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37. We dedicate this paper to the memory of Harrison (Hatch) Echols, whose clear thinking about, and elegant descriptions of, fidelity mechanisms in DNA replication significantly inspired our studies and interpretations. Supported, in part, by USPHS research grants GM-15792 and GM-29158 (P.H.vH.), by USPHS postdoctoral fellowships GM-12915 (D.A.E.) and AI-07568 (M.C.Y.), by a grant from the Lucille P. Markey Charitable Trust, and by an American Cancer Society Research Professorship of Chemistry (P.H.vH.). We thank C. Chan, R. Landick, and G. Eichhorn for sharing unpublished manuscripts; E. Baldwin, C. Chan, M. Chamberlin, G. Eichhorn, and R. Landick, as well as several laboratory colleagues, for helpful discussions; and C. Chan, G. Feng, and R. Landick for providing us with purified GreA and GreB proteins.

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Guanidinium Chloride Induction of Partial Unfolding in Amide Proton Exchange in RNase A

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Amide (NH) proton exchange rates were measured in 0.0 to 0.7 M guanidinium chloride (GdmCl) for 23 slowly exchanging peptide NH protons of ribonuclease A (RNase A) at pH* 5.5 (uncorrected pH measured in D_2O), 34°C. The purpose was to find out whether GdmCl induces exchange through binding to exchange intermediates that are partly or wholly unfolded. It was predicted that, when the logarithm of the exchange rate is plotted as a function of the molarity of GdmCl, the slope should be a measure of the amount of buried surface area exposed to GdmCl in the exchange intermediate. The results indicate that these concentrations of GdmCl do induce exchange by means of a partial unfolding mechanism for all 23 protons; this implies that exchange reactions can be used to study the unfolding and stability of local regions. Of the 23 protons, nine also show a second mechanism of exchange at lower concentrations of GdmCl, a mechanism that is nearly independent of GdmCl concentration and is termed "limited structural fluctuation."

Whether hydrogen-deuterium (H-D) exchange in proteins occurs by partial unfolding has been debated hotly (*1*). Classic experiments (*2*) indicate that the NH proton exchange rates of bovine pancreatic trypsin inhibitor (BPTI) depend on a global thermodynamic property of the protein. The exchange rates of individual NH protons were compared for a family of proteins homologous to BPTI, either from different species or from chemically modified variants produced, for example, by reducing and blocking a specific disulfide bond. The exchange rates of all measured NH protons could be correlated closely with T_m, the midpoint of the thermal transition for global unfolding, suggesting that exchange rates depend on global thermodynamic behavior. In contrast, experiments over many years have suggested that some exchange reactions may occur by partial unfolding (*1*). Still other experiments and lines of reasoning (*3*) have suggested (*1*) that exchange reactions may occur by limited structural fluctuations involving solvent penetration, analogous to the mechanism by which oxygen penetrates myoglobin to reach its binding site on the heme iron.

To obtain new evidence on this question, we used two-dimensional proton nuclear magnetic resonance (2D ^{1}H-NMR) to measure exchange rates of individual NH protons in RNase A as a function of the concentration of guanidinium chloride (GdmCl). Guanidinium chloride causes protein unfolding by interacting with the unfolded protein, and the change in Gibbs energy upon unfolding varies linearly with the molarity (M) of GdmCl. The slope, $-m$, of the plot of Gibbs energy as a function of the molarity of GdmCl is proportional to the number of GdmCl interaction sites exposed on unfolding (*4*), which is taken to be proportional to the surface area exposed. We make use of this fact to ask whether H-D exchange of individual peptide NH protons can be caused to occur by partial unfolding by means of adding GdmCl. For our conditions, exchange is known to occur by the EX2 exchange mechanism (*1, 5*) that is defined below. This means that the apparent change in Gibbs energy, ΔG_i^*, for the conformational reaction that permits exchange can be obtained for each proton by the relation

$$\Delta G_i^* = -RT\ln\left(\frac{k_{HX}(i)}{k_c(i)}\right) \quad (1)$$

where $k_{HX}(i)$ is the observed rate constant for exchange of proton i and $k_c(i)$ is the chemical exchange rate when proton i is fully solvent exposed, obtained from model peptide data (*6*). The conversion of H-D exchange rates to Gibbs energies is possible because in the EX2 exchange mechanism

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the equilibrium between the open and closed forms is fast compared to the exchange step. Hence, the observed exchange rates are a measure of the equilibrium fraction of molecules that are available for exchange. If exchange is caused by a partial unfolding mechanism, the relation

$$\Delta G_i^* = \Delta G_i^*(0) - m_i M \quad (2)$$

should hold, as it does in global unfolding, and m_i, the m value for an individual proton i (termed the micro-m value), should be proportional to the surface area exposed on partial unfolding. $\Delta G_i^*(0)$ is the apparent change in Gibbs energy at zero denaturant for proton i, and M is the denaturant concentration. The micro-m values for different individual protons can be compared with each other and with m for global unfolding measured by techniques such as fluorescence, absorbance, or circular dichroism.

