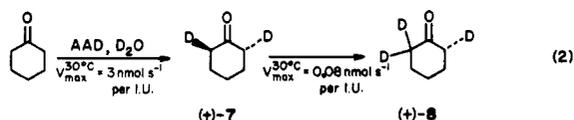


cyclohexanones were then converted to labeled 1-hexanols (Scheme II), diluted with 1-[1-¹⁴C]hexanol as internal standard, and oxidized enzymically with NAD⁺ and horse liver alcohol dehydrogenase (HLADH).¹¹ The ¹⁴C/³H ratios for the *N*-phenylcarbamate of 1-hexanol and the semicarbazone of hexanal produced by HLADH-catalyzed oxidation were compared for each sample. Upon enzymic oxidation, the stereorandomly labeled sample lost 23% of its tritium label, the sample from "exchanged in" [2-³H]cyclohexanone lost 39% of its label, and the sample from "exchanged out" lost none of its label. Assuming that the Baeyer-Villiger oxidation in Scheme II proceeds with retention of configuration at the migrating center,¹² HLADH catalyzes removal of the *pro-R* hydrogen from hexanol,² and approximately half the original label in [2-³H]cyclohexanone ultimately appears in an unexchangeable methylene group,¹³ we conclude that AAD catalyzes exchange of *pro-R* hydrogens at the α positions of cyclohexanone.¹⁴

We have confirmed this assignment by preparing optically active α -deuterated ketones via AAD-catalyzed exchange of cyclopentanone, 4-isopropylcyclohexanone, and 4-thiacyclohexanone (**6**) with D₂O. Absolute configurations for α -deuterated cyclopentanone¹⁵ and 4-isopropylcyclohexanone¹⁶ have been reported and are consistent with our assignment. Both enantiomers of α -deuterated **6** were prepared and absolute configurations assigned by conversion to the known optically active [2-²H]propionic acid¹⁷ via α -deuterated 3-pentanone,¹⁸ as shown in Scheme III. The assignment of absolute stereochemistry to **3** agrees with the octant rule¹⁹ and independent work from Djerassi's laboratory.²⁰ The *R* configuration was assigned to (-)-**4** by its conversion to (-)-[2-²H]propionic acid,²¹ shown in Scheme III. This assignment was confirmed by preparation of (+)-2-[3-²H]butanone by chromic acid oxidation of an authentic sample of 2-[3-²H]butanol known to have *3S* configuration.²²

We have studied the kinetics of AAD-catalyzed exchange of the α -hydrogens of cyclohexanone and 2-butanone by polarimetry and mass spectrometric analysis of the extent of deuteration. Surprisingly, we observe that AAD catalyzes a slow but detectable exchange of the *pro-S* hydrogens at a rate 1-3% that of the exchange of *pro-R* hydrogens.²³ Exchange reaction **2** gives excellent yields of (+)-**7**,²⁴ but at longer times (+)-**8** can be seen

both by mass spectrometry and ²H NMR.²⁵ The *pro-S* and *pro-R*



exchange are both inhibited completely by 5 μ M acetopyruvate, a known inhibitor of the decarboxylation reaction.^{5b} Both exchanges are slowed by a factor of 5 in 10 μ M 5-nitrosalicylaldehyde, a competitive inhibitor of AAD.^{5a} Furthermore, perchlorate,²⁶ in concentrations from 1 to 10 μ M, inhibits in parallel both the *pro-R* and *pro-S* exchanges. These experiments strongly suggest that *pro-S* exchange occurs at the active site and proceeds via the same intermediate responsible for stereospecific *pro-R* exchange.

The broad substrate specificity of AAD makes this enzyme valuable in the synthesis of a wide variety of compounds that are optically active by virtue of asymmetric isotopic substitution. The small amount of nonspecific exchange is especially interesting, since it appears to be an exception to the general rule that enzyme stereoselectivity is absolute once a substrate is bound.^{10,27} For AAD, unlike many enzymes, stereochemical infidelity is not easily understood in terms of alternative modes of binding.² If both the predominant *pro-R* exchange and the minor *pro-S* exchange occur by the attack of a proton on the same enamine intermediate, we are forced to conclude that two stereochemically distinct reactions can occur at the same active site on the same bound intermediate to yield the same chemical result. We know of no other case where this has been shown for an enzyme.

