Hydrogen Exchange in Native and Alcohol Forms of Ubiquitin†

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ABSTRACT: Ubiquitin adopts a non-native folded structure in 60% methanol solution at low pH. Two-dimensional nuclear magnetic resonance (2D NMR) was used to measure the hydrogen-exchange rates of backbone amide protons of ubiquitin in both native and methanol forms, and to characterize the structure of ubiquitin in the methanol state. Protection factors (the ratios of experimentally determined exchange rates to the rates calculated for an unfolded polypeptide) for protons in the native form of ubiquitin range from less than 10 to greater than 10^5. Most of the protons that are protected from exchange are located in regions of hydrogen-bonded secondary structure. The most strongly protected backbone amide protons are those of residues comprising the hydrophobic core. Hydrogen exchange from ubiquitin in methanol solution was too rapid to measure directly by 2D NMR, so a labeling scheme was employed, in which exchange with solvent occurred while the protein was in methanol solution. Exchange was quenched by dilution with aqueous buffer after the desired labeling time, and proton occupancies were measured by ^{1}H NMR of the native form of the protein. Protection factors for protons in the methanol form of ubiquitin range from 2.6 to 42, with all protected protons located in hydrogen-bonded structure in the native form. Again, the most strongly protected protons are those of residues in the hydrophobic core. Comparison of the patterns of the hydrogen-exchange rates in the native and methanol forms indicates that almost all of the native secondary structure persists in the methanol form, but that it is almost uniformly destabilized by 4–6 kcal/mol. Some key tertiary interactions indicative of association of the α helix and β sheet are also evident. The structure of ubiquitin in the methanol form is similar, but not identical, to intermediates on the refolding pathway of ubiquitin (Briggs, M. S., & Roder, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2017–2021).

A structural description of protein-folding intermediates is essential for a mechanistic understanding of the folding process. However, kinetic intermediates are generally too short-lived for the application of high-resolution structure determination tools such as two-dimensional nuclear magnetic resonance (2D NMR)[1] and X-ray diffraction; consequently these intermediates have been characterized primarily by various lower resolution methods (Dobson, 1991, 1992). An alternative route to the structural description of folding intermediates is to find experimental conditions under which the intermediate state is stable and can be studied at equilibrium, thus allowing the application of techniques that require large amounts of time for data collection.

The molten globule state, a partially folded form of protein structure that is intermediate between the native and completely unfolded states, can be induced and stabilized under a variety of non-native conditions, depending on the protein (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989). These conditions include extremely low or high pH, high salt concentration, high temperature, or moderate concentrations of strong denaturants or organic solvents. The properties of the molten globule state have been described for a number of proteins (Dolgikh et al., 1984, 1985; Kuwajima et al., 1985; Ikeguchi et al., 1986; Ptitsyn, 1987; Semisotnov et al., 1987; Ptitsyn et al., 1990; Ptitsyn & Semisotnov, 1991). Its characteristics include a volume that is intermediate between the unfolded and native states, as judged by hydrodynamic studies and small-angle X-ray scattering, the presence of significant amounts of nativelike secondary structure, as judged by far UV circular dichroism and infrared spectroscopy, and greater flexibility than the native state and/or lack of stable tertiary structure, indicated by near-UV CD spectroscopy and NMR (Ptitsyn & Semisotnov, 1991).

The participation of a compact intermediate similar to the molten globule state in the folding pathway has been demonstrated for a number of proteins. Various hydrodynamic studies indicate the accumulation of a compact intermediate during folding of bovine carbonic anhydrase B, *Staphylococcus aureus* β-lactamase, and human interleukin 1β (Zerovnik & Pain, 1987; Ptitsyn et al., 1990; Ptitsyn & Semisotnov, 1991). Kinetic studies utilizing stopped-flow CD and fluorescence spectroscopy have also shown that an intermediate similar to the molten globule state is formed during the folding of a variety of proteins (Ptitsyn & Semisotnov, 1991, and references therein). On the basis of these studies, Ptitsyn et al. (1990) have suggested that the molten globule state is a general kinetic intermediate in protein folding. In this work we test this hypothesis by comparing the structure of a known kinetic intermediate in the folding pathway of ubiquitin with that of its molten globule state.

Ubiquitin, a small cytoplasmic protein (76 residues, 8565 molecular weight) (reviewed by Rechsteiner, 1988), takes on a non-native folded structure in alcohol/water solutions, as reported by Wilkinson and Mayer (1986). CD spectra of the alcohol-induced structure indicate a large increase in the α helical content of the protein, leading the authors to postulate that ubiquitin undergoes a major change in conformation in alcohol. Harding et al. (1991) studied the structure of ubiquitin in 60% methanol/40% water at pH 2 by 2D NMR. Their results show that a gross structural reorganization of

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[1] Abbreviations: UV, ultraviolet; CD, circular dichroism; 2D NMR, two-dimensional nuclear magnetic resonance; H-exchange, hydrogen exchange; MW, molecular weight; COSY, *J*-correlated spectroscopy; PDLA, poly(D,L-alanine); 1D NMR, one-dimensional nuclear magnetic resonance; pD*, uncorrected pH meter reading in D_2O solutions.

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the protein has not occurred, and that the methanol-induced structure contains a subset of the interactions present in the native state. They conclude that the hydrophobic face of the β sheet in the methanol-induced state is covered by a partially structured α helix, which is more flexible than the α helix in the native state. They report that the turn-rich area of the protein (residues 37–62) does not appear to be structured in the methanol state.

These studies indicate similarities between the methanolic form of ubiquitin and the molten globule. Since the folding process of ubiquitin has been described (Briggs & Roder, 1992), knowledge of the structure of the alcohol form of ubiquitin will allow comparison of a putative molten globule structure with structures on the folding pathway. In this work, we have used hydrogen-exchange and 2D NMR techniques to characterize the structure of the alcohol-induced form of ubiquitin. The principle of hydrogen exchange is that amide protons of the polypeptide backbone that are involved in hydrogen-bonded secondary structure or tertiary H-bonding, or are sequestered within the protein, have solvent exchange rates that are slowed relative to exchange rates in an unfolded polypeptide (Woodward et al., 1982; Englander and Kallenbach, 1984; Rashin, 1987). Thus by monitoring the H-exchange rate of individual amide protons, detailed structural information can be obtained for many locations along the polypeptide chain.