The term "apparent change in Gibbs energy" refers to the fact that $k_c(i)$, given by model peptide data, may not represent accurately the exchange rate in the intermediate conformation in which exchange occurs because: (i) proton i may not be fully exposed to solvent in the intermediate that permits exchange, (ii) dipeptide data do not in some cases reflect all the factors that affect exchange rates in unfolded proteins (*7, 8*), and (iii) no account is taken here of the dependence of $k_c(i)$ on GdmCl concentration (9). (The last effect may be small, because only a narrow range of GdmCl concentration is considered.)

The effect of a denaturant (8 M urea) on the exchange rates of NH protons in another protein (BPTI) was studied earlier (*10, 11*), but conflicting results were reported. The two sets of experiments were made at different temperatures, 55°C (*10*) versus 20°C and 35°C (*11*), and the results may reflect the effects of 8 M urea on two different exchange mechanisms. When exchange is measured at 55°C in the presence of moderate concentrations of different denaturing agents (*10*), the exchange rates of seven core NH protons are accurately correlated with the T_m for overall unfolding in the given solvent.

Two mechanisms controlling hydrogen exchange. Examination of 23 curves of Gibbs energy as a function of denaturant concentration reveals two types of plots. The first type (observed for residues Asp^{14}, Met^{29}, Met^{30}, Asn^{44}, Ala^{56}, Cys^{58}, Ser^{59}, Gln^{60}, Lys^{61}, Val^{63}, Tyr^{73}, Gln^{74}, Ala^{102}, and Glu^{111}) is characterized by having a single linear region (Fig. 1A and Table 1), indicating that hydrogen exchange for each of these residues is controlled by a single mechanism for each residue over the denaturant concentration range studied. We define the exchange behavior of these protons as class 1 exchange behavior. The slopes, micro-m values, for the class 1 protons range from 97 cal mol^{-1} M^{-1} for Met^{29} to 3005 cal mol^{-1} M^{-1} for Cys^{58}.

The second type of exchange behavior (observed for residues Gln^{11}, His^{12}, Met^{13}, Glu^{49}, Cys^{72}, Cys^{84}, Lys^{98}, Thr^{100}, and His^{119}) is characterized by plots with two linear regions that typically meet at a well-defined transition point (Fig. 1B and Table 1). The GdmCl dependence in the linear region at low denaturant concentrations is in all cases significantly less than the GdmCl dependence observed at higher denaturant concentrations. The micro-m values for the initial regions at low denaturant concentration for these protons in class 2a range from 4 cal mol^{-1} M^{-1} for Gln^{11} to 629 cal mol^{-1} M^{-1} for His^{119}. The micro-m values for the later regions at higher denaturant concentration for the protons in class 2b range from 1263 cal mol^{-1} M^{-1} for Glu^{49} to 3027 cal mol^{-1} M^{-1} for Cys^{84}. Hydrogen exchange for the residues that show this second type of behavior is controlled by two distinct mechanisms for each residue. The first mechanism (shown by residues in class 2a) occurs at low denaturant concentration and is characterized by small micro-m values. The second mechanism (residues in class 2b) occurs at higher denaturant concentration and is characterized by larger micro-m values.

Linear regression analysis of $\Delta G_i^*(0)$ as a function of micro-m values for the data corresponding to all residues in both class 1 and class 2b results in Eq. 3 (Fig. 2A).

$$\Delta G_i^*(0) = 6.07 + 0.00124 m_i \quad (3)$$

Because of the high degree of correlation shown by these data ($r = 0.94$), we conclude that all these residues undergo exchange by the same kind of mechanism. In contrast, the data corresponding to residues in class 2a cluster above the regression curve and represent a separate exchange mechanism.

We should point out that the distinction between class 2a and 2b residues was not made on the basis of whether their $\Delta G_i^*(0)$ and m_i data fit Eq. 3, but solely on their biphasic behavior in plots of ΔG_i^* as a function of the concentration of GdmCl.