Acknowledgment. We thank Professor Frank H. Westheimer, in whose laboratories this work was performed, and Dr. Walter Blättler for helpful discussions. The support of the NSF (to J.D.R. through a predoctoral fellowship), the Xerox Corporation (to S.A.B. through a postdoctoral fellowship), the NIH (through Grants NS-14773 to T.H.M. and GM-04-71223 to F.H.W.), and the Rockefeller Foundation (to T.H.M. as an IPH Visiting Scholar) are gratefully acknowledged.

(24) Exchange of 1.5 g of cyclohexanone in 30 mL of buffered D₂O containing 1 mg (800 IU) of AAD at 38 °C reached peak optical activity after 1 h, and ethereal extraction followed by distillation afforded 1.1 g of (+)-**7**, [α]_D²⁵ +3.7° (neat), which showed a *d*₁:*d*₂:*d*₃ ratio of approximately 2:9:1 by mass spectrometry.

(25) The proton-decoupled 41.44-MHz deuterium NMR spectrum of (+)-**7** in CHCl₃ shows a single peak at δ 1.62, while a mixture of (+)-**7** and (+)-**8** shows two singlets at δ 1.60 (CD₂) and 1.62 (CHD). We are indebted to Mr. Peter Demou of Yale University for recording these spectra at the Southern New England High Field NMR Facility, supported by the Biotechnology Resources Program of the NIH (RR-798).

(26) Fridovich, I. *J. Biol. Chem.* **1963**, *238*, 592-598.

(27) The high degree of stereospecificity often encountered in enzymes is illustrated by fumarase, which is at least 99.99% stereospecific with respect to the β center: Fisher, H. F.; Frieden, C.; McKinley McKee, J. S. *J. Am. Chem. Soc.* **1955**, *77*, 4436.

(11) Enzymic oxidations were carried out under nitrogen in 0.1 M ammonium carbonated buffer (pH 10.1) containing 0.2 M acetaldehyde, 0.8 mM NAD⁺, and 0.18 mg mL⁻¹ HLADH. Experimental details are reported in: Polavarapu, P. L.; Nafie, L. A.; Benner, S. A.; Morton, T. H. *J. Am. Chem. Soc.*, submitted for publication.

(12) Mislow, K.; Brenner, J. *J. Am. Chem. Soc.* **1953**, *75*, 2318-2322.

(13) The presence of tritium in both the 1 and 5 positions of the radiolabeled hexanols was confirmed by chemical oxidation to hexanoic acid, by which ~40% of the ³H label was lost.

(14) Degradation of (-)-**7**, [α]_D²⁸ -3.9° (neat), which was prepared by AAD-catalyzed exchange of cyclohexanone-2,2,6,6-*d*₄ with H₂O, to *n*-pentyl camphanate, followed by NMR analysis (Gerlach, H.; Zagalak, B. *J. Chem. Soc., Chem. Commun.* **1973**, 274-275) confirms our assignment. We are grateful to Professor John M. Schwab (*J. Am. Chem. Soc.*, submitted for publication) for communicating these results to us prior to publication.

(15) Hine, J.; Li, W.-S. *J. Am. Chem. Soc.* **1980**, *102*, 4403-4409.

(16) Sundararaman, P.; Djerassi, C. *Tetrahedron Lett.* **1978**, 2457-2460; **1979**, 4120.

(17) Zagalak, B.; Frey, P. A.; Karabatsos, G. L.; Abeles, R. H. *J. Biol. Chem.* **1966**, *241*, 3028-3035.

(18) α -Deuterated **6**, [α]_D³⁷ +6.4° (*c* 2, D₂O) was converted to α -deuterated 3-pentanone, [α]_D²⁵ -1.6° (*c* 4, ether). Levorotatory α -deuterated 3-pentanone was also prepared by AAD-catalyzed exchange of 3-pentanone with D₂O.

(19) Lightner, D. A.; Gawronski, J. K.; Bouman, T. D. *J. Am. Chem. Soc.* **1980**, *102*, 1983-1990.

(20) Sundararaman, P.; Barth, G.; Djerassi, C. *J. Org. Chem.* **1980**, *45*, 5231-5236. We are grateful to Professor Djerassi for communicating these results to us prior to publication.

(21) Retej, J.; Umani-Ronchi, A.; Arigoni, D. *Experientia* **1966**, *22*, 72-73.

(22) We are grateful to Professor Duilio Arigoni for supplying a sample of (2*R*,3*S*)-2-[3-²H]butanol.