A combination of hydrogen exchange and NMR has been used to characterize destabilized forms of two proteins. Hughson et al. (1990) used this method to characterize the structure of a partially folded apomyoglobin intermediate induced by removing the heme group, and decreasing pH. Their results suggest that the A, G, and H helices are present in the partially folded form while the B and E helices are predominantly unfolded. They propose that the A, G, and H helices comprise a compact domain that may resemble an intermediate on the folding pathway. Jeng et al. (1991) applied the same methods to obtain a structural description of acid-denatured cytochrome *c*. Their results show that the three major α helices of cytochrome *c* remain folded in the acid-destabilized state at high salt concentrations, but the majority of the amide protons involved in nonhelical H-bonds are only marginally protected, suggesting a dramatic destabilization of these tertiary interactions.

Here we report the results of hydrogen-exchange studies of both native and methanol forms of ubiquitin. Forty-one of the backbone amide protons in native ubiquitin exchange slowly enough to measure the exchange rate using 2D NMR. All but five of these are involved in hydrogen bonds in the crystal structure (Vijay-Kumar et al., 1987). Thirty of the backbone amide protons are protected from exchange in the methanol-induced structure. Overall, the extent of protection is much reduced from the native levels, indicating substantial destabilization of structure in the methanol form. However, the pattern of protection is very similar to that found in the native protein, suggesting that nativelike structure persists in the methanol form, but is highly dynamic. This molten globule form of ubiquitin structure is similar to a kinetic folding intermediate previously identified (Briggs & Roder, 1992), but differs from it in having more structure in the highly turn-rich, proline-rich region from Ile36 to Arg42.

MATERIALS AND METHODS

Ubiquitin, deuterated solvents, and poly(D,L-alanine) (PDLA) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

1D ^{1}H NMR spectra were recorded on a Varian Gemini 300 at room temperature, using a spectral width of 4500 Hz, 128 transients, and a 1.5-s relaxation delay, with presaturation of the water signal during the relaxation delay. The ubiquitin samples were 25 mg/mL ubiquitin (2.9 mM) in D_2O adjusted to pD* 2.0 with DCl, and contained methanol or urea as indicated. (pD* is the uncorrected pH electrode reading in D_2O solutions.)

2D *J*-correlated (COSY) spectra were recorded on a Varian XL 400 at room temperature with a spectral width of 4800 Hz. A total of 128 transients of 2048 complex data points were recorded for each of 256 t_1 increments. The total acquisition time was 12 h. For fast COSY spectra, 16 transients of 2048 complex data points were recorded for each of 256 t_1 increments with a total acquisition time of 2 h. The water signal was suppressed by irradiation during a 1-s relaxation delay. The data were processed on a micro Vax II computer using the program FTNMR provided by Dennis Hare (Hare Research Inc., Woodinville, WA). Unshifted sine-squared multiplication was applied in both dimensions before Fourier transformation. Cross-peak intensities were measured by volume integration using a radius of two points. Cross-peak volumes were corrected for concentration variations by normalization to the volumes of cross peaks between nonexchangeable protons. Proton occupancy was calculated by dividing normalized cross-peak volume by the cross-peak volume at zero exchange time, obtained by extrapolation.

For measurement of H-exchange rates of native ubiquitin, 10 mg of ubiquitin was dissolved in 0.6 mL of 50 mM CD_3CO_2D in D_2O, pD* 3.5, just prior to taking the COSY spectrum. The final protein concentration was 2.0 mM. The pD* of the solution was readjusted to 3.5 after dissolving of the protein. The sample was kept at room temperature and 12 COSY spectra were taken on the same sample within 6 months. For fast COSY spectra, the concentration of ubiquitin was increased to 40 mg/mL (4.7 mM).

Samples for measurement of H-exchange rates from the methanol-induced state of ubiquitin were prepared as follows: 10 mg of ubiquitin was dissolved in 0.4 mL of D_2O previously adjusted to pD* 2.0 by addition of DCl. A 0.6-mL sample of perdeuterated methanol was added to induce the methanol form. The final pD* was 2.7, determined in a separate control. After the desired exchange period, the solution was diluted with 9 mL of 50 mM CD_3CO_2D in D_2O, pD* 3.5, to return the protein to the native state. The diluted solution was concentrated by ultrafiltration, using a 3000 MW cutoff membrane (Amicon Corp.). The solvent was exchanged for 50 mM CD_3CO_2D in D_2O, pD* 3.5, by ultrafiltration. To minimize hydrogen exchange during the ultrafiltration steps, the sample temperature was kept at 0–4 °C. Samples were stored at –20 °C prior to NMR analysis.

The H-exchange rate of PDLA was measured spectrophotometrically, as described by Englander et al. (1979). Samples contained approximately 0.25 mg/mL of PDLA in D_2O, yielding an initial absorbance at 220 nm of about 1.0. Absorbance changes were measured at 220 nm, and calculations were performed as described by Englander et al. (1979). At pD* from 1.5 to 3.0, solutions were buffered with 50 mM phosphate, and at pD* from 3.5 to 5.5, with 50 mM acetate. Solutions with pD* <1.5, were unbuffered; DCl was used to adjust the pD*.

