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Temperature Dependence of Anisotropic Protein Backbone Dynamics

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Abstract: The measurement of ^{15}N NMR spin relaxation, which reports the $^{15}N-^{1}H$ vector reorientational dynamics, is a widely used experimental method to assess the motion of the protein backbone. Here, we investigate whether the $^{15}N-^{1}H$ vector motions are representative of the overall backbone motions, by analyzing the temperature dependence of the $^{15}N-^{1}H$ and $^{13}CO-^{13}C_\alpha$ reorientational dynamics for the small proteins binase and ubiquitin. The latter dynamics were measured using NMR cross-correlated relaxation experiments. The data show that, on average, the $^{15}N-^{1}H$ order parameters decrease only by 2.5% between 5 and 30 °C. In contrast, the $^{13}CO-^{13}C_\alpha$ order parameters decrease by 10% over the same temperature trajectory. This strongly indicates that there are polypeptide-backbone motions activated at room temperature that are not sensed by the $^{15}N-^{1}H$ vector. Our findings are at variance with the common crank-shaft model for protein backbone dynamics, which predicts the opposite behavior. This study suggests that investigation of the ^{15}N relaxation alone would lead to underestimation of the dynamics of the protein backbone and the entropy contained therein.

Introduction

The dynamics of the polypeptide backbone are thought to contain a substantial fraction of protein configurational entropy.[1−4] A powerful method to assess these dynamics is measurement of the ^{15}N NMR spin relaxation, which essentially monitors $^{15}N-^{1}H$ vector reorientation at the nano- to picosecond time scale.[5−8] Here, we investigate whether the $^{15}N-^{1}H$ vector dynamics are representative of the overall backbone motions. This is carried out by comparing the average dynamics as measured for the $^{15}N-^{1}H$ vector to the dynamics as measured for the $^{13}CO-^{13}C_\alpha$ vector for two small proteins. Because the $^{15}N-^{1}H$ and $^{13}CO-^{13}C_\alpha$ vectors point in different directions, reorientational motions of the peptide plane that cannot be sensed by the one vector may be sensed by the other, and vice versa.[9,10] The temperature dependence, rather than the absolute magnitude of the dynamics for the different vectors, is studied here to avoid the difficulties of relative quantification which thus far have somewhat obfuscated the comparisons.[9−11] Our results strongly indicate that there are polypeptide-backbone motions activated at room temperature that are not sensed by the $^{15}N-^{1}H$ vector. Significantly, this indicates that motions sensed by the $^{15}N-^{1}H$ vector alone underrepresent the dynamics of the protein backbone and the entropy contained therein. It is recommended that additional backbone dynamics experiments be conducted.

Methods

^{15}N Relaxation Rate Measurements. The spin−lattice relaxation rate $^{15}N-R_1$, spin−spin relaxation rate $^{15}N-R_2$, and ^{15}N NOE were measured at 278, 293, 303, and 318 K for ubiquitin. For binase, $^{15}N-R_1$, $^{15}N-R_2$, and ^{15}N NOE were measured at 303 K. We assumed the large-molecule limit for all residues for the binase ^{15}N NOE at 278 K because the ^{15}N NOE is already featureless at 303 K. Data processing was carried out with NMRPipe.[12] ^{15}N-relaxation rates and uncertainty estimations in those rates were determined using the NMRView program.[13] The $^{15}N-^{1}H$ order parameter analysis was carried out using MODELFREE.[14] A $\Delta\sigma$ value of −172 ppm was used for the ^{15}N CSA tensor with an $^{15}N-^{1}H$ bond length of 0.102 nm.[15−18] There was no statistically significant difference in the average order parameters whether overall anisotropic rotation was considered or not for both binase and ubiquitin.[10,19]

$^{13}CO-^{13}C_\alpha$ Cross-Correlated Relaxation Rate Measurements. Transverse cross-correlated relaxation rates between ^{13}CO chemical shift