(23) After reaching a peak value, the optical rotation of a reaction mixture slowly declines and ultimately vanishes. The *pro-S* exchange has also been monitored by loss of radioactivity from (2*S*)-[2-³H]cyclohexanone. Both the *pro-R* and *pro-S* exchange rates are independent of pH in the range pH 5.2-6.8. Both rates are also independent of phosphate buffer concentration from 15 to 50 mM at pH 5.95.

Increase of ¹³C NMR Relaxation Times in Proteins due to Picosecond Motional Averaging[†]

Ronald M. Levy[§] and Martin Karplus[§]

Department of Chemistry, Harvard University
Cambridge, Massachusetts 02138
and Department of Chemistry
Rutgers, The State University of New Jersey
New Brunswick, New Jersey 08903

J. Andrew McCammon

Department of Chemistry, University of Houston
Houston, Texas 77004
Received July 8, 1980

The nature of atomic motions in the interior of proteins is currently a topic of great interest.¹⁻³ It has been shown by

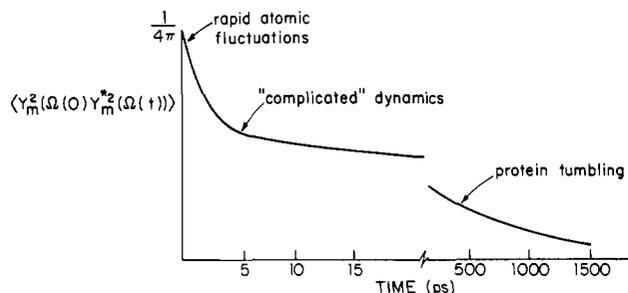


Figure 1. Schematic diagram of the general behavior of the decay of NMR correlation functions in proteins. The physical origins for the decay in correlation resulting from the different kinds of protein motions are indicated in the figure.

molecular dynamics simulations² that, in spite of the close-packed structure of native proteins, significant atomic fluctuations occur on a picosecond time scale. Protonated ¹³C NMR measurements of identified resonances are particularly suited for studying the internal dynamics because the relaxation parameters (T_1 , T_2 , and NOE) are dominated by the dipolar interaction between the ¹³C nucleus and the directly bonded protons.⁴⁻⁸ In this communication we make use of a recent molecular dynamics simulation of the pancreatic trypsin inhibitor (PTI)^{9,10} to demonstrate the effects of picosecond fluctuations on the observed ¹³C T_1 values. We show that picosecond averaging has to be included in relating measured T_1 values to other motional properties of the protein (e.g., overall tumbling) and, under suitable conditions, ¹³C relaxation data can serve to probe the picosecond reorientation dynamics of the ¹³C-H bond vector.

The spectral density $J(\omega)$, as a function of frequency ω , which determines the T_1 , T_2 , and NOE values is given by

$$J(\omega) = \int_0^\infty \langle Y_m^2[\Omega(0)]Y_m^{*2}[\Omega(t)] \rangle \cos \omega t dt \equiv \int_0^\infty \langle A(0)A(t) \rangle \cos \omega t dt \quad (1)$$

where $Y_m^2[\Omega(t)] \equiv A(t)$ is a second-order spherical harmonics and $\langle \rangle$ represents a time correlation function; the angle Ω specifies the orientation of the CH internuclear vector with respect to the external magnetic field. For the atoms of a rigid, isotropically tumbling protein, the spectral density function is

$$J(\omega) = \langle A^2(0) \rangle \tau_0 / [1 + (\omega\tau_0)^2] \quad (2)$$

where τ_0 is the rotational correlation time; $\tau_0 = 1/(6D)$ where D is the diffusion coefficient. Since proteins are not rigid, the $J(\omega)$ for individual nuclei do not generally obey eq 2, and the general form expected for $\langle A(0)A(t) \rangle$ is shown schematically in Figure 1. There is a rapid initial loss of correlation in the first few picoseconds, often leading to a plateau value, $\langle A(0)A(t_p) \rangle$,

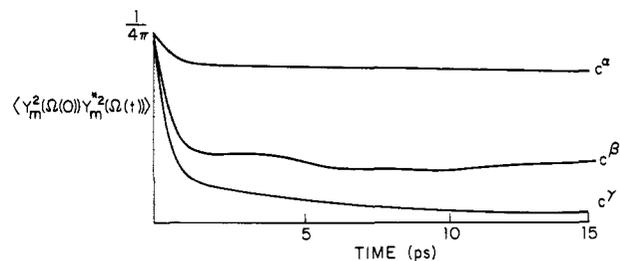
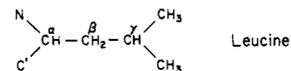


