peaks in the quartet are separated by the J-coupling constant for C–H bonds in a methyl group ($J_{\text{CH}} = 125$ Hz). The quartet represents the four coherences of $C_xH_1$,$H_2$,$H_3$, $C_x$($H_1$,$H_2$,$H_3$,$H_9$ + $H_1$,$H_2$,$H_9$ + $H_1$,$H_2$,$H_3$) and $C_x$($H_1$,$H_2$,$H_3$,$H_9$ + $H_1$,$H_2$,$H_3$) and their intensity ratio ($I_{\alpha}$ : $I_{\beta}$ : $I_{\gamma}$ : $I_{\delta}$) is 3:1:1:3 in the absence of relaxation.\(^22,24\) With the contribution of the transverse relaxation rate ($R_{2,\lambda}$),\(^25\) the intensities can be expressed as:

\[
\begin{align*}
I_{\alpha} &\propto 3e^{-R_{2,\lambda} \Delta} \\
I_{\beta} &\propto e^{-R_{2,\lambda} \Delta} \\
I_{\gamma} &\propto e^{-R_{2,\lambda} \Delta} \\
I_{\delta} &\propto 3e^{-R_{2,\lambda} \Delta}
\end{align*}
\]

where $\Delta$ is the length of the constant time period.

By considering the dipolar coupling and chemical shift anisotropy (CSA), $R_{2,\lambda}$, can be expressed as:\(^20,25\)

\[
\begin{align*}
R_{2,\lambda,\alpha} &= \lambda + 3\sigma + 2\eta \\
R_{2,\lambda,\beta} &= \lambda - \sigma + \frac{2}{3}\eta \\
R_{2,\lambda,\gamma} &= \lambda - \sigma - \frac{2}{3}\eta \\
R_{2,\lambda,\delta} &= \lambda + 3\sigma - 2\eta
\end{align*}
\]

where $\lambda$ is the rate of the autorelaxation and $\sigma$ and $\eta$ are the rate of the CCR between dipolar couplings of two CH bonds and between dipolar coupling of CH and CSA of the $^{13}$C nucleus, respectively. Thus, the CCR rate between dipolar couplings of two CH bonds can be determined experimentally from the intensities of the quartet,\(^20\)

\[
\sigma_{\text{obs}} = \frac{1}{8\Delta} \ln \frac{9I_{\alpha}I_{\delta} I_{\gamma}I_{\beta}}{I_{\beta}I_{\gamma}}.
\]

Here, we report the temperature dependence of $\sigma_{\text{obs}}$ for the methyl groups of uniformly $^{15}$N, $^{13}$C-labeled human ubiquitin extracted from a series of 2D constant time $^{13}$C, $^1$H HSQC measurements at fourteen temperatures: 275, 278, 281, 283, 286, 288, 291, 293, 296, 298, 301, 303, 305, and 308 K. Ubiquitin possesses 50 methyl groups residing in 30 residues.
Figure 1. Constant time $^{13}$C, $^1$H HSQC spectrum of uniformly $^{15}$N,$^{13}$C-labeled human ubiquitin recorded at a proton frequency of 700 MHz and a temperature of 275 K. The concentration of ubiquitin was 3.6 mM in 90%/10% H$_2$O/D$_2$O, containing 50 mM sodium phosphate at pH 6.8, 100 mM NaCl, and 0.1% NaN$_3$. The constant time duration and INEPT delays were set to 27.8 and 2 ms, respectively. The spectrum was recorded with 1024 and 128 complex points in the direct ($t_2$) and indirect ($t_1$) dimensions, respectively, with eight scans per $t_1$ increment. The $t_{1,\text{max}}$ and $t_{2,\text{max}}$ were 24.3 ms and 113 ms, respectively. Frequency discrimination in the indirectly detected dimension was achieved with the States-TPPI scheme. The spectrum was processed with NMRPipe software. 1D slices of selected quartets are illustrated together with the corresponding coherences for each peak in the multiplet.

Immediately clear from the measurement at 275 K (see Fig. 1), the peaks in the highlighted quartets are well resolved, despite the increase in $\tau_c$ accompanied with lowering the temperature. In addition, even at 275 K, a significant amount of motion is evident for the methyl groups of ubiquitin, especially for L851 whose quartet approaches the ideal 3:1:1:3 intensity ratio.