RESULTS

Methanol-Induced State. Ubiquitin takes on a non-native partially folded form in alcoholic solution at low pH. Wilkinson et al. (1986) and Harding et al. (1991) have characterized this structure by CD spectroscopy and by NMR, respectively. We have obtained 1D NMR spectra of ubiquitin

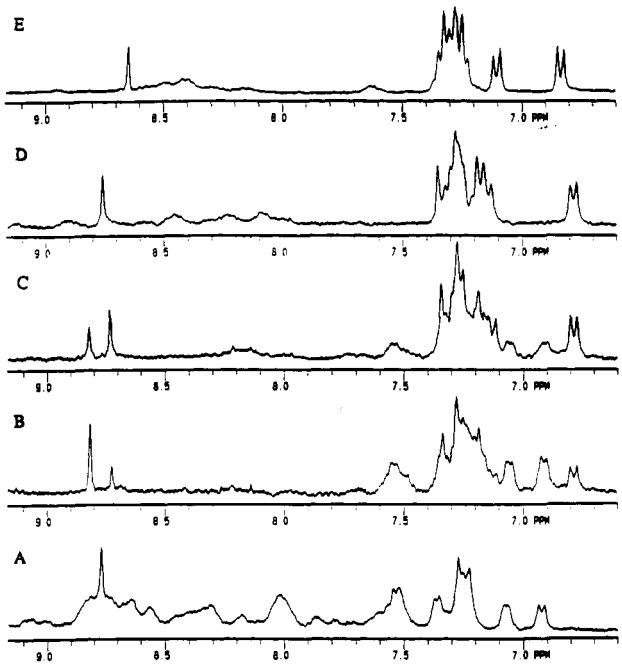

FIGURE 1: The aromatic region (6.6–9.2 ppm) of the 1D ^{1}H NMR spectra of ubiquitin. Spectra were obtained on a Varian Gemini 300 spectrometer. All spectra were obtained at ambient temperature (23–25 °C). Sample conditions: 25 mg/mL ubiquitin in DCl, D_2O, pD* 2.0. A, 0% CD_3OD (native state); B, 44% CD_3OD; C, 47% CD_3OD; D, 60% CD_3OD (methanol state); E, 8 M urea (denatured state).

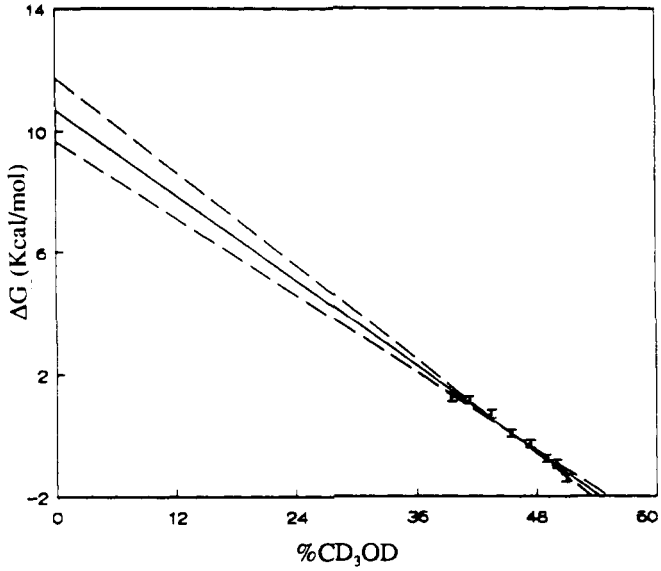

FIGURE 2: Dependence of $\Delta G°$ for the native to methanol transition on methanol concentration (%, v/v). $\Delta G°$ was determined from equilibrium constants derived from the ratio of the areas of the His68 C_2H peaks, as described in the text. The error in $\Delta G°$ was derived by estimating a maximum error of 10% in the areas of the resonances.

in the native, denatured (8 M urea), and methanol-induced states. The spectra are consistent with those published by Harding et al. (1991). Conformational differences are most apparent in the downfield region where the backbone amide and aromatic side chain resonances are found, shown in Figure 1. For example, the C_2 ring proton resonance of His68 appears at 8.82 ppm in the native protein, at 8.74 ppm in the methanol-induced form, and at 8.65 ppm in the urea-denatured protein. The Tyr59 3,5 ring protons are also resolved in all three states, appearing at 6.93 ppm in the native spectrum, at 6.79 ppm in the methanol state, and at 6.84 ppm in the urea-denatured protein. The resonances of the Phe4 C_α proton and the Leu50 and Ile61 side-chain methyl protons are resolved in the 1D NMR spectrum of the native protein, but merge into extremely overlapped regions of the spectrum in the methanol and urea states (data not shown).

Fractions of methanol-induced and native structures were calculated from the areas of the corresponding His68 C_2H and Tyr59 3,5 ring proton resonances, and from the normalized areas of the native Phe4 $C_\alpha H$ and Leu50 and Ile61 side-chain methyl resonances. For all of these resonances, at 23 to 25 °C, the transition to the methanolic form begins at about 40% methanol and is complete by about 53% methanol, with a midpoint ranging from 45% methanol to 46.9% methanol, depending on the resonance examined. The average transition midpoint is 45.9% methanol at this temperature. There is no change in the spectra from about 53% methanol to 85% methanol. Methanol concentrations greater than 85% cause the protein to precipitate. Varying pD* between 1 and 4 in the presence of 60% methanol had no effect on the 1D NMR spectrum (data not shown).

Unfolding free energy was calculated from the ratio of methanol-induced structure to native structure, determined as described above. In Figure 2 methanol concentration is plotted versus unfolding free energy using the His68 C_2H resonance data. Plots using data derived from the Tyr59 3,5 ring protons, Phe4 $C_\alpha H$, or Leu50 or Ile61 side-chain methyl protons yield similar results. Extrapolation to 0% methanol, using the linear model (Pace, 1986), yields $\Delta G° = 10.7 \pm 1.0$ kcal/mol for the native to methanol state transition. For comparison, the denaturant-induced transition to the unfolded state occurs with $\Delta G° = 5.0$ kcal/mol, when the transition is induced by guanidine hydrochloride at pH 3.5 (Briggs & Roder, 1992), and $\Delta G° = 3.9$ kcal/mol, when the transition is initiated by urea at pH 2 (Pan & Briggs, unpublished data). The free energies for the two unfolding transitions are of comparable magnitude, implying that the unfolded states induced by guanidine hydrochloride and urea are structurally similar. The very different $\Delta G°$ for the native to methanol state transition suggests that the alcohol state is indeed not identical to the unfolded state. However, it is surprising that the methanol-induced structure is so apparently destabilized relative to the unfolded state. The significance of this finding is still unclear; however, these unexpected thermodynamics may indicate that use of the linear extrapolation model is not appropriate when studying structural transitions in organic solvents, although it appears to be valid for the investigation of guanidine- and urea-induced unfolding (Santoro & Bolen, 1992).