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Table 1. Average Relaxation Data and Order Parameters for the $^{15}N-^{1}H$ and $^{13}CO-^{13}C_\alpha$ Vectors at 278 and 303 K for Binase[a,b]

	temp (K)		
	278	303	ratio 303/278
$\langle R_1(NH)\rangle$ (s^{-1})	1.28 ± 0.03	2.45 ± 0.04	1.91 ± 0.05
$\langle R_2(NH)\rangle$ (s^{-1})	16.88 ± 0.61	7.73 ± 0.15	0.46 ± 0.02
τ_c (ns)	13.44 ± 0.25	5.50 ± 0.11	0.41 ± 0.01
$\langle S^2(NH)\rangle$[c]	$0.903 \pm 0.002(0.019)$	$0.884 \pm 0.001(0.011)$	$0.979 \pm 0.003(0.024)$
$\langle CO-C_\alpha(CC)\rangle$ (s^{-1})	$-3.382 \pm 0.031(0.279)$	$-1.281 \pm 0.014(0.136)$	$0.379 \pm 0.005(0.05)$
$\langle S^2(CO-C_\alpha)\rangle$[d]	$0.899 \pm 0.008(0.075)$	$0.807 \pm 0.008(0.082)$	$0.898 \pm 0.013(0.117)$
$\langle CO-C_\alpha(CR)\rangle$ (s^{-1})	$-0.215 \pm 0.005(0.039)$	$-0.0756 \pm 0.005(0.0045)$	$0.352 \pm 0.008(0.06)$
$\langle S^2(CO-C_\alpha)\rangle$[e]	$0.878 \pm 0.020(0.157)$	$0.807 \pm 0.005(0.048)$	$0.919 \pm 0.022(0.174)$

[a] The averages were calculated for all resolved resonances of binase. [b] The uncertainties in the averages were calculated from the average uncertainty per residue in the raw data (given in parentheses) divided by $\sqrt{N}$, where N is the number of residues considered (typically 80). [c] The S^2 values of the $^{15}N-^{1}H$ vector were obtained using the MODELFREE program. [d] The S^2 values of the $^{13}CO-^{13}C_\alpha$ vector were obtained using eq 1, $\sigma_{11} - \sigma_{33} = 156$ ppm, $\sigma_{22} - \sigma_{33} = 80$ ppm, $\theta_{11,CO-C\alpha} = 156°$, $\theta_{22,CO-C\alpha} = 246°$, which may be seen as the average tensor parameters accommodating the approximations in eq 1. The values obtained correspond very closely to solid-state NMR data ($\sigma_{11} - \sigma_{33} = 154$ ppm, $\sigma_{22} - \sigma_{33} = 81$ ppm, $\theta_{11,CO-C\alpha} = 156°$, $\theta_{22,CO-C\alpha} = 246°$).[23−25] The ratio of the order parameters is more significant than the absolute values (see text). [e] The S^2 values of the $^{13}CO-^{13}C\alpha$ vector were obtained using eq 2; the ratio is more significant than the absolute values.

anisotropy and the $^{13}CO-^{13}C_\alpha$ dipolar interaction were measured for both proteins at all temperatures, using 3D HNCO experiments without $^{13}C_\alpha$ decoupling in a constant-time ^{13}CO evolution time of 36 ms[20−22] (see Supporting Information). The cross-correlation rates were obtained from the data using an in-house written time-domain fitting program. The uncertainties in the rates were evaluated from a Monte Carlo analysis around the fitted parameters. The cross-correlation rates can be given as

$$\Gamma^{CSA/DD}_{CO/CO-C\alpha} = \frac{1}{30}\left(\frac{\mu_0}{4\pi}\right)\frac{\hbar\omega_C\gamma_C^2}{r^3_{CO-C\alpha}} \times [(\sigma_{11} - \sigma_{33}) \times (3\cos^2\theta_{11,CO-C\alpha} - 1) + (\sigma_{22} - \sigma_{33}) \times (3\cos^2\theta_{22,CO-C\alpha} - 1)] \times \tau_c \times \left\{4S^2_{CO-C\alpha} + \frac{3S^2_{CO-C\alpha}}{1 + (\omega_C\tau_c)^2}\right\} \quad (1)$$

