Comparison of the Refolding of Hen Lysozyme from Dimethyl Sulfoxide and Guanidinium Chloride†

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ABSTRACT: The folding of hen egg white lysozyme is complex, involving parallel pathways and distinct folding domains [Radford, S. E., Dobson, C. M., & Evans, P. A. (1992) *Nature 358*, 302−307]. In the present work the refolding of this protein from two denatured states that have different conformational properties, one generated by the presence of guanidinium chloride (GdmCl) and the other by dimethyl sulfoxide (DMSO), has been examined. Refolding was initiated by rapid dilution and followed by hydrogen-exchange pulse labeling, stopped-flow circular dichroism (CD) in the near-ultraviolet region, and stopped-flow fluorescence experiments. When the final refolding conditions were identical (545 mM GdmCl, 8% (v/v) DMSO, and 20 mM sodium acetate, pH 5.5, 20 °C), the folding behavior from the different denatured states monitored by near-UV CD and hydrogen-exchange pulse labeling was indistinguishable. These experiments indicate that the folding process of hen lysozyme is not significantly dependent on the nature of the two denatured states. The complexities in the pathway, therefore, appear to arise from properties of the collapsed state which is formed within the first few milliseconds of refolding. The kinetics of folding were found to be dependent on the concentration of DMSO in the final refolding buffer, although the fundamental properties of the pathway, including the existence of parallel events and distinct folding domains, are preserved under all the conditions studied. Inclusion of DMSO in the refolding buffer increases the rate of formation of native-like structure and of the native state itself. This could result from destablization of species formed early in folding, allowing them to rearrange more rapidly to permit productive folding to proceed. The results indicate that examination of a wide range of conditions will contribute substantially to a more complete understanding of protein folding pathways.

Hen egg white lysozyme is a globular protein of 129 amino acid residues. The structure of the protein in the crystalline state has been solved by X-ray diffraction (Blake et al., 1967; Imoto et al., 1974; Grace, 1980) and in solution by NMR spectroscopy (Redfield & Dobson, 1988; Smith et al., 1993). It comprises two lobes, separated by a cleft containing the active site residues (Figure 1). The first lobe consists of four α-helices and a 3_{10}-helix and is referred to as the α-domain, whereas the second lobe, referred to as the β-domain, contains a triple-stranded antiparallel β-sheet, a short 3_{10}-helix, and a long loop region. The protein structure is stabilized by four disulfide bridges, which are maintained in all the studies described here.

Hydrogen-exchange labeling studies of the refolding of lysozyme from 6 M guanidinium chloride (GdmCl)[1] have shown that the two lobes are separate folding domains with distinct folding kinetics (Miranker et al., 1991, 1993; Radford et al., 1992). A number of techniques in addition to hydrogen-exchange labeling have been applied to elucidate the events taking place during the refolding of lysozyme from

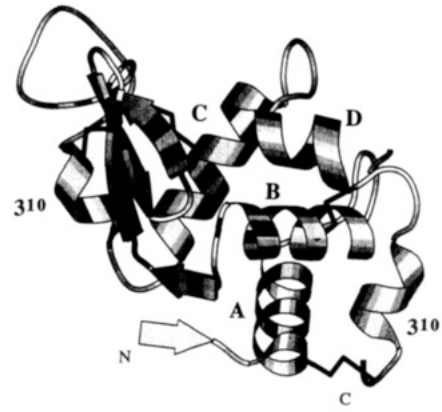

FIGURE 1: Schematic representation of the structure of hen egg white lysozyme (Blake et al., 1967). A cleft divides the molecule into two domains: the α-domain is shown on the right, and the β-domain is on the left. The four disulfide bridges are also shown. Residues used as probes in the hydrogen-exchange pulse-labeling experiments are shown in black. The diagram was produced using the program Molscript (Kraulis, 1991).