For nearly 50% of all methyl groups in ubiquitin, Eq. (3) was used to calculate $\sigma_{\text{obs}}$ at each of the 14 temperatures. The results are compiled in Supporting Information Table SII. For the remaining 28 methyl groups, either spectral overlap becomes problematic due to the chemical shift differences between methyl group carbons being similar to $J_{\text{CH}}$, $2J_{\text{CH}}$, or $3J_{\text{CH}}$ and/or strong coupling is present between the $\delta$ and $\gamma$ carbons in leucine as reported for L1561, L4361, L5061, L5651, and L6982. In addition, we only analyzed methyl groups whose quartets were not overlapped with other methyl group quartets over the entire temperature range (275–308 K).

As evident from the range of $\sigma_{\text{obs}}$ at each temperature, the methyl groups exist in a wide array of environments. Variations in the mobility of the methyl group contribute to the observed differences in $\sigma_{\text{obs}}$. It should be noted that studying the temperature dependence of methyl group dynamics with deuterium relaxation studies $^{17–19,26}$ though more time consuming, enables many more methyl groups to be analyzed due to significantly less spectral overlap. Nevertheless, with the wide availability of residue selective methyl group labeling $^{27–29}$ which now even includes stereospecific selection of methyl groups, $^{30}$ we anticipate this approach being applicable to proteins larger than ubiquitin.

Quantification of methyl group dynamics in ubiquitin

The experimental CCR rate can be used to extract the methyl group order parameter ($S_{\text{axis}}^2$) by comparing $\sigma_{\text{obs}}$ with the theoretical value of the CCR ($\sigma_{\text{rigid}}$) in the absence of local motions $^{20,25,31}$:

\[ S_{\text{axis}}^2 = \frac{\sigma_{\text{obs}}}{\sigma_{\text{rigid}}} \] (4)

\[ \sigma_{\text{rigid}} = \frac{1}{45} \left( \frac{\mu_0 \gamma_H \gamma_C}{8 \pi^2 r_{\text{CH}}^2} \right)^2 2 \tau_c + \frac{3 \tau_c}{2(1 + (\omega_c \tau_c)^2)} \] (5)

where $\mu_0$ is the permeability of a vacuum, $h$ is the Planck constant, $\gamma_H$ and $\gamma_C$ are the gyromagnetic ratios of $^1$H and $^{13}$C, respectively, $r_{\text{CH}}$ is the CH bond length, $\omega_c$ is the Larmor frequency of $^{13}$C and $\tau_c$ is the rotational correlation time. $S_{\text{axis}}^2$ is a dimensionless quantity utilized for describing the amplitude of motions for the methyl group. $^{20}$ Values for $S_{\text{axis}}^2$ range from 0 to 1, where 1 represents a rigid methyl group and zero represents unrestricted local motion.