Global unfolding. Experiments conducted with optical techniques yield an m value for RNase A of approximately 3000 cal mol^{-1} M^{-1} (*12*). The micro-m values for Cys^{58} and Cys^{84} are 3005 and 3027 cal mol^{-1} M^{-1}, respectively. The good agreement between the m value for global unfolding and the micro-m values for Cys^{58} and Cys^{84} strongly suggests that the equilibrium governing exchange in these two cases must be the global unfolding equilibrium

$$N \rightleftharpoons U \quad (4)$$

where N represents native protein and U represents unfolded protein. The $\Delta G_i^*(0)$ values for Cys^{58} and Cys^{84} (both 9.6 kcal mol^{-1}) are, however, significantly larger than the stabilization energy of roughly 6 kcal mol^{-1} determined by optical techniques (*12*). The offset of 3.6 kcal mol^{-1} (that is, $\Delta G_i = \Delta G_i^* - 3.6$), which has been found independently (*13*), arises from a substantial difference (a factor of approximately 10^3) between the rate of exchange in an unfolding intermediate and in an unstructured peptide (*5*). Another protein for which values of $k_{23}(i)$ appear to be much smaller than corresponding values of $k_c(i)$ is cytochrome c (oxidized form) (*14*). An offset of 1 kcal

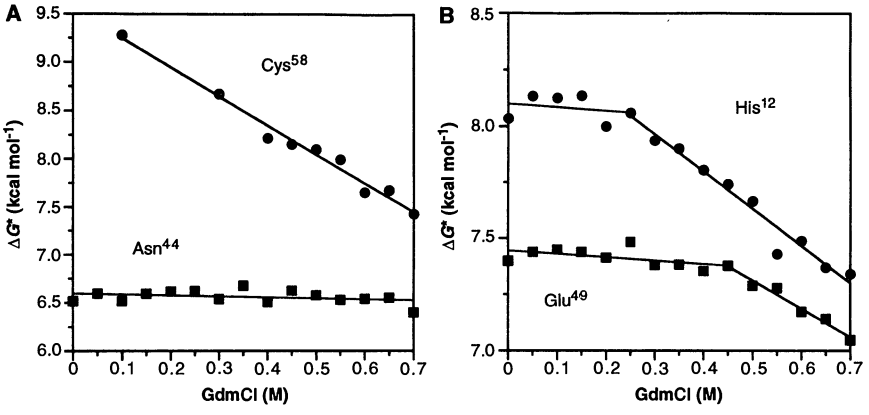

Fig. 1. Difference in apparent Gibbs energy between the native protein and the intermediate undergoing exchange for slowly exchanging ("core") NH protons of RNase A plotted as a function of molarity of GdmCl at pH* 5.5, 34°C. (**A**) Type I plots. (**B**) Type II plots. Apparent Gibbs energies were computed from exchange rates with Eq. 1. Exchange rate data were collected as described (*21*). The intrinsic chemical exchange rate for each residue except Val was computed as described (*6*). These rates were adjusted for pH*, the dissociation constant of D_2O (*22*), and temperature as described (*7, 23*). In calculating values of $k_c(i)$, the pK_a's of His^{12} and His^{119} were taken as 6.0 and 6.1, respectively (*24*), and the pK_a's of Asp^{14}, Glu^{49}, and Glu^{111} were all taken as 4.0. The value of k_c for Val is that given (*6*) divided by four (*7*). The correlation between intrinsic chemical exchange rate and peptide sequence and structure has been reexamined recently (*8*).

mol^{-1} has been reported for pancreatic trypsin inhibitor (*10*).

Partial unfolding versus limited structural fluctuation. The equilibrium permitting hydrogen exchange for Cys^{58} and Cys^{84} (at 0.7 M denaturant) is one that involves global unfolding. This observation suggests that other exchange data that fall on the same regression line (Fig. 2A) as Cys^{58} and Cys^{84} (the data for residues in class 1 and class 2B) belong to residues that also undergo exchange by an unfolding mechanism. Because the micro-m values for most class 1 and class 2b protons are smaller than the global unfolding m-value seen for Cys^{58} and Cys^{84}, it follows that these other unfolding reactions must represent only partial unfolding. The equilibrium process controlling exchange for these NH protons must be of the form

$$N \rightleftharpoons I_k \quad (5)$$

where I_k represents some partially unfolded structure. The degree to which I_k is unfolded is directly proportional to the magnitude of the micro-m value of proton i, which undergoes exchange from the intermediate I_k. For example, given a micro-m value corresponding to two-thirds of the global unfolding m value, the structure of I_k must retain one-third of the buried surface area of N. Thus, Cys^{58} and Cys^{84} are special cases of the partial unfolding mechanism for which $I_{Cys^{58}} = I_{Cys^{84}} = U$.