where the symbols have their usual meaning; $\theta_{11,CO-C\alpha}$ and $\theta_{22,CO-C\alpha}$ describe the angle between the (σ_{11} and σ_{22}) principal axes of the ^{13}CO CSA tensor and the $^{13}CO-^{13}C_\alpha$ vector.[21] Equation 1 was simplified from the general equation in two ways. First, it assumed that the two cross-correlation order parameters associated with each tensor component can be replaced by a single autocorrelation order parameter. This is justified because the cross-correlation order parameter belonging to the first term reports on the reorientational dynamics of the combined $^{13}CO-^{13}C_\alpha$ and σ_{11} vectors which are within 30° of collinearity, and because the contribution of the second tensor component in its entirety is expected to be only 14%.[23−25] The error made with this approximation is small and systematic and, together with uncertainties in CSA tensor magnitude and directions, drops out of the ratios of cross-correlation rates considered in this paper. Second, eq 1 is written for the limit of fast local motion, containing only two dynamic variables, τ_c and $S^2_{CO-C\alpha}$. τ_c is obtained from the ^{15}N relaxation data, and hence $S^2_{CO-C\alpha}$ can be determined (see Discussion).

$^{13}CO-^{13}C_\alpha$ Cross-Relaxation Rate Measurements. These experiments were carried out for binase only, at temperatures of 278 and 303 K. The cross-relaxation rates ($\sigma_{CO-C\alpha}$) due to the $^{13}CO-^{13}C_\alpha$ dipole−dipole interaction were obtained by measuring the steady-state $^{13}CO-^{13}C_\alpha$ NOE and the ^{13}CO T_1 relaxation rate.[21,26] The ^{13}CO R_1 rates were fitted using the NMRView program, and the NOE effects upon $^{13}C_\alpha$-saturation were determined in NMRPipe. The details of the $^{13}CO-^{13}C\alpha$ cross-relaxation measurements and their interpretation have been described in detail elsewhere.[21,26] In the limit of fast local motion (as the only assumption), one can write:

$$\sigma_{CO-C\alpha} = -\left(\frac{\mu_0}{4\pi}\right)^2\frac{\gamma_c^4\hbar^2}{10}\langle r^{-3}_{CO-C\alpha}\rangle^2 \times \tau_c \times \left\{S^2_{CO-C\alpha} - \frac{6S^2_{CO-C\alpha}}{1 + (2\omega_c\tau_c)^2}\right\} \quad (2)$$

τ_c is obtained from the ^{15}N relaxation data, and hence $S^2_{CO-C\alpha}$ can be determined.

Results

The Protein Binase. The ^{15}N relaxation data for the 109 residue ribonuclease binase were presented elsewhere.[10] The R_1 and NOE data are relatively featureless despite the fact that the protein contains several surface loops. The ^{15}N R_2 data show extensive exchange broadening in the active site areas, related to the functioning of the protein.[2] The order parameters of the $^{15}N-^{1}H$ vector at different temperatures were extracted from the data using the MODELFREE[14] program at the different temperatures and are available in the Supporting Information. They have uncertainties of about 2% per residue. The average order parameters at two temperatures are listed in Table 1. They were obtained from averaging over about 80 residues and are therefore much more precise; their estimated uncertainty is 2%/$\sqrt{80}$, or about 0.2%. Inspection of Table 1 shows that very little change occurs in $^{15}N-^{1}H$ detected dynamics over the temperature range investigated: the ratio of the average order parameters $\langle S^2_{NH}(303)/\langle S^2_{NH}\rangle(278)$ is near unity (0.979 ± 0.003). Expressed differently, the temperature coefficient of the $^{15}N-^{1}H$ order parameter for binase is 8×10^{-4} K^{-1}, which corresponds closely to the values found for ribonuclease H1 (5.9×10^{-4} K) and troponin C (8×10^{-4} K) by others.[27,28]