Figure 2. Leucine-29 NMR correlation functions for α , β , and γ carbons calculated from 96-ps PTI atomic trajectory.

after t_p ps. This fast decay results from the combined effect of the vibrational potential of the residue containing the nucleus and collisions between the atoms of the residue and those of the surrounding cage in the protein. For residues that are involved in a larger scale, more complex fluctuations, the initial decay is followed by a much slower loss of correlation over the next tens to hundreds of picoseconds. Finally, $\langle A(0)A(t) \rangle$ relaxes to zero due to the overall tumbling motion of the protein. Figure 2 shows the first 15 ps of the correlation function $\langle A(0)A(t) \rangle$ for the C^α , C^β , and C^γ carbons of Leu-29 of PTI. For the C^α and C^β carbons the motional averaging is essentially complete after 2 ps [$\langle A(0)A(2) \rangle \approx \langle A(0) \rangle^2$], while for the C^γ carbon the NMR correlation function continues to decay beyond 2 ps.

Since the initial decay occurs in a very short time, the spectral density at the Larmor frequency ω is reduced uniformly.^{11,12} For the situation where the correlation function displays a plateau value after t_p ps ($t_p\omega \ll 1$), we have

$$J(\omega) \approx \langle A(0)A(t_p) \rangle \tau_0 / [1 + (\omega\tau_0)^2] \quad (3)$$

and

$$T_1 \approx [\langle A^2(0) \rangle / \langle A(0)A(t_p) \rangle] T_1^R \quad (4a)$$

where T_1^R is the rigidly tumbling protein relaxation time value obtained from eq 2. The effect of the picosecond motion is, thus, to increase the measured T_1 over the rigid value by the scale factor $\langle A^2(0) \rangle / \langle A(0)A(t_p) \rangle$. An approximate upper limit on the picosecond averaging in the dynamic simulation is obtained by assuming that the decay time of the correlation function is less than or equal to the length of the dynamics run, t_d ; then $\langle A(0)A(t_d) \rangle = \langle A(0) \rangle^2$, and we have for $t_d\omega \ll 1$ and $\tau_0 \gg \tau_d$,

$$T_1 \approx [\langle A^2(0) \rangle / \langle A(0) \rangle^2] T_1^R \quad (4b)$$

For an examination of the effect of fast motional averaging on T_1 values, we make use of the fluctuations in atomic coordinates calculated from the 96-ps molecular dynamics simulation for PTI at 300 K. Sixty-two protonated carbons were studied, including all of the protonated carbons on the eight aromatic residues (Tyr-10, -21, -23, and -35 and Phe-4, -22, -33, and -45), two of the glycine C^α carbons (Gly-28 and -57), and the methine and methylene carbons of the aliphatic residues (Leu-6 and -29 and Ile-18 and -19). These residues provide favorable examples because they are well buried (the aliphatic methyl groups not discussed here are on the protein surface), and their coupling to the surrounding protein matrix is dominated by van der Waals interactions. Examples of the ratios T_1/T_1^R due to motional averaging obtained from eq 4a with $t_p = 2$ ps and from eq 4b are listed in Table I. Also listed are the NT_1 values calculated from

[†] Supported in part by grants from the National Science Foundation and the National Institute of Health.

* Address correspondence to Harvard University.

[‡] Address as of September 1, 1980, Rutgers.

- (1) F. R. N. Gurd and M. Rothgeb, *Adv. Protein Chem.*, **33**, 74 (1979).
- (2) M. Karplus and J. A. McCammon, *CRC Crit. Rev. Biochem.*, in press.
- (3) I. A. Campbell and C. M. Dobson, *Methods Biochem. Anal.*, **25**, 1 (1979).
- (4) A. Allerhand, D. Doddrell, and R. Komorski, *J. Chem. Phys.*, **55**, 189 (1971).
- (5) E. Oldfield, R. Norton, and A. Allerhand, *J. Biol. Chem.*, **250**, 6368 (1975).
- (6) K. Wüthrich and R. Baumann, *Org. Magn. Reson.*, **8**, 532 (1976).
- (7) R. J. Wittebort, M. Rothgeb, A. Szabo, and F. R. N. Gurd, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1059 (1979).
- (8) L. W. Jelinski and D. A. Torchia, *J. Mol. Biol.*, **138**, 255 (1980).
- (9) J. A. McCammon, B. R. Gelin, and M. Karplus, *Nature (London)*, **267**, 585 (1977).
- (10) M. Karplus and J. A. McCammon, *Nature (London)*, **277**, 578 (1979).