Hydrogen-Exchange Rates of Ubiquitin in the Native State. The hydrogen-exchange rates of protons in native ubiquitin were measured as described in Materials and Methods. Forty-one backbone amide protons exchange with solvent deuterons slowly enough to be detected by 2D NMR, due to sequestration of these amide protons from the solvent and their involvement in hydrogen bonds. The fingerprint regions of COSY spectra taken at four exchange time points are shown in Figure 3A. Proton occupancies were determined as described, and are plotted versus time for representative protons in Figure 4A. Hydrogen-exchange rates were obtained by fitting a single exponential decay curve to the data. The measured hydrogen-exchange rates, as well as calculated intrinsic exchange rates (see below), are listed in the supplementary material.

Protection factors for the slowly exchanging protons in native ubiquitin are given in Table I, and are plotted versus residue number in Figure 5. The protection factor, P, is defined as the ratio of k_c to k_{ex}, where k_c is the intrinsic exchange rate of a given amide proton in a random coil polypeptide and k_{ex} is the experimentally determined exchange rate. The solvent

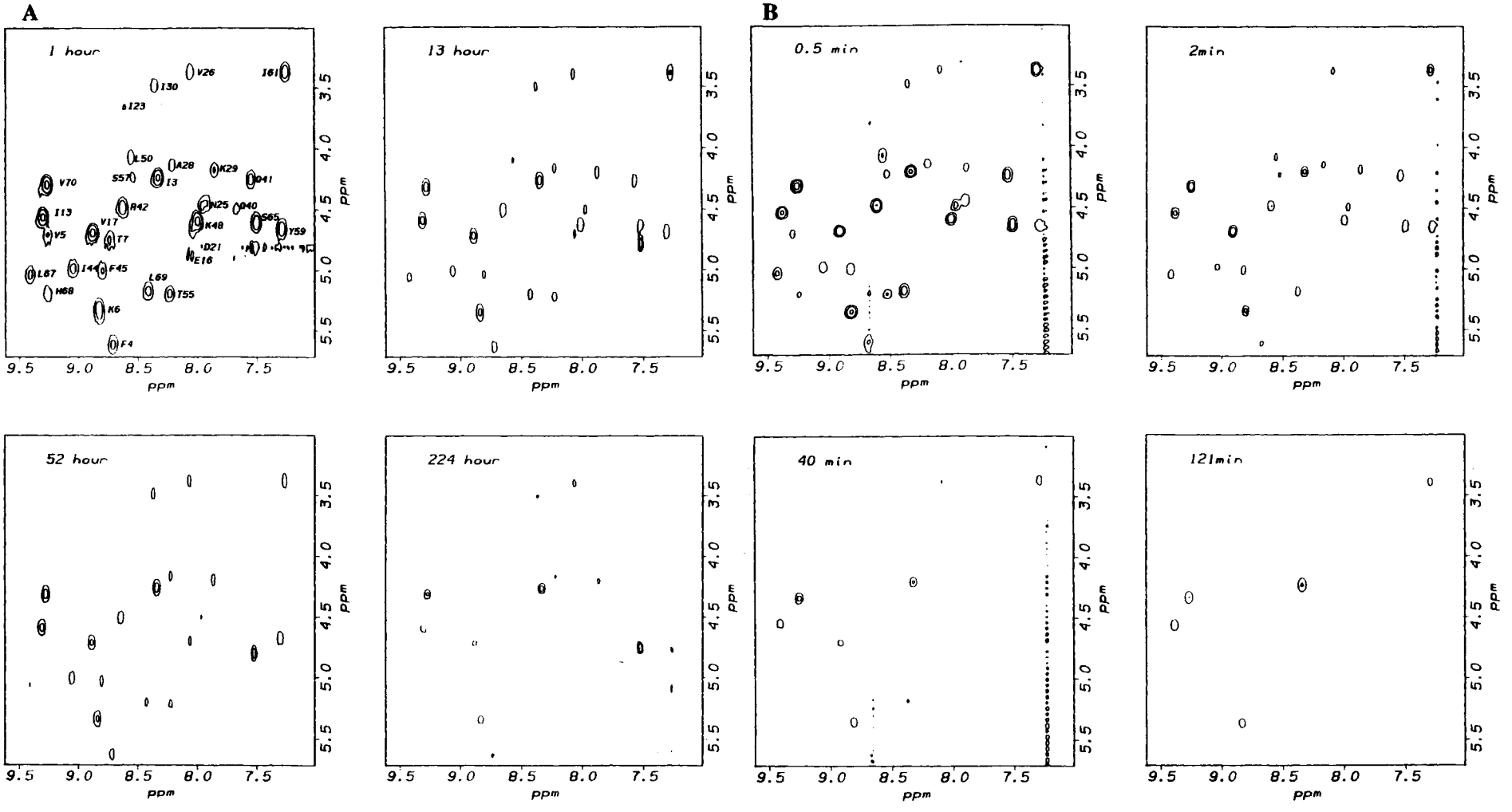

FIGURE 3: Fingerprint regions ($NHC_\alpha H$) of magnitude of COSY spectra of ubiquitin. In part A, ubiquitin was dissolved in 50 mM CD_3CO_2D, pD* 3.5, and allowed to exchange for the indicated times. The sample was stored at room temperature (~23 °C) between spectra. In part B, ubiquitin was dissolved in DCl, D_2O, pD* 2.0, and methanol was added to induce the methanol form for the indicated times. After the desired exchange time the solution was diluted with 50 mM CD_3CO_2D, pD* 3.5, to return the protein to the native state, and the diluted sample was concentrated by ultrafiltration. Cross-peak assignments were derived from the data of DiStefano et al. (1987), taking into account minor chemical shift differences due to differences in sample temperature and pH.