Figure 1 presents the $^{13}CO-^{13}C_\alpha$ cross-correlated relaxation rates for binase at 303 and 278 K. The data are quite articulated, especially at lower temperature; however, no secondary structure

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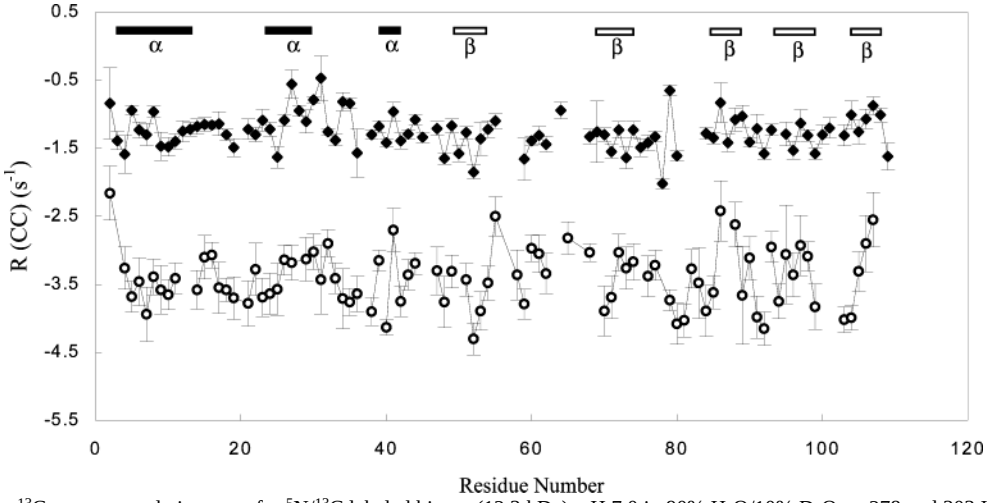

Figure 1. The $^{13}CO-^{13}C_\alpha$ cross-correlation rates for $^{5}N/^{13}C$ labeled binase (12.3 kDa), pH 7.0 in 90% H_2O/10% D_2O, at 278 and 303 K, are presented with ○ and ◆, respectively. The approximate locations of secondary structure elements are indicated above. The data were obtained with a Bruker Avance 500 MHz spectrometer with pulse sequences described in refs 20−22 and given in the Supporting Information. The internal sample temperatures in this and all other experiments used in this work were verified from the chemical shift difference between the H_2O resonance and the resonance of an internal DSS reference, and were found to be within 2 °C from the spectrometer settings.

trends are visible in the data, except for the more mobile N- and C-termini at the lower temperature. The individual order parameters, extracted from the data using eq 1 (see Methods), have experimental uncertainties of about 10%; the average order parameters, which were obtained from about 80 residues, have an uncertainty of $10/\sqrt{80}$, or about 1%. The average $^{13}CO-^{13}C_\alpha$ order parameter as determined from cross-correlation ($S^2_{CO-C\alpha}$ (CC)) decreases substantially over the temperature trajectory investigated: $\langle S^2_{CO-C\alpha}$ (CC)$\rangle(303)/\langle S^2_{CO-C\alpha}$ (CC)$\rangle(278) = 0.898 \pm 0.013$. This behavior is very different from the $^{15}N-^{1}H$ order parameters as listed above. According to a standard *t*-test,[29] we obtain that the difference in the ratio $\langle S^2_{CO-C\alpha}$(CC)$\rangle(303)/\langle S^2_{CO-C\alpha}$ (CC)$\rangle(278)$ and the corresponding ratio $\langle S^2_{NH}\rangle(303)/\langle S^2_{NH}\rangle(278)$ could happen by chance in less than 1×10^{-6} cases for the 80 points of data considered (using the uncertainties of the individual order parameters). The difference is thus statistically very significant.