If the residues in class 2b (Fig. 2A), have partial unfolding as their controlling mechanism, then residues in class 2a should exchange by some other mechanism involving more limited breakage of hydrogen bonds. We use "limited structural fluctuation" as a name for this second mechanism of hydrogen exchange.

The change in mechanism observed for residues with biphasic exchange plots (Fig. 1B) is, therefore, a transition from limited structural fluctuation to partial unfolding. Because limited structural fluctuation is seen to have only a weak dependence on denaturant concentration (small micro-m values) and partial unfolding is seen to have a stronger dependence on denaturant concentration (larger micro-m values), it follows that partial unfolding is induced by exposure to GdmCl, as expected a priori. That is, increasing the GdmCl concentration leads to stronger interaction between the denaturant and the partially unfolded exchange intermediates and ultimately leads to unfolding (including both partial and global unfolding) as the dominant hydrogen exchange mechanism. For many NH protons, but not all, limited structural fluctuation provides a lower energy alternative to partial unfolding at 0 M GdmCl. Limited structural fluctuation can provide a mechanism to bypass prohibitively high energy barriers to partial unfolding.

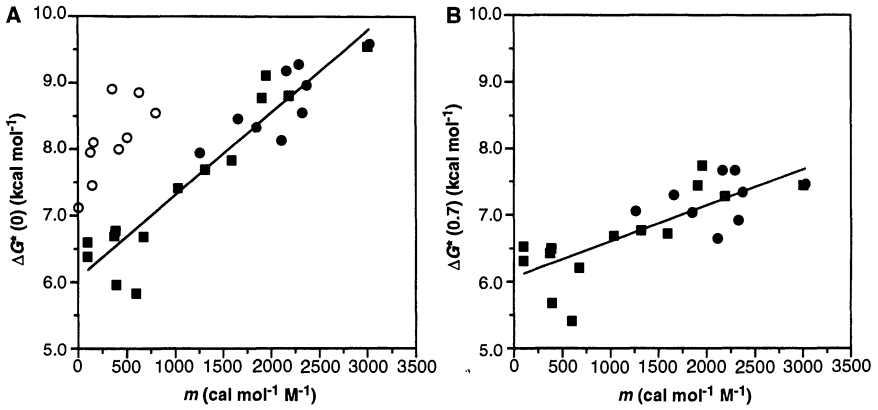

Fig. 2. The apparent change in Gibbs energy, ΔG_i^* at 0.0 M (**A**) and 0.7 M GdmCl (**B**) as a function of m_i. Class 1 residues (filled squares), class 2a residues (open circles), and class 2b residues (filled circles).

Table 1. Values of m_i and $\Delta G_i^*(0)$ for all protons studied. See Eq. 2 and text for definitions of m_i and $\Delta G_i^*(0)$. For the nine protons that show a transition between two different mechanisms of exchange (Fig. 1B), the GdmCl concentration at which the transition occurs is given as well as the values of m_i and $\Delta G_i^*(0)$ for the two lines. Residues are indicated with the one-letter abbreviations (*20*).

Residue	m_i (cal mol^{-1} M^{-1})	$\Delta G_i^*(0)$ (kcal mol^{-1})	Transition (M)	m_i† (cal mol^{-1} M^{-1})	$\Delta G_i^*(0)$‡ (kcal mol^{-1})
Q11	4	7.12	0.45	2113	8.13
H12	158	8.10	0.25	1659	8.46
M13	417	8.00	0.25	1849	8.33
D14	395	5.96			
M29	97	6.38			
M30	1315	7.69			
N44	98	6.60			
E49	147	7.45	0.45	1263	7.94
A56	371	6.69			
C58	3005	9.55			
S59	382	6.77			
Q60	675	6.68			
K61	597	5.83			
V63	1951	9.11			
C72	802	8.55	0.45	2163	9.18
Y73	1907	8.77			
Q74	2189	8.81			
C84	353	8.91	0.30	3027	9.59
K98	127	7.95	0.25	2328	8.55
T100	503	8.18	0.45	2373	8.97
A102	1592	7.83			
E111	1032	7.41			
H119	629	8.86	0.25	2293	9.28

†Posttransition m_i values for those protons showing two mechanisms of exchange. ‡Posttransition $\Delta G_i^*(0)$ values for those protons showing two mechanisms of exchange.