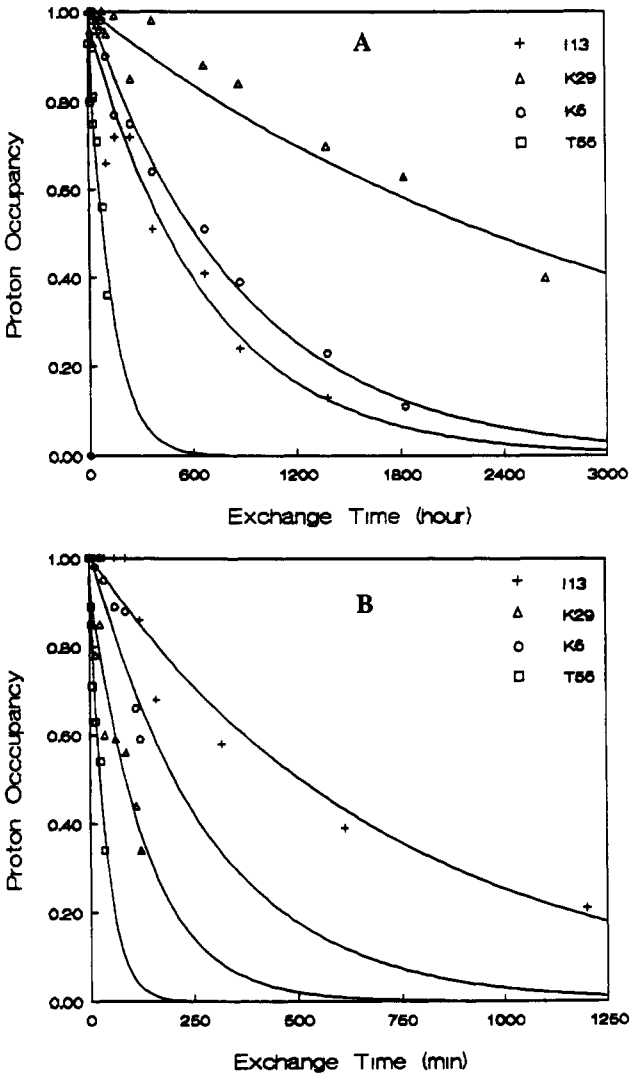

FIGURE 4: Time course of H–D exchange for representative ubiquitin backbone amide sites in the native state. Proton occupancy is plotted versus exchange time. H–D exchange rates were obtained by fitting the data to a single exponential decay curve. Panel A shows hydrogen exchange from the native state. Panel B show hydrogen exchange from the methanol structure.

exposed exchange rate k_c was calculated using the measured exchange rate constant for Ala-Ala (Jeng & Englander, 1991), modified to reflect local sequence effects according to the method of Molday et al. (1972), and depends only on pH, temperature, and local sequence. Thus the protection factor reflects the retardation of hydrogen exchange due to the presence of structure in the protein. Protection factors are large for those protons that are involved in folded or partially folded structure and small for those that are predominantly unfolded.

The measured P values range from 22 to about 10^5 for the slowly exchanging backbone amide protons in native ubiquitin. Protons that could not be detected in a 2-h COSY spectrum have a minimum exchange rate of about 2.5×10^{-2} min^{-1}, which corresponds to a maximum protection factor of about 10. Although the protection factors for these protons thus range from 1 to 10, each was arbitrarily assigned a P value of 1 ($\log P = 0$) for plotting in Figure 5. Most of the amide protons with a P value larger than 10^4 are in the hydrophobic core formed by three strands of β sheet and the α helix, while those with P values less than 10^2 are located in regions of irregular structure, or on the surface of the protein (Vijay-Kumar et al., 1987). The most slowly exchanging protons, whose signals are still visible in a COSY spectrum taken after 10 months of exchange, are the backbone amide protons of residues I3, F4, V26, I30, and I44. All are located in the protein's hydrophobic core: V26 and I30 are on one side of the α helix adjacent to the face of the β sheet where I3, F4, and I44 are found. The backbone amide protons of I23 and L56, which are involved in a hydrogen-bonding network located between the major elements of secondary structure and are indicative of interactions between them, are also among the most slowly exchanging protons. All but five of the 41 slowly exchanging protons are involved in hydrogen bonds in the crystal structure (Vijay-Kumar et al., 1987); all of these (Q2, T12, E16, T22, and Q49) have protection factors smaller than 500.

Hydrogen-Exchange Rates of Ubiquitin in 60% Methanol Solution. Since hydrogen-exchange rates from the methanol form are significantly faster than those in the native state, an indirect technique was used to measure the hydrogen-exchange rates in methanol solution. Ubiquitin was dissolved in D_2O at pD* 2 and room temperature. Predeuterated methanol was added to a final concentration of 60% (v/v) to initiate the transition to the alcohol state. After the desired exchange time, which ranged from 30 s to 2 h, aqueous buffer was added to a final concentration of 10% (v/v) methanol to return the protein to the native state. The solution was then concentrated and the exchange buffer was replaced with NMR buffer by ultrafiltration. The fingerprint regions of COSY spectra taken at four exchange time points are shown in Figure 3B. Proton occupancies versus time for representative backbone amide protons of ubiquitin in 60% methanol are plotted in Figure 4B. Hydrogen-exchange rates were obtained by fitting a single exponential decay curve to the data. The measured hydrogen-exchange rates and calculated intrinsic exchange rates are listed in the supplementary material. Protection factors, calculated as described above, are given in Table I and plotted versus residue number in Figure 5. A correction for the effect of methanol on the intrinsic rates was not necessary, as explained below.