To assess whether the temperature dependence $S^2_{CO-C\alpha}$ determined from cross correlation is biased by an (unexpected) temperature dependency of the ^{13}CO CSA, or other assumptions associated with the use of eq 1 (see Methods), we also investigated the temperature dependence of the $^{13}CO-^{13}C_\alpha$ cross-relaxation rate (carbon−carbon NOE). These latter rates are independent of the ^{13}CO tensor and have a different dependency on the bond length (see eq 2). The data of the $^{13}CO-^{13}C_\alpha$ cross-relaxation rate measurements for binase, recorded at two temperatures, are given in the Supporting Information; the averages are given in Table 1. While the experimental uncertainties in the individual $^{13}CO-^{13}C_\alpha$ cross-relaxation rates are rather large especially at low temperature (18%), the average cross-relaxation rates for the entire protein can still be obtained with good precision (averaging over 80 residues reduces the uncertainty of the average to 2%).

The average $^{13}CO-^{13}C_\alpha$ order parameter derived from the cross relaxation ($S^2_{CO-C\alpha}$ (CR)) decreases, like the order parameter derived from the cross correlation, substantially over the temperature trajectory investigated: $\langle S^2_{CO-C\alpha}$ (CR)$\rangle(303)/\langle S^2_{CO-C\alpha}$ (CR)$\rangle(278) = 0.919 \pm 0.022$. Expressed differently, the temperature coefficient of $S^2_{CO-C\alpha}$ (CR) is 0.0027 K^{-1}. The $^{13}CO-^{13}C_\alpha$ cross-relaxation data thus strongly validate the $^{13}CO-^{13}C_\alpha$ cross-correlation data, and vice versa. Both show a much stronger dependence of $S^2_{CO-C\alpha}$ on temperature than the S^2_{NH}. Because of the inherent better experimental precision, we elected to only use $^{13}CO-^{13}C_\alpha$ cross correlation in the studies of ubiquitin.

The Protein Ubiquitin. The ^{15}N relaxation data obtained for ubiquitin (a 76 residue protein associated with the intracellular proteolysis pathway) at 303 K compare well with the values reported in the literature.[19] The derived average order parameters at four temperatures are listed in Table 2; residue-specific rates are given in the Supporting Information. The average $^{15}N-^{1}H$ order parameters decrease monotonically over the trajectory investigated. Very little change occurs over the temperature range investigated: for instance, the ratio of the average order parameters is $\langle S^2_{NH}\rangle(303)/\langle S^2_{NH}\rangle(278) = 0.968 \pm 0.002$, which agrees very closely with the data obtained for binase.

The temperature dependence of the ^{13}CO CSA/$^{13}CO-^{13}C_\alpha$ DD cross-correlation rate for ubiquitin is shown in Figure 2. The data are relatively featureless, except for a smooth decrease of the rates toward the C-terminus at all temperatures as expected for these unstructured residues. The "jagged" behavior around residue 5, seen at all temperatures, is currently inexplicable in terms of the structure of the protein. The figure indicates that excellent experimental reproducibility was achieved. The average $^{13}CO-^{13}C_\alpha$ order parameters decrease monotonically over the entire temperature trajectory investigated (see Table 2). The average $^{13}CO-^{13}C_\alpha$ order parameter decreases even more strongly than for binase over the temperature trajectory investigated, for example, $\langle S^2_{CC}\rangle(303)/\langle S^2_{CC}\rangle(278) = 0.890 \pm 0.011$. The difference in the decrease of the $^{15}N-H$ and $^{13}CO-^{13}C_\alpha$ order parameters is, as for binase, statistically very significant (the *t*-test gives less than a 1×10^{-6} chance for a coincidental difference).

Discussion

The extensive NMR relaxation data obtained for binase and ubiquitin reveal an apparently inherent dynamical property.