For determination of $\sigma_{\text{rigid}}$ with Eq. (5), the methyl group C–H bond length ($r_{\text{CH}}$) was taken as 1.095 angstroms, tetrahedral geometry assumed and the $\tau_c$ of ubiquitin at each temperature is reported in Supporting Information Table SII. With $\sigma_{\text{rigid}}$, we calculated $S_{\text{axis}}^2$ with Eq. (4) for all 14 temperatures, presented in the Supporting Information Table SII. Figure 2 illustrates the correlation of calculated $S_{\text{axis}}^2$ at a selected set of temperatures. Readily apparent from the figure is the high degree of correlation for $S_{\text{axis}}^2$ over these temperatures. For every pair-wise combination of $S_{\text{axis}}^2$, the Pearson correlation coefficient is $r \geq 0.98$. Since $S_{\text{axis}}^2$ has a linear dependence on $T$, the high correlation between $S_{\text{axis}}^2$ at all the temperatures suggests that uncertainties arising...
from each experimental measurement are consistent over the entire data set. Figure 3(A) presents the correlation plot of $S^2_{\text{axis}}$ values from CCR measurements versus $S^2_{\text{axis}}$ extracted from the rates of multiple spin coherences involving $^2\text{H}$ in the methyl group, both at 303 K. Figure 3(B) is the correlation plot of the currently reported $S^2_{\text{axis}}$ at 301 K versus $S^2_{\text{axis}}$ extracted from methyl group $^{13}\text{C}$ spin-lattice ($T_1$) relaxation rates and $\sigma$ modulated by the one bond $\text{C}^\text{A}$-$\text{H}$ coupling constant ($J_{\text{CH}}$) measured at 300 K. For both comparisons, the Pearson correlation coefficient is high, 0.96 and 0.97, respectively. The agreement between $S^2_{\text{axis}}$ obtained by three independent studies coupled with the high correlation for $S^2_{\text{axis}}$ over the entire temperature range provides strong confirmation that the temperature dependence of $S^2_{\text{axis}}$ can be studied using this method. It is important to point out that deviations from the diagonal most likely indicate small differences in experimental setup, such as sample conditions, slight temperature variations, and/or measurement error. Most importantly, we have significantly reduced the total amount of time required to obtain this information. With methods involving $^2\text{H}$ labeling of methyl groups, $R_1$ and $R_2$ relaxation rates are typically measured in order to extract $S^2_{\text{axis}}$ at one temperature. As for $\sigma$ modulated by the one bond $\text{C}^\text{A}$-$\text{H}$ coupling constant ($J_{\text{CH}}$), $S^2_{\text{axis}}$ determined at one temperature needs a series of 2D constant time $^{13}\text{C}$-$^1\text{H}$ HSQC measurements with increasing delay times. However, with the present method, one temperature point requires only one 2D constant time $^{13}\text{C}$-$^1\text{H}$ HSQC measurement.

Finally, the time-scale of motion encompassed by $S^2_{\text{axis}}$ is faster than the $\tau_c$ of ubiquitin, describing ps to
Table I. Thermal Coefficients Extracted from Methyl Group Order Parameters (S°{sub axis}) Within Ubiquitin

<table>
<thead>
<tr>
<th></th>
<th>( \kappa \times 10^{-3} \text{ K}^{-1} )</th>
<th>( \Lambda^c )</th>
<th>( C_p \text{ (J K}^{-1}\text{mol}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3/2</td>
<td>-2.9 ± 0.4</td>
<td>7.5 ± 1.4</td>
<td>52.7 ± 11.9</td>
</tr>
<tr>
<td>I361</td>
<td>-2.5 ± 0.4</td>
<td>3.2 ± 0.5</td>
<td>27.3 ± 4.5</td>
</tr>
<tr>
<td>T7/2</td>
<td>-0.8 ± 0.5</td>
<td>1.0 ± 0.8</td>
<td>8.5 ± 7.9</td>
</tr>
<tr>
<td>L861</td>
<td>-2.3 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>13.1 ± 1.1</td>
</tr>
<tr>
<td>T9/2</td>
<td>-3.0 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>23.4 ± 2.8</td>
</tr>
<tr>
<td>T12/2</td>
<td>-4.4 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>41.5 ± 4.9</td>
</tr>
<tr>
<td>I1331</td>
<td>-3.2 ± 0.3</td>
<td>2.5 ± 0.2</td>
<td>22.4 ± 2.3</td>
</tr>
<tr>
<td>V17/2</td>
<td>-3.1 ± 0.4</td>
<td>4.0 ± 0.4</td>
<td>34.7 ± 4.3</td>
</tr>
<tr>
<td>T22/2</td>
<td>-3.3 ± 0.5</td>
<td>8.1 ± 0.4</td>
<td>59.7 ± 14.1</td>
</tr>
<tr>
<td>I23/2</td>
<td>-2.3 ± 0.4</td>
<td>5.5 ± 1.1</td>
<td>43.9 ± 11.4</td>
</tr>
<tr>
<td>I2331</td>
<td>-4.3 ± 0.3</td>
<td>3.1 ± 0.2</td>
<td>27.6 ± 2.6</td>
</tr>
<tr>
<td>I362</td>
<td>0.1 ± 0.4</td>
<td>-0.8 ± 0.3</td>
<td>-6.9 ± 3.7</td>
</tr>
<tr>
<td>I3661</td>
<td>-2.8 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>19.8 ± 2.4</td>
</tr>
<tr>
<td>L4332</td>
<td>-3.6 ± 0.4</td>
<td>2.6 ± 0.2</td>
<td>23.4 ± 2.4</td>
</tr>
<tr>
<td>I4461</td>
<td>-2.6 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>L5062</td>
<td>-5.4 ± 0.6</td>
<td>7.5 ± 0.6</td>
<td>64.5 ± 7.7</td>
</tr>
<tr>
<td>L5662</td>
<td>-5.2 ± 0.5</td>
<td>5.7 ± 0.4</td>
<td>49.3 ± 4.9</td>
</tr>
<tr>
<td>I611/2</td>
<td>-2.5 ± 0.3</td>
<td>4.7 ± 0.3</td>
<td>39.5 ± 6.6</td>
</tr>
<tr>
<td>I6161</td>
<td>-5.7 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td>40.4 ± 3.0</td>
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<tr>
<td>L6732</td>
<td>-2.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>11.8 ± 1.2</td>
</tr>
<tr>
<td>V70/2</td>
<td>-2.3 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>13.4 ± 1.5</td>
</tr>
<tr>
<td>L7381</td>
<td>0.0 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Average</td>
<td>-2.9 ± 1.5</td>
<td>3.4 ± 2.4</td>
<td>28.5 ± 18.9</td>
</tr>
</tbody>
</table>