From the time scale in Figure 4B it is evident that the H-exchange rates from the methanol form of ubiquitin are much faster than those in the native state, but we were able to observe 30 amide protons that exchange slowly enough to be detected. All of these protons also exchange slowly in the native state. The other 11 amide protons that exchange slowly enough to be observed in the native state exchange too quickly for detection in the methanol form. All five of the protons that are not hydrogen bonded in the crystal structure, but are protected from exchange in the native state, exchange quickly, and are not protected in the methanol form. Six other amides that are protected in the native structure also exchange at the intrinsic rate in the methanol state: T7 and L15 are located at the end and outer edge, respectively, of the N-terminal β hairpin, D32 is at the C-terminal end of the α helix, D58 and N60 are found in 3_{10} helix/turn-rich region, and H68 is in the middle of the central β strand. The 30 observable amide protons are distributed throughout all of the secondary structure elements; there is no α helix or β strand in which no amide proton can be detected. In addition, there are several protected protons in the turn-rich region of irregular structure (E40, Q41, N60, and I61) and in the 3_{10} helix. The backbone amide protons I23 and L56, key indicators of the association of the α helix and β sheet, are also protected in the methanol state.

The protection factors for amide protons in the methanol form range from 2.6 to 42, 3 orders of magnitude faster than in the native state, on average, indicating a dramatic destabilizing effect due to the presence of methanol. However,

Table I

secondary structure	residue	P^a P(native)[b]	P^a P(60% CD_3OD)[c]	secondary structure	residue	P^a P(native)[b]	P^a P(60% CD_3OD)[c]
β1	Gln2	(2.2×10^1)			Gln40	9.2×10^1	15
β1	Ile3	4.4×10^4	22	β3	Gln41	1.0×10^3	4.3
β1	Phe4	4.2×10^4	23	β3	Arg42	2.0×10^3	2.6
β1	Val5	2.1×10^4	13	β3	Ile44	4.2×10^4	24
β1	Lys6	4.1×10^3	10		Phe45	1.5×10^3	12
β1	Thr7	3.0×10^4		β4	Lys48	2.3×10^3	3.6
β2	Thr12	(1.5×10^2)		β4	Gln49	(6.6×10^1)	
β2	Ile13	6.5×10^3	33	β4	Leu50	6.7×10^2	7.0
β2	Leu15	(7.1×10^1)		3_{10}	Thr55	3.4×10^3	6.4
β2	Glu16	3.3×10^2		3_{10}	Leu56	2.7×10^4	14
β2	Val17	1.2×10^4	11	3_{10}	Ser57	3.3×10^2	6.7
	Thr22	(3.9×10^1)		3_{10}	Asp58	(1.5×10^2)	
α1	Ile23	1.7×10^4	6.0	3_{10}	Tyr59	1.3×10^4	19
α1	Asn25	1.4×10^3	6.3		Asn60	(6.0×10^1)	
α1	Val26	4.4×10^5	14		Ile61	2.4×10^3	42
α1	Ala28	1.1×10^4	7.4		Ser65	1.2×10^3	2.6
α1	Lys29	2.1×10^4	3.9	β5	Leu67	7.6×10^2	10
α1	Ile30	1.8×10^5	11	β5	His68	1.7×10^3	
α1	Gln31	2.8×10^3	5.6	β5	Leu69	8.2×10^3	28
α1	Asp32	(3.0×10^1)		β5	Val70	2.9×10^3	16
α1	Ile36	1.2×10^2	2.7				

[a] Amide proton protection factors. Protection factors $P = k_c/k_{ex}$ determined from measured H–D exchange rate, k_{ex}, and the corresponding intrinsic exchange rate, k_c, calculated according to Jeng and Englander (1991) and Molday et al. (1972). The estimated errors in k_{ex}, ±10%. [b] Protection factors for ubiquitin in the native state, 50 mM acetate, pD* = 3.5, 22 °C. Values in parentheses were determined from only three or four time points. [c] Protection factors for ubiquitin in the methanol state, 60% CD_3OD/40% D_2O (v/v), 50 mM phosphate, pD* = 2.7, 22 °C.

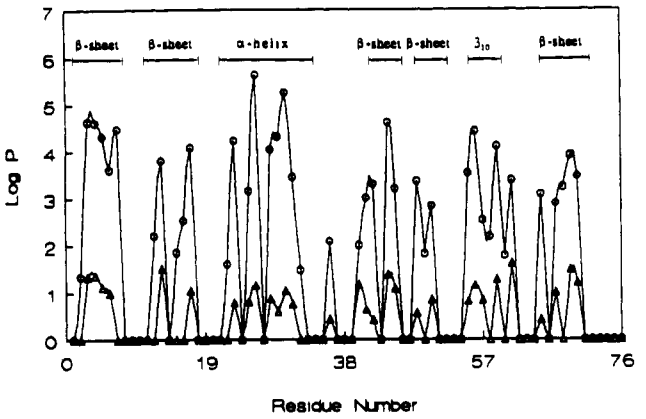

FIGURE 5: Logarithm of protection factors (log P) in the native state (○) and methanol-induced state (▲) are plotted versus residue number. Protons that could not be detected in 2-h COSY spectrum are assigned a P value of 1 (log P = 0).