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Table 2. Average Relaxation Data and Order Parameters for the $^{15}N-^{1}H$ and $^{13}CO-^{13}C_{\alpha}$ Vectors at 278, 293, 303, and 318 K for Ubiquitin[a,b]

	temp (K)						
	278	293	303	318	ratio 318/278	ratio 303/278	ratio 293/278
$\langle R_1(NH)\rangle$ (s^{-1})	1.82 ± 0.02	2.42 ± 0.02	2.63 ± 0.01	2.61 ± 0.04	1.43 ± 0.03	1.44 ± 0.02	1.33 ±0 .02
$\langle R_2(NH)\rangle$ (s^{-1})	10.55 ± 0.22	7.32 ± 0.10	5.77 ± 0.13	4.12 ± 0.10	0.39 ± 0.01	0.55 ± 0.02	0.69 ± 0.02
τ_c (ns)	8.35 ± 0.12	5.31 ± 0.07	3.96 ± 0.10	2.54 ± 0.11	0.30 ± 0.01	0.47 ± 0.01	0.64 ± 0.01
$\langle S^2(NH)\rangle^c$	0.865 ± 0.001 (0.010)	0.857 ± 0.001 (0.007)	0.837 ± 0.001 (0.005)	0.816 ± 0.001 (0.009)	0.943 ± 0.002 (0.015)	0.968 ± 0.002 (0.013)	0.991 ± 0.002 (0.014)
$\langle CO-C_{\alpha}(CC)\rangle$ (s^{-1})	−1.992 ± 0.009 (0.074)	−1.226 ± 0.009 (0.071)	−0.885 ± 0.010 (0.082)	−0.576 ± 0.007 (0.057)	0.289 ± 0.004 (0.031)	0.444 ± 0.006 (0.044)	0.615 ± 0.005 (0.042)
$\langle S^2(CO-C_{\alpha})\rangle^d$	0.844 ± 0.004 (0.031)	0.798 ± 0.006 (0.046)	0.751 ± 0.009 (0.070)	0.710 ± 0.000 (0.071)	0.841 ± 0.011 (0.090)	0.890 ± 0.011 (0.089)	0.945 ± 0.008 (0.062)

[a] The averages were calculated for all resolved resonances of ubiquitin except for the C-terminal residue 5. [b] The uncertainties in the averages were calculated from the average uncertainty per residue in the raw data (given in parentheses) divided by $\sqrt{N}$, where N is the number of residues considered (typically 62). [c] The S^2 values of the $^{15}N-^{1}H$ vector were obtained using the MODELFREE program. [d] The S^2 values of the $^{13}CO-^{13}C\alpha$ vector were obtained using eq 1 (see Table 1 footnote).

Between 5 and 30 °C, motional modes become activated that significantly decrease the order parameter of the $^{13}CO-^{13}C\alpha$ vector, but hardly affect the order parameter of the $^{15}N-^{1}H$ vector. We have not been able to discern statistically significant differences in temperature dependence of order parameters between helices, sheets, and loop areas. We therefore assume for now that the effects are inherent to the nature of the peptide plane.

Because of the inferences that our findings may have, it behooves us to carefully consider possible statistical and systematic uncertainties in our measurements and interpretation. As outlined in the sections above, we evaluated the statistics of the measured differences in temperature dependence. Standard *t*-tests indicate that the difference in temperature dependence cannot arise by chance. Moreover, the data on ubiquitin display a monotonic, but very different, temperature dependence of both order parameters over a range of 40 °C (Table 2). We conclude that our results are statistically significant beyond reasonable doubt.