\(^a\) All errors were determined from 500 Monte Carlo simulation runs.

\(^b\) The temperature dependencies of \( S^2\text{ axis} \) (\( \kappa = \frac{dS^2\text{ axis}}{dT} \)) were obtained from the slope of a linear fit of \( S^2\text{ axis} \) versus temperature (\( T \)).

\(^c\) The characteristic thermal coefficient \( \Lambda \) was obtained from the slope of a linear fit of \( (1 - \sqrt{S^2\text{ axis}}) \) versus \( \ln T \).

\(^d\) The heat capacities \( C_p \) were obtained from the slope of a linear fit of the conformational entropy \( S_{\text{conf}} \) versus \( \ln T \).

\(^e\) The Boltzmann constant and Avogadro’s number, respectively.

\(^f\) For these methyl groups, only \( S^2\text{ axis} \) from temperatures between 283 and 308 K were used for the fitting procedure due to the requirement of \( S^2\text{ axis} < 0.95 \) for determining \( S_{\text{conf}} \).

\(^g\) For this methyl group, only \( S^2\text{ axis} \) from temperatures between 278 and 308 K were used for the fitting procedure due to the requirement of \( S^2\text{ axis} < 0.95 \) for determining \( S_{\text{conf}} \).

\(^h\) ns dynamics. An earlier study from our group has determined the methyl group RDC-based order parameters \( S^2\text{ RDC} \) for ubiquitin, which reflect time-scales from ps to ms. When comparing the two sets of order parameters at 308 K, the average values of \( S^2\text{ axis} \) and \( S^2\text{ RDC} \) are 0.59 ± 0.21 and 0.43 ± 0.25, respectively, with a Pearson correlation coefficient of \( r = 0.84 \). Clearly, the methyl groups possess additional dynamics on a slower time-scale than encapsulated by \( S^2\text{ axis} \).

Analysis of the temperature dependence of \( S^2\text{ axis} \) in ubiquitin

To quantify the temperature dependencies of \( S^2\text{ axis} \), the thermal coefficient \( \kappa \) was taken from the linear fit of \( S^2\text{ axis} \) versus temperature (\( \kappa = \frac{dS^2\text{ axis}}{dT} \)). Table I and Figure 4 present the results of the fitting procedure. An overall decrease is observed in \( S^2\text{ axis} \) as the temperature increases from 275 to 308 K. The average value of \( \kappa \) is \(-2.9 \pm 1.5 \times 10^{-3} \text{ K}^{-1} \), which is quite comparable to the value of \(-2.6 \pm 1.1 \times 10^{-3} \text{ K}^{-1} \) reported previously for ubiquitin over the larger temperature range of 278–328 K. Furthermore, similar trends in the deviation of \( S^2\text{ axis} \) with temperature are also observed for calmodulin bound to a peptide. Figure 5A illustrates the distribution of \( \kappa \) within ubiquitin (1UBQ). The largest \( \kappa \) values are clustered around 16151, spatially near the N-terminus of ubiquitin, and progressively decrease toward the C-terminal region of the protein.