(11) M. Elwenspoek, *Mol. Phys.*, **37**, 689 (1979).

(12) O. W. Howarth, *J. Chem. Soc., Faraday Trans. 2*, **75**, 863 (1979).

Table I. Calculated Increase in Spin-Lattice Relaxation Time in PTI due to Picosecond Motional Averaging

atom class	residue no.	ring carbon no.	motional averaging scale factor		$NT_1^{a,b}$
			eq 4b	eq 4a	
α Carbons					
Gly	28		1.19	1.19	0.058
Leu	29		1.21	1.16	0.057
Phe	22		1.13	1.10	0.054
β Carbons					
Leu	6		3.48	1.54	0.075
Leu	29		3.20	2.58	0.126
Ile	18		2.67	1.31	0.064
Ile	19		1.14	1.13	0.055
γ Carbons					
Leu	6		4.0	1.86	0.091
Leu	29		11.4	4.21	0.206
Ile	18		3.64	1.78	0.087
Ile	19		1.95	1.95	0.096
Ring Carbons					
Phe	4	2,6	3.51	1.56	0.077
		3,5	3.28	1.54	0.075
Phe	22	2,6	4.21	1.67	0.081
		3,5	5.71	1.70	0.083
Tyr	10	2,6	1.48	1.43	0.070
		3,5	1.70	1.70	0.083
Tyr	21	2,6	1.16	1.14	0.056
		3,5	1.16	1.13	0.055

^a Calculated with $t_p = 2$ ps. ^b T_1 in seconds with $T_1^R = 0.049$ s at 25 MHz with $\tau_0 = 1.7$ ns.

eq 4a with $T_1^R = 0.049$ s; T_1^R is obtained with a CH bond length of 1.09 Å, $\tau_0 = 1.7$ ns, and a resonance frequency of 25 MHz. The cases for which T_1 from eq 4b is significantly greater than that from eq 4a suggest that the lower frequency internal motions are important and may require more extensive calculations to obtain a quantitatively reliable result for T_1 .

In 13 out of 14 α carbons studied, motional averaging increased the T_1 's by less than 20% ($1.05 \leq T_1/T_1^R \leq 1.11$); only for Phe-45 is the increase slightly greater ($T_1/T_1^R = 1.31$). The glycine carbons do not exhibit significantly greater motional averaging than other C^α 's, which have bulky side chains attached. Close agreement was found between the results from eq 4a and 4b for the α carbons (see Table I). This indicates that the NMR correlation functions do approach a plateau within 2 ps. It is therefore unlikely that the lower frequency internal fluctuations contribute significantly to the relaxation of these atoms. For the 12 residues studied that have side chains, all of the β carbons exhibit more motional averaging than the α carbons to which they are attached. The behavior of the β carbons of aromatic residues is, however, very similar to the attached α carbons. An approximate plateau value appears within 2 ps ($1.11 \leq T_1/T_1^R \leq 1.23$) except for Phe-22 and -45 ($T_1/T_1^R \approx 1.33$) and Tyr-35 ($T_1/T_1^R = 1.67$). The β carbons of the aliphatic residues exhibit greater individual variation (see Table I), with the longest NT_1 corresponding to Leu-29. Of all the carbons studied, the aliphatic γ carbons had the greatest increase in T_1 : the T_1 's for the two Leu and two Ile γ carbons in PTI are predicted by eq 4a to increase over the rigid protein values by 100–300%. For three of the four aliphatic γ carbons (Ile-19 is the exception) the motional averaging does not

plateau within 2 ps, and lower frequency fluctuations contribute to the NMR relaxation. By contrast, the eight aromatic rings in PTI are highly hindered in their picosecond dynamics. For five of the rings (Tyr-21, -23, -25 and Phe-33, -45) there is a plateau in the NMR correlation function within 2 ps and $1.13 \leq T_1/T_1^R \leq 1.27$. Three of the rings (Phe-4, -22, Tyr-10) are calculated to have longer T_1 's by eq 4a, and Phe-4 and Phe-22 have NMR correlation functions that continue to decay beyond 2 ps (see Table I).