Systematic uncertainties may arise from several sources; we argue in the following that neither of these can cause the effects observed. First, the structures of the proteins did not change over the temperature trajectories investigated as proven by the observation that the ^{13}CO and $^{1}H-^{15}N$ NMR resonance frequencies remained virtually unchanged. Second, all relaxation experiments used are (partially) dependent on dipolar relaxation that is very sensitive with respect to the bond length separating the nuclei. The temperature dependency of the $^{15}N-^{1}H$ bond length (it becomes longer with increasing temperature) is reported to be larger than that of the $^{13}CO-^{13}C_{\alpha}$ bond length in non-hydrogen bonded peptides.[11] If not accounted for in the data analysis (as we do not), this effect would lead to an apparent stronger temperature dependence of the $^{15}N-^{1}H$ order parameter than of the $^{13}CO-^{13}C_{\alpha}$ order parameter, opposite to observation. Third, we consider the possible role of conformational exchange broadening, which may affect the ^{15}N relaxation results, but not the ^{13}CO cross-correlation and cross-relaxation results. For instance, conformational exchange broadening in ubiquitin could not be completely suppressed at the lowest temperature (average R_{ex} 2.50 ± 0.29 s^{-1} at 278 K as compared to 0.55 ± 0.13 s^{-1} at 303 K). Potential failure to separate residual exchange broadening and true R_2 relaxation by the MODELFREE data analysis should, if anything, result in an overestimation of the order parameter at low temperature, thus increasing the apparent dependency of the $^{15}N-^{1}H$ order parameter on temperature. This is contrary to the effects observed. Fourth, we need to consider that both the $^{13}CO-^{13}C_{\alpha}$ cross-correlation and the $^{13}CO-^{13}C_{\alpha}$ cross-relaxation rate experiments, in contrast to the suite of ^{15}N experiments, are isolated experiments from which one can determine only one unknown dynamical parameter per residue. Given that the rotational correlation time is known from the ^{15}N data, the ^{13}CO data are thus sufficient to determine an order parameter for a simplified spectral density function [$J(\omega) = S^2\tau_m/(1 + \omega^2\tau_m^2)$], which assumes that local motions are either fast, small, or both. The correlation times for the local motions as sensed by the $^{15}N-^{1}H$ vectors are a few tens of picoseconds for both proteins except for the termini (data not shown), suggesting that this may also be the case for the $^{13}CO-^{13}C_{\alpha}$ detected motions. If not, and if slower local motions would increasingly contribute to the spectral density function with

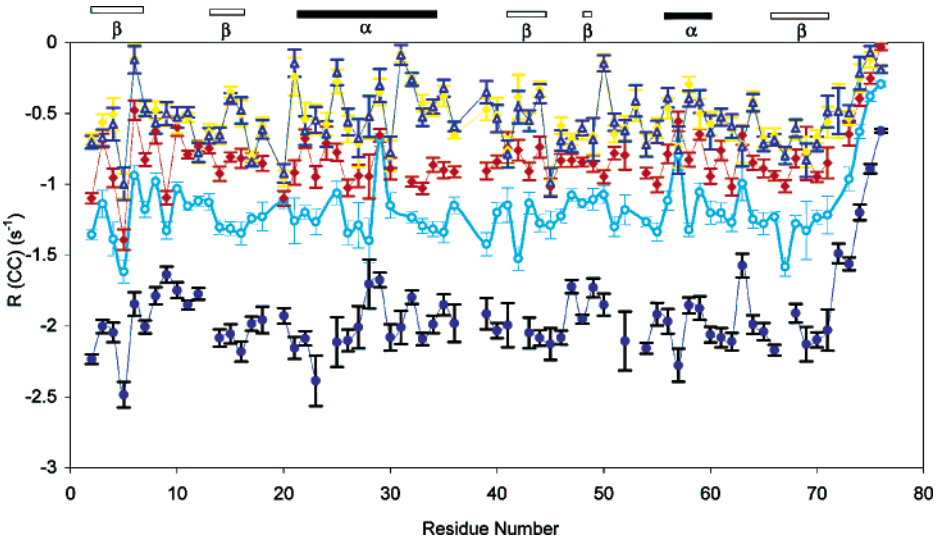

Figure 2. The $^{13}CO-^{13}C_\alpha$ cross-correlation rates[20−22] for 1 mM $^{15}N/^{13}C$ labeled ubiquitin in 90% H_2O/10% D_2O, deuterated acetic acid buffer, pH 4.7, at 278, 293, 303, and 318 K, are presented with black, green, red, and blue colors, respectively. The yellow color is a repeat experiment at 318 K. The approximate locations of secondary structure elements are indicated above.