Global unlocking. That the data for class 1 and class 2b residues (Fig. 2) are linearly correlated supports the interpretation that exchange of these residues occurs by partial unfolding, because the change in Gibbs energy on unfolding should be approximately proportional to the surface area exposed on unfolding, just as the micro-m value is proportional to the surface area exposed. What is unexpected in this plot is the intercept of 6.0 kcal mol^{-1} at $m = 0$; it implies that a global unlocking step is coupled to partial unfolding, and unlocking must occur without exposing significant buried surface area. Then, as global unlocking occurs, a wide range of partial unfolding reactions can occur, each with its own set of values for m_i and $\Delta G_i^*(0)$. The fact that the intercept at $m = 0$ has the same value (6.0 kcal mol^{-1}) at 0.7 M GdmCl (Fig. 2B) as at 0.0 M GdmCl (Fig. 2A) shows that the globally unlocked state does not expose new surface area for interaction with GdmCl. The intercept in the linear free energy plot (Fig. 2) should be corrected for the offset of 3.6 kcal mol^{-1} between ΔG^* of exchange and ΔG of unfolding (5). Applying this correction reduces the barrier in Gibbs energy between N and the globally unlocked state from 6.0 kcal mol^{-1} to 2.4 kcal mol^{-1}.

What is the nature of global unlocking? An interesting possibility is that the un-

locked intermediate precedes and is close to, both in structure and Gibbs energy, the transition state intermediate for unfolding. A study of the unfolding transition state of hen lysozyme (*15*) shows that little buried surface area in the native protein becomes exposed to solvent in the unfolding transition state, as measured either by the negligible change in heat capacity, $\Delta Cp_{N\ddagger}$, between the native protein (N) and the transition state (‡) or by the small dependence of the activation enthalpy on the concentration of various ionic denaturants, including GdmCl. The activation enthalpy, $\Delta H_{N\ddagger}$, is large and temperature-independent; it may arise from breaking hydrogen bonds and van der Waals interactions in moving from the native state to the transition state (*15*). The properties deduced for the globally unlocked intermediate are reminiscent of a model proposed for "dry" molten globule intermediates (*16*).

The existence of a global unlocking step may provide the key to understanding how rates of exchange controlled by partial unfolding reactions can be correlated with a global thermodynamic property such as T_m (*2*). Each partial unfolding reaction is coupled to overall unfolding by global unlocking. It should be emphasized that we are studying here the exchange rates of NH protons that are among the most slowly exchanging protons of RNase A. More rapidly exchanging protons may undergo exchange by limited structural fluctuations or possibly by partial unfolding reactions that do not involve global unlocking.

Mutational effects on the relative exchange rates of NH protons in the oxygenated (R) versus deoxygenated (T) forms of human hemoglobin provide a remarkable example of a correlation between an overall thermodynamic property of a tetrameric molecule (the equilibrium constant for the R ⇌ T reaction) and the exchange rates of local clusters of NH protons (*17*). When the exchange rates are converted to changes in Gibbs energy (Eq. 1) and the ΔΔG* values for the two alpha and two beta subunits are added together to give ΔΔG for the effect of mutation on the hemoglobin tetramer, the same value of ΔΔG is found as when the equilibrium constant for the R ⇌ T reaction is measured directly by determining the appropriate association constants for subunit assembly and ligand binding. This striking correlation has been explained (*17*) as arising from the cooperativity of the R ⇌ T reaction.

Structural correlations. Two observations can be made that relate the structure of native RNase A (*18*) to the values of m_i and $\Delta G_i^*(0)$ for protons undergoing exchange by partial unfolding. First, Asp^{14}, which is just outside helix 1, shows m = 395 cal mol^{-1} M^{-1} and $\Delta G^*(0)$ = 6.0 kcal mol^{-1}, whereas Gln^{11}, His^{12}, and Met^{13}, which are hydrogen-bonded in helix 1, show significantly higher values of m_i and $\Delta G_i^*(0)$. Likewise Lys^{61}, which is outside helix 3, has m = 597 cal mol^{-1} M^{-1} and $\Delta G^*(0)$ = 5.8 kcal mol^{-1}, whereas Ala^{56}, Cys^{58}, Ser^{59}, and Gln^{60}, which are in the COOH-terminal end of helix 3, show higher values of $\Delta G^*(0)$. Second, the two protons that show values of m_i and $\Delta G_i^*(0)$ expected for global unfolding, Cys^{58} and Cys^{84}, are cysteine residues whose side chains form large disulfide-bonded loops. Apparently these loops act as anchors to prevent partial unfolding. And Cys^{72}, which forms a small disulfide-bonded loop to Cys^{65}, has smaller values of m and $\Delta G^*(0)$ than Cys^{58} and Cys^{84}.