The characteristic thermal coefficient \( \Lambda = \frac{d(1 - \sqrt{S^2\text{ axis}})}{d \ln T} \) relates the temperature dependencies of the generalized order parameter, in this case \( S^2\text{ axis} \), to the characteristic temperature (\( T^* \)). The term \( T^* \) describes the density of thermally accessible conformational states. Table I presents the results for the determination of \( \Lambda \), which correlates with \( \kappa \) (Pearson coefficients of \( r = 0.7 \)). The average value of \( \Lambda \) (3.4 ± 2.4 compared with 2.3 ± 1.0 previously reported for ubiquitin over the larger temperature range of 278–328 K) indicates that there is significant contributions from rotameric state dynamics. In Figure 5B, \( \Lambda \) is plotted on the structure of ubiquitin. As with \( \kappa \), the largest values of \( \Lambda \) are located in the N-terminal region of ubiquitin. Toward the C-terminal region a progressive decline of \( \Lambda \) is visible.

**Determination of the \( C_p \) from the temperature dependence of the conformational entropy**

An approximate relationship between the conformational entropy \( S_{\text{conf}} \) and \( S^2\text{ axis} \) is

\[ S_{\text{conf}} = k_B N_A \left( \ln \left( 3 - \sqrt{1 + 8 \sqrt{S^2\text{ axis}}} \right) \right)^{\frac{3}{2}}. \]

where \( k_B \) is the Boltzmann constant and \( N_A \) is Avogadro’s number. The heat capacity \( C_p \) obtained from the temperature dependence of \( S_{\text{conf}} \) is defined as

\[ C_p = \frac{dS_{\text{conf}}}{d\ln T}. \]

Table I presents the values of \( C_p \) for the methyl groups in ubiquitin for the temperature interval of 275–308 K. For 22 methyl groups in ubiquitin, we report an average \( C_p \) of 28.5 ± 18.9 J K\(^{-1}\) mol\(^{-1}\). For the drkN SH3 domain, similar values for the methyl group \( C_p \) were obtained: 17 ± 12 J K\(^{-1}\) mol\(^{-1}\) and 33 ± 23 J K\(^{-1}\) mol\(^{-1}\) for the temperature intervals 287–303 K and 278–287 K, respectively.
As measured by differential scanning calorimetry, the global \( C_p \) for ubiquitin was \( \sim 12.6 \) kJ K\(^{-1}\) mol\(^{-1}\) at 298 K.\(^{35}\) In this study, the summation of all the individual methyl group \( C_p \) equals 626 J K\(^{-1}\) mol\(^{-1}\). Keeping in mind that this value does not include the contributions of 28 additional methyl groups where \( C_p \) could not be determined, these results suggest that the total methyl group \( C_p \) makes a \( \sim 10\% \) contribution to the total \( C_p \) of ubiquitin. It should be noted that a majority \( C_p \) can be determined from the primary sequence of proteins.\(^3\) Since 16\% (200 out of 1231) of all atoms in ubiquitin are from the methyl groups, the \( \sim 10\% \) contribution for the methyl groups to the total ubiquitin \( C_p \) seems to be a reasonable estimate. Figure 5(C) presents the distribution of \( C_p \) in ubiquitin. The largest values of \( C_p \), as with \( \kappa \) and \( \Lambda \), are located spatially near the N-terminus of the protein, which is the part of the hydrophobic core of the protein.