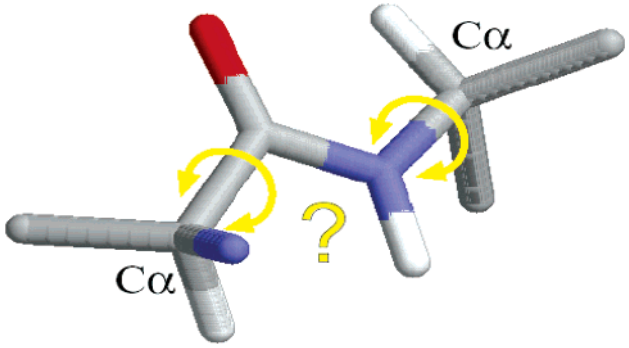

Figure 3. Cartoon of a peptide plane depicting motions predicted from molecular dynamics simulations along the dihedral angles Φ and Ψ commonly referred to as crank-shaft motions.[30−32] These motions would be preferentially detected by the $^{15}N-^{1}H$ bond vector, and less by the $^{13}CO-^{13}C_\alpha$ vector. Because the opposite is observed in the current studies, it is proposed that this model should be revisited (see text).

increasing temperature, the $^{13}CO-^{13}C_\alpha$ order parameter would decrease even faster than calculated.

In summary, we submit that the difference in temperature dependence for the order parameters is not introduced by artifacts in the experiments or by problems with extraction of dynamical parameters from the relaxation data. Our experimental findings are at variance with predictions from molecular dynamics calculations. The latter studies predict the dominance of anticorrelated dihedral angle Φ−Ψ wobbles,[30−32] which rotate the peptide plane around an axis close to the $^{13}CO-^{13}C_\alpha$ director (see Figure 3). These so-called crankshaft motions would cause large reorientations of the $^{15}N-^{1}H$ vector and small reorientations of the $^{13}CO-^{13}C_\alpha$ vector. Hence, it would predict smaller $^{15}N-^{1}H$ order parameters than $^{13}CO-^{13}C_\alpha$ order parameters, opposite to the trends seen in our observations. Probably this discrepancy between theory and experiment is caused by the assumption, used in most molecular dynamics force fields, that the $^{15}N-^{1}H$ vector is rigidly affixed to and within the peptide plane.[33] Ab initio quantum mechanical calculations on small molecules[34,35] and high-resolution protein crystal structures indicate that this assumption may not be entirely correct.[36,37] The added degree of freedom would allow the $^{15}N-^{1}H$ vector to move either more or less than the $^{13}CO-^{13}C\alpha$ vector, the latter possibility being strongly indicated by our data. However, given the complexity of the local environment of $^{15}N-^{1}H$ vectors in proteins, which includes hydrogen bonding and crowding effects, it is difficult to predict intuitively what exact effect the nonrigidity may have on $^{15}N-^{1}H$ dynamics. More studies, both experimentally and theoretically, are needed to address these issues.

Our experimental data strongly indicate that the $^{13}CO-^{13}C_\alpha$ dynamics are more related to the more active side chain dynamics than to the more sedate $^{15}N-^{1}H$ dynamics. This follows from the correspondence of the average temperature dependence of the S^2 ($^{13}CO-^{13}C_\alpha$ vector) of 0.0027 K^{-1} with the temperature dependency (dS^2_{axis}/dT) of about 0.0025 K^{-1} as determined for the methyl groups of a calmodulin−peptide complex.[38]

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Supporting Information Available: Pulse sequence, ^{15}N order parameters, and $^{13}CO-^{13}C_\alpha$ cross-relaxation rates (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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