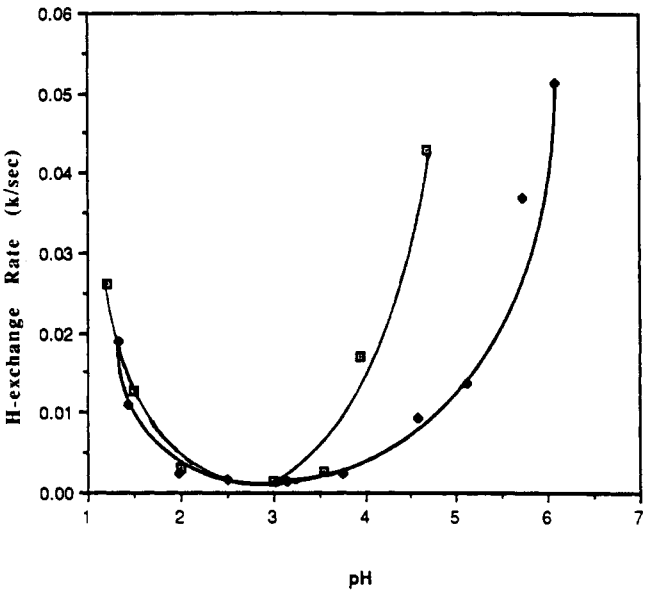

FIGURE 6: pH dependence of amide proton exchange of poly(D,L-alanine) in aqueous solution and in 60% methanol solution. H–D exchange was monitored by the change in absorbance at 220 nm as described in Englander et al. (1979): ⊡, exchange rates in aqueous solvent; ♦, exchange rates in 60% methanol.

comparison of the protection patterns in Figure 5 indicates that the structures of the native and methanol forms are remarkably similar: areas of strong protection in the native form, which are primarily regions of secondary structure, are also protected from exchange in the alcohol form, while relatively fast-exchanging protons in the native protein are also fast in the alcohol form. Most of the protons that are protected from exchange in the native form but not in the methanol form are located at the edges or ends of the β sheet or α helix, showing that the destabilization is more pronounced in these areas. The similarity of the protection factor patterns demonstrates that the major secondary structures present in the native protein also exist in the alcohol form, but are significantly less stable, presumably due to disruption of tertiary interactions within the hydrophobic core that allow mutual stabilization of secondary structural elements.

Measurement of the Intrinsic H-Exchange Rate of Poly(D,L-alanine) in 60% Methanol Solution. The published intrinsic exchange rates for peptide and protein backbone amide protons, k_c, were measured in aqueous solution (Jeng & Englander, 1991; Molday et al., 1972). Interpretation of our exchange data for the methanol form of ubiquitin requires knowledge of the effect of methanol on k_c. We measured the exchange rates of a model unstructured polypeptide, poly(D,L-alanine), in 60% methanol at various pH's using the spectrophotometric technique described by Englander et al. (1979). This method relies on the approximately 5% increase in A_{220} of the peptide bond on exchange of deuterium for the amide proton. Figure 6 shows that at pD* ≤3.0, PDLA has about the same H-exchange rates in D_2O and in 60% CH_3OD/D_2O. The large difference at higher pD* values is probably caused by the effect of large concentrations of organic solvent on K_w (Englander & Kallenbach, 1984). Since we carried out our experiment at pD* <3, we used the published k_c values (Jeng & Englander, 1991) without correction for the presence of 60% methanol.

DISCUSSION

Thermodynamics of the Native ⇔ Methanol Transition. The unexpectedly large value of $\Delta G°$ for the native ⇔ methanol state transition, compared to the $\Delta G°$ for the unfolding transition in guanidine hydrochloride or urea, leads us to postulate that the linear extrapolation method does not accurately reflect the energetics of this process. Another approximation for $\Delta G°$ may be derived from the calculated protection factors listed in Table I. According to the local unfolding model for hydrogen exchange, the exchange process is comprised of two steps: local unfolding, which transiently exposes the amide proton to solvent, and the exchange event itself, which occurs only from the locally unfolded structure. The rate constants for these processes are

$$\text{folded} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{open} \xrightarrow{k_c} \text{hydrogen exchanged}$$

The observed exchange rates are determined by a competition between k_c, the intrinsic exchange rate for an unfolded polypeptide, and k_{-1}, the rate of refolding from the open form. In the case where k_c is much smaller than k_{-1}, such as at low pH, the measured exchange rate is $K_1 k_c$, where K_1 is k_1/k_{-1}, the equilibrium constant for the opening process. So K_1 is k_{ex}/k_c, or $1/P$. Assuming that the protection factors for the most slowly exchanging protons reflect the largest unfolding events, and thus that the derived K_1's approach K_{den}, the free energy difference between the native and methanol forms of ubiquitin may be estimated from the change in the protection factors of these protons (Englander & Kallenbach, 1984).

Calculating $\Delta G°$ from the protection factors for V26 and I30, the most highly protected protons, gives a free energy difference of 6 kcal/mol between the native and methanol structures. The same calculation based instead on the protection factors of I23 and L56, two protons involved in crucial tertiary hydrogen bonds, yields $\Delta G°$ of 4.6 kcal/mol. These figures seem more reasonable than that obtained from the linear extrapolation model. It is intriguing that the calculated $\Delta G°$ for the slowly exchanging protons in the α helix (V26 and I30) is significantly higher than that for protons that indicate tertiary structure (I23 and L56). This is consistent with the description of the molten globule as a structure with substantial amounts of secondary structure and destabilized tertiary structure.

Hydrogen Exchange from Native Ubiquitin. Our results are consistent with aspects of both the local unfolding model and the solvent-penetration model for hydrogen exchange (Woodward et al., 1982; Englander & Kallenbach, 1984). All but five of the protected protons in ubiquitin are involved in hydrogen bonds in the crystal structure (Vijay-Kumar et al., 1987); these five have the smallest protection factors of the slowly exchanging amides. Thirteen backbone amides are hydrogen bonded in the crystal structure, but exchange too quickly to be detected in 2D NMR experiments. Of these, three are backbone-to-side-chain H-bonds, which frequently are not protected from hydrogen exchange (Wlodawer et al., 1984). Nine are located at the ends of secondary structure elements, or in the turn-rich region of irregular structure, and consequently might be expected to undergo conformational changes more readily than amides in the middle of the secondary structure (Rohl et al., 1992; Wand et al., 1986; Dempsey, 1986). The last, K27, is in the middle of the α helix sequence, but faces the outside of the protein. Faster exchange of protons on the exterior surface of α helices relative to those on the buried surface has been observed for destabilized forms of proteins (Hughson et al., 1990; Jeng et al., 1991), and may reflect conformational fluctuations such as helix bending that could weaken hydrogen-bonding interactions.