**Insight into the thermal stability of ubiquitin from the distribution of thermal coefficients**

A striking feature regarding the distribution of the thermal coefficients is the concentration of the largest values near the N-terminus of the protein. For these methyl groups to be located within the core of the protein may appear at first to be counterintuitive. Flexible regions tend to populate additional states more readily (especially upon an increase in thermal energy) and thus, would potentially possess larger than average \( C_p \) values. In addition, several studies indicate that the N-terminal region of ubiquitin, specifically the \( \alpha \)-helix and the turn between \( \beta \)-strands 1 and 2, is quite resilient to temperature fluctuations\(^{36,37}\) and comprises the core structural elements that form first during ubiquitin folding.\(^{38,39}\) However, investigations into the temperature dependence of NH order parameters for several thermo-stable proteins reveal some of the larger \( C_p \) values to be located within the more rigid regions of secondary structure.\(^{11–13}\) Furthermore, the magnitude of \( C_p \) depends on the change in \( S_{conf} \) [see Eq. (7)], and, as calculated by three independent groups,\(^7,8,11\) the steepest change for \( S_{conf} \) occurs as the order parameter, \( S^2 \), varies between 0.7 and 0.95. Perhaps the high melting temperature of \( \sim 363 \) K for ubiquitin\(^{35}\) and other thermo-stable proteins can be attributed in part to the relatively large local \( C_p \) of the methyl groups and NH bonds in the folded proteins reducing the magnitude of \( \Delta C_p \) upon unfolding.

![Image of thermal stability of ubiquitin](image-url)

**Figure 4.** Temperature dependence of \( S^2_{\text{ax}} \) in ubiquitin (\( \kappa = dS^2_{\text{ax}}/dT \)). All data were fit with a linear regression line in order to obtain \( \kappa \) (solid line).\(^9\) For many of the data points, the error bars are smaller than the symbol size. A: Plot of the temperature dependence of \( S^2_{\text{ax}} \) for the following leucine \( \delta \) methyl groups: 881 (○), 4362 (□), 5032 (○), 5662 (◇), 6762 (●), and 7361 (▲). B: Plot of the temperature dependence of \( S^2_{\text{ax}} \) for the following isoleucine \( \delta 1 \) methyl groups: 3 (○), 13 (○), 23 (◇), 36 (▲), 44 (●), and 61 (□). C: Plot of the temperature dependence of \( S^2_{\text{ax}} \) for the following isoleucine \( \gamma 2 \) methyl groups: 3 (○), 23 (○), 36 (▲), and 61 (□). D: Plot of the temperature dependence of \( S^2_{\text{ax}} \) for the following threonine \( \gamma 2 \) methyl groups: 7 (○), 9 (○), 12 (□), and 22 (▲). E: Plot of the temperature dependence of \( S^2_{\text{ax}} \) for the following valine \( \gamma 2 \) methyl groups: 17 (○) and 70 (□).
One methyl group possesses a slightly negative $C_p$, I36\text{c}2. Two other studies have reported negative $C_p$ for NH bonds located in secondary structural regions.\textsuperscript{12,15} Enhancements in the strength of hydrophobic interactions with increasing temperature were given as a possible explanation for this phenomenon.\textsuperscript{40} In the case of I36\text{c}2, previous work has demonstrated that the hydrophobic interaction between the side chain of I36 and I30 at the C-terminus of the $\alpha$-helix is important for the stability of ubiquitin.\textsuperscript{41} Thus, the negative $C_p$ signifying a decrease in the flexibility and a reduction in the available conformational space for this methyl group may be related to the strengthening of this important interaction with increasing temperature.

**Conclusion**

In this study, we analyzed the temperature dependence for the methyl group order parameters in ubiquitin. With the requirement of only one 2D constant time $^{13}$C, $^1$H HSQC measurement without decoupling of $^1$H during $^{13}$C-chemical shift evolution, we have significantly reduced the total amount of experimental time required to obtain this information. From these experiments, the extracted thermal coefficients provide a glimpse into the location of ubiquitin thermo-stability near the N-terminus of the protein. Clearly, methyl groups contribute quite appreciably to the total heat capacity of ubiquitin, as well as of other proteins,\textsuperscript{9,11} through the regulation of local conformational entropy. Taken together with the temperature dependence of NH order parameters, a per residue gauge of local protein thermodynamics offers a powerful complement to the already well-established methods for determining global thermodynamic parameters in proteins.