Not all the values of m_i and $\Delta G_i^*(0)$ can be given simple structural explanations. One of the main goals of future work is to find detailed structural explanations for the results.

Note added in proof: After this paper was submitted, two articles appeared proposing that hydrogen exchange in BPTI occurs by two separate EX2 mechanisms that might be termed limited structural fluctuation and unfolding. The authors propose that the unfolding exchange mechanism occurs by total unfolding (*19*).

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5. Because exchange in our conditions is known to occur by the EX2 exchange mechanism (*1*), the observed exchange rate, k_{HX}, is as indicated below since $k_{21} \gg k_{23}$.

$$k_{HX} = \left(\frac{k_{12}}{k_{21}}\right) k_{23} \quad (6)$$

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} I \overset{k_{23}}{\rightarrow} I^* \quad (7)$$

In the EX2 mechanism, the native protein (N) is resistant to exchange but is in rapid equilibrium with a conformation (I) that permits exchange, and the slow rate-limiting step is the direct exchange step, which has the rate constant k_{23}. Consequently, we obtain an apparent equilibrium constant, K_{12}^*, for the N ⇌ I reaction by dividing k_{HX} by k_c, the "chemical" exchange rate which can be estimated with model peptides (*6*).

$$\frac{k_{HX}}{k_c} = \left(\frac{k_{12}}{k_{21}}\right)\left(\frac{k_{23}}{k_c}\right) = K_{12}^* \quad (8)$$

If k_c equals k_{23}, then K_{12}^* equals K_{12}, the true equilibrium constant of the N ⇌ I reaction; otherwise, $K_{12}^* < K_{12}$ because k_{23} may be smaller than k_c but cannot be larger. Therefore, we compute the apparent standard Gibbs energy of the N ⇌ I reaction from

$$\Delta G_{12}^* = -RT \ln K_{12}^* = -RT \ln\left(\frac{k_{HX}}{k_c}\right) \quad (9)$$

In the main text, we omit the subscripts 1 and 2 and consider the exchange rates [$k_{HX}(i)$ and $k_c(i)$] and Gibbs energies (ΔG_i^*) for individual protons i. The offset of 3.6 kcal mol^{-1} between ΔG_i^* of exchange and ΔG_i of unfolding for RNase A (see main text) indicates that $k_{23}(i) = 2.7 \times 10^{-3}\, k_c(i)$. Exchange involves both breaking the hydrogen bond made by proton i and gaining access for hydroxyl ion to the site of exchange. The work of breaking the hydrogen bond is expressed by the equilibrium constant K_{12}, whereas the low effective concentration of hydroxyl ion at the site of exchange (which, according to our analysis, should be the same for all 23 protons) is thought to be the primary factor responsible for the low value of $k_{23}(i)/k_c(i)$.
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20. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; H, His; K, Lys; M, Met; N, Asn; Q, Gln; S, Ser; T, Thr; V, Val; and Y, Tyr.
21. The NMR data for exchange rate determinations were obtained by collecting back-to-back, absolute-value mode ^1H-^1H two-dimensional spin correlated spectra (COSY) on a General Electric GN-Omega 500 operating at a proton frequency of 500.13 MHz without removing the sample from the spectrometer. For GdmCl concentrations less than 0.45 M, a total of ten spectra were collected (one every three hours); a total of seven spectra were collected for all other GdmCl concentrations. The raw NMR data were processed with FELIX version 1.1 (provided by Hare Research, Inc.). The N-H, Cα-H crosspeak assignments were taken from earlier work [A. D. Robertson, E. O. Purisima, M. A. Eastman, H. A. Scheraga, *Biochemistry* **28**, 5930 (1989); M. Rico *et al.*, *Eur. J. Biochem.* **183**, 623 (1989)]. Crosspeak heights (normalized to the nonexchanging aromatic crosspeaks of Tyr^{25}) were used to determine exchange rates. All experiments were performed at 34°C with 5 mM RNase A in 0.1 M sodium chloride (pH* 5.5). Samples containing 0.0 to 0.7 M deuterated GdmCl were prepared immediately before being inserted into the NMR spectrometer.
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