We have not analyzed the hydrogen-exchange rates in terms of solvent-accessible surface area, as has been done for other proteins (Rashin, 1987), but inspection of the crystal structure indicates that the fast-exchanging protons are located throughout the protein, both exposed at the surface and sequestered in regions that would need to undergo a conformational fluctuation to come in contact with solvent. Most are not hydrogen bonded. The slowly exchanging amides are also located in both buried and exposed areas, although the very slowest are in the interior of the hydrophobic core.

Thus, our data support a hydrogen-exchange mechanism that requires both hydrogen-bond breakage and contact with solvent, mediated either by local unfolding or smaller conformational fluctuations that allow solvent to enter the interior of the protein. Neither hydrogen bonding, location in secondary structure, nor burial in the interior of the protein appears sufficient to ensure protection from hydrogen exchange, nor is lack of any of these factors an unambiguous indicator of fast exchange. The relationship between hydrogen-exchange rates and protein structure is influenced by many factors whose effects are interdependent.

Hydrogen Exchange from the Methanol Form of Ubiquitin. The hydrogen-exchange rates for protons in the methanol form of ubiquitin are much faster than those in the native state. However, the patterns of hydrogen-exchange rates and protection factors are very similar in the two structures, as shown in Figure 5. The increase in exchange rates, coupled with the correspondence of the protection patterns, leads us to conclude that all secondary structural elements of the native structure are present in the methanol state, but are destabilized by several kilocalories/mole, as discussed above. Tertiary structure in the form of the α helix–β sheet interface is also still present, as indicated by protection of I23 and L56 from exchange. The protons that are protected from exchange in the native form, but not in the methanol state, are located at the ends and edges of secondary structure, or in regions of irregular structure, with the exception of H68.

This result is different from that reported by Harding et al. (1991), who observed fewer amide proton signals than we do, after a 30-min exchange period. They concluded that three of five β strands and the α helix are present, while other parts of molecule, including the 3_{10} helix and the turn-rich region of irregular structure, are more likely disordered. The discrepancy may be explained by differing experimental procedures. In the work by Harding et al. (1991), ubiquitin was dissolved in 60% methanol at pH 2.0, room temperature, for 30 min. The methanol was then removed by centrifugation under vacuum to give the refolded protein. Thus, the protein was in the presence of a high concentration of methanol for longer than the stated 30-min exchange period, during which time some of the protected amide protons will exchange with solvent. However, not all of the slowly exchanging protons observed by Harding et al. are those that exchange most slowly in our hands, raising the possibility that the protein's structure was perturbed during the evaporation process. In addition, the conclusions of the Harding et al. paper are based on only one relatively long exchange time point, while in this study we report exchange rates based on a wide range of exchange times, allowing us to detect marginally stable structural elements.

Our conclusion and that of Harding et al. are also in disagreement with the conclusion reached by Wilkinson and Mayer (1986) based on the CD spectrum of ubiquitin in alcohol solution. The CD spectrum clearly implies a large increase

in the amount of helical structure present, which is not supported by the NMR results. The reason for this discrepancy is not yet clear. However, it is interesting that some studies of protein folding by stopped-flow CD techniques find that the CD signal in the early stages of folding becomes greater than that of native protein, followed by a slower decay to the native ellipticity (Radford et al., 1992; Sugawara et al., 1991).

Comparison of the Methanol State of Ubiquitin with a Kinetic Folding Intermediate. In studies of early hydrogen-bonding events in the folding process of ubiquitin, Briggs and Roder (1992) studied the time course of protection from exchange for 26 backbone amide protons that form stable H-bonds upon refolding and that exchange slowly under native conditions. Most of these protons exhibit a very fast protection phase with a time constant of about 10 ms. Association of elements of secondary structure appears to be important in driving this early folding event. Somewhat slower protection rates for residues 59, 61, and 69 provide evidence for the subsequent stabilization of a surface loop. Only two of the observed residues, Gln41 and Arg42, display a second, slower protection phase with significant amplitude. The slow phase has been interpreted in terms of a native-like folding intermediate containing cis peptide bonds preceding Pro37 and/or Pro38. This kinetic folding intermediate (the cis-containing form) has some structural similarities to the methanol-induced state of ubiquitin: in both the intermediate and the methanol-induced structure, most of the native secondary structural elements are present, and association of the α helix and β sheet is important in stabilizing interactions between them. In the structure observed on the folding pathway, the region from about Pro37 to Arg42 has not yet attained native hydrogen bonding. However, we find that in the methanol-induced structure this area has the same pattern of hydrogen-exchange rates as in the native protein, and therefore conclude that this part of the structure is present in the methanolic form. This is not unexpected since in the kinetic folding intermediate the protein had just been shifted from a denaturing environment in which the proline residues could attain the equilibrium cis–trans distribution favored by the unfolded form, while in the methanol-induced state the "loosened" structure is apparently not sufficiently unstructured to allow trans to cis isomerization of peptidyl–prolyl bonds. Another difference between the kinetic intermediate and the methanol-induced form is that the ends of the β strands and the α helix are destabilized in the methanol state. This effect is not observed in the kinetic folding intermediate.

CONCLUSIONS

We have characterized the methanol-induced structure of ubiquitin by hydrogen-exchange methods, measured by 2D NMR. The striking similarity of exchange-rate patterns of the native and methanol forms indicates that the alcohol-induced structure has essentially all of the structure present in the native structure. The overall increase in exchange rates in the methanol-induced state is consistent with a molten globulelike structure, with nativelike but highly dynamic secondary and tertiary structure.

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SUPPLEMENTARY MATERIAL AVAILABLE

The measured and calculated exchange rates for ubiquitin in the native and methanol states (2 pages). Ordering information is given on any current masthead page.

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