**Materials and Methods**

**NMR spectroscopy and processing**

Uniformly $^{15}$N,$^{13}$C-labeled human ubiquitin was expressed and purified as described previously.\textsuperscript{42} For the NMR measurements, a 3.6 mM sample of the protein was prepared in 350 $\mu$L of 50 mM sodium phosphate buffer, 100 mM NaCl, 0.1% NaN\textsubscript{3}, and pH 6.8 in 10% D\textsubscript{2}O/90% H\textsubscript{2}O. A series of 2D constant time $^{13}$C, $^1$H HSQCs without decoupling of $^1$H during $^{13}$C-chemical shift evolution were measured at 14 temperature points: 275, 278, 281, 283, 286, 288, 291, 293, 296, 298, 301, 303, 305, and 308 K. All NMR experiments were performed in succession on a 700 MHz Avance-III Bruker spectrometer equipped with a triple resonance probe head. The constant time duration and INEPT delays were set to 27.8 and 2 ms, respectively. The spectrum was recorded with 1024 and 128 complex points in the direct ($t_2$) and indirect ($t_1$) dimensions, respectively, with eight scans per $t_1$ increment. The $t_{1,\text{max}}$ and $t_{2,\text{max}}$ were 24.3 ms and 113 ms, respectively. Frequency discrimination in the indirectly detected dimension was achieved with the States-TPPI scheme.\textsuperscript{43} Each measurement required 59 min.

All time domain data were processed in the same manner with NMRPipe software.\textsuperscript{44} The data were zero-filled to 8 and 16 k in $t_1$ and $t_2$, respectively. After implementing a time domain solvent correction, a sine-bell window function was applied in the direct dimension, followed by Fourier transformation of $t_2$. For the indirect dimension, a mirror image linear prediction algorithm was used to increase the resolution in $t_1$. Next, a Gaussian window function was employed followed by Fourier transformation of $t_1$. Finally, a polynomial baseline correction in the frequency domain was applied in the direct dimension.
**Methods for error determination**

The intensities of each peak in the quartet were taken from the program CARA\textsuperscript{15} and the dipolar-dipolar CCR rates (\(\sigma_{obs}\)) were calculated according to Eq. (3). To determine the errors in \(\sigma_{obs}\), the noise levels \((q)\) for each measurement were estimated using NMRPipe and the error \((\Delta \sigma_{obs})\) was obtained from the following relation,

\[
\Delta \sigma_{obs} = q \frac{1}{8 \Delta t} \left( \frac{1}{I_i} + \frac{1}{I_j} + \frac{1}{I_k} + \frac{1}{I_l} \right),
\]

where \(\Delta t\) is the length of the constant time period and \(I_i\) is the intensity of each peak in the quartet \((i = x, y, z\) or \(\beta^\prime\)). For the methyl group order parameters \((S^2_{axis})\), the errors \((\Delta S^2_{axis})\) were calculated by propagating the error from \(\Delta \sigma_{obs}\) using the equation,

\[
\Delta S^2_{axis} = \frac{\Delta \sigma_{obs}}{\sigma_{rigid}},
\]

where \(\sigma_{rigid}\) is derived from Eq. (5). Finally, the errors in the conformational entropy \((\Delta S_{conf})\) were determined by propagating the error from \(\Delta S^2_{axis}\),

\[
\Delta S_{conf} \approx 4k_B N_A A \left( \frac{1}{3 - B} \right),
\]

where

\[
A = \frac{\Delta S^2_{axis}}{2 \sqrt{S^2_{axis}}},
\]

\[
B = 1 + 8 \sqrt{S^2_{axis}^*},
\]

\(k_B\) is the Boltzmann constant and \(N_A\) is Avogadro’s number. To estimate the error in the temperature dependency of \(S^2_{axis}\) \((\kappa = \frac{ds_{out}}{dT})\), the characteristic thermal coefficient \((\Lambda = \frac{dn}{dnT})\), and the heat capacity \((C_p = \frac{ds_{out}}{dt})\), Monte Carlo simulations on 500 randomly generated data sets were performed.

**References**

23. Muller N, Bodenhausen G, Ernst RR (1987) Relaxation-induced violations of coherence transfer selection-