The analysis by atom class of the effect of calculated picosecond dynamics on ^{13}C relaxation times in PTI is in general agreement with published^{4,13–18} ^{13}C NMR studies at the present level of assignment. The majority of α carbons and ring carbons in PTI are predicted to have T_1 's close to the rigid protein values, while the aliphatic residues are predicted to have larger T_1 's due to picosecond motional averaging. Wüthrich et al.¹⁷ report that the envelopes representing the α -carbon and tyrosine ϵ -carbon resonances have about the same relaxation time, 0.045 ± 0.003 s at 25.1 MHz and 312 K. The exact T_1^R value for those conditions is not known, and the observed value represents an average of many resonances; nevertheless, the measured value is in qualitative accord with the dynamics simulation. Rapid methyl spinning increases a methyl carbon T_1 by ~ 3 over a rigid methine carbon; this is the magnitude of the increase observed for alanine methyl carbons.^{16,17} The increase in Ile methyl carbon T_1 's is considerably larger (as much as ~ 8 for Ile-18 C ^{β}). These results are expected from the substantial picosecond motional averaging of the Ile C ^{β} and C ^{γ} carbons observed in the molecular dynamics study.

Sources of variation in T_1 unrelated to internal dynamics include uncertainties in the protein tumbling time, anisotropic tumbling,¹⁹ and variations in the CH bond length.²⁰ For rigid PTI, NT_1 varies by 25% at 25 MHz and 10% at 45 MHz over the estimated range of τ_0 values (1–3 ns). Anisotropy in the tumbling (the axial ratio of PTI is approximately 3:1) can also lead to a variation in T_1 ; for rigid carbons with their CH vector parallel and perpendicular to the long axis of PTI, the difference in the relaxation rates due to orientation is less than 20% between 25 and 90 MHz and 35% at 15 MHz. For CH bond lengths in the range 1.09–1.11 Å, the variation in T_1 is 12%. From these results it is clear that a variety of effects, including picosecond averaging, must be considered in using experimental T_1 values to determine protein properties such as the tumbling time.

By analysis of a large series of ^{13}C T_1 values, it should be possible to isolate the effects of picosecond motional averaging. Care is necessary, however, since for many ^{13}C nuclei longer time fluctuations (up to nanoseconds) can make significant contributions to the relaxation. To avoid this problem in PTI, spectrometer frequencies below 45 Hz can be used, since below this frequency internal motions can only increase ^{13}C dipolar T_1 's over the values for the rigidly tumbling protein (τ_0 is located to the left of the minimum in the T_1 vs. τ curve at this Larmor frequency); thus contributions from longer times cannot reduce the effect of motional averaging.

The present analysis of the picosecond dynamics of atoms in the protein interior has demonstrated the importance of these motions in increasing ^{13}C relaxation times. Further, the highly hindered character of the calculated motions suggests that simplified models which take account of the excluded volume effects due to the close packing of the protein interior (e.g., restricted diffusion model)^{21,22} may be suitable for the interpretation of the resulting relaxation times. Additional measurements of T_1 values for assigned ^{13}C resonances will be useful for testing the detailed dynamical results reported in this paper.²³

(13) O. Jardetsky, A. Ribeiro, and R. King, *BBRC*, **92**, 883 (1980).

(14) B. D. Sykes, W. E. Hull, and G. H. Snyder, *Biophys. J.*, **21**, 138 (1978).

(15) K. Wüthrich, "NMR in Biological Research: Peptides and Proteins", North Holland, Amsterdam, 1976.

(16) R. Richarz and K. Wüthrich, *Biochemistry*, **17**, 2263 (1978).

(17) K. Wüthrich, G. Wagner, R. Richarz, and W. Braun, *Biophys. J.*, in press.

(18) A. Ribeiro, R. King, C. Restivo, and O. Jardetsky, *J. Am. Chem. Soc.*, **102**, 4040 (1980).

(19) D. T. Wilbur, R. S. Norton, A. D. Clouse, R. Addleman, and A. Allerhand, *J. Am. Chem. Soc.*, **98**, 8250 (1976).

(20) K. Dill and A. Allerhand, *J. Am. Chem. Soc.*, **101**, 4376 (1979).

(21) R. J. Wittebort and A. Szabo, *J. Chem. Phys.*, **69**, 1722 (1978).

(22) R. E. London and J. Avitable, *J. Am. Chem. Soc.*, **100**, 7159 (1978).

(23) R. M. Levy, C. M. Dobson, and M. Karplus, work in progress.