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[1] Abbreviations: GdmCl, guanidinium chloride; DMSO, dimethyl sulfoxide; CD, circular dichroism; FT, Fourier transform; NMR, nuclear magnetic resonance; MeU-diNAG, 4-methylumbelliferyl-*N*,*N*′-diacetyl-β-chitobiose, UV, ultraviolet; ANS, 1-anilinonaphthalene sulfonic acid; HSQC, heteronuclear single quantum coherence, TMS, tetramethylsilane.

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its GdmCl-denatured state (Dobson et al., 1994). Stopped-flow CD studies in the far-UV have shown that substantial secondary structure is present very early in the folding process (Chaffotte et al., 1992; Radford et al., 1992), although hydrogen-exchange labeling studies have indicated that this structure is not persistent and is presumably rapidly fluctuating (Radford et al., 1992). Tertiary structure, as measured by fluorescence-detected inhibitor binding (Itzhaki et al., 1994) and by near-UV CD of aromatic chromophores (Kuwajima et al., 1985; Ikeguchi et al., 1986; Chaffotte et al., 1992; Radford et al., 1992), develops much later. These experiments suggest that the earliest detected folding intermediates have properties akin to a molten globule-like structure with substantial, but unstable, secondary structure formed prior to the fixing of tertiary interactions. In accord with this, these early intermediates show maximal fluorescence enhancement with the hydrophobic dye 1-anilinonaphthalenesulfonic acid (ANS) (Itzhaki et al., 1994). The folding pathway of lysozyme is complex and involves several alternate routes (Radford et al., 1992; Miranker et al., 1993). In the majority of molecules an intermediate is populated in which the α-domain is substantially protected from exchange in the absence of a persistently structured β-domain. In a smaller number of molecules, however, an alternative route involving rapid protection of amides in both domains in a cooperative manner is taken (Miranker et al., 1993).

There is now firm evidence that the denatured states of proteins are often not well described as random coils but that they may possess significant residual structure (Evans et al., 1991; Dill & Shortle, 1991; Logan et al., 1994; Wüthrich, 1994). Furthermore, the conformational properties of these states may depend on the denaturation conditions employed (Evans et al., 1991; Shortle, 1993). An important question, therefore, arises as to the influence of these residual interactions on folding processes occurring *in vitro*. Studies of lysozyme unfolded under different conditions have suggested that the enzyme denatured thermally and in high concentrations of GdmCl and urea possesses significant residual clustering of hydrophobic residues (Dobson et al., 1984; Broadhurst et al., 1991; Evans et al., 1991), although in these cases no evidence exists for highly persistent interactions involving secondary structural elements (Buck et al., 1994). By contrast, the enzyme denatured in DMSO solution appears to be substantially less structured than these denatured states, at least as judged by the dispersion of side-chain chemical shifts in the NMR spectrum and the reactivity of aromatic side chains to photoexcited flavin radicals (Evans et al., 1991; Broadhurst et al., 1991). Kinetic refolding data from the denatured states of lysozyme in 6 M GdmCl and in 90% (v/v) DMSO have, therefore, been compared in order to examine the importance of the unfolded state in determining the folding behavior of the enzyme. In this paper we show that the folding pathway of lysozyme is not significantly influenced by the nature of the denatured state in the cases explored here, although the final refolding conditions were found to be of major importance in influencing the details of the folding process.

MATERIALS AND METHODS

Enzyme and Chemicals. Hen lysozyme (Sigma) was dialyzed exhaustively at pH 3.0, lyophilized, and stored at −20 °C. Deuterated GdmCl (Sigma) was prepared by three consecutive cycles of dissolution and lyophilization from D_2O (99.9%, Fluorochem Ltd.). Lysozyme deuterated (>99%) at all exchangeable sites was obtained by dissolving the protein (20 mg/mL) in D_2O, pH 3.8, and heating the solution to 80 °C for 10 min. The protein was then lyophilized. This procedure was repeated three times. Deuterated DMSO (DMSO-d_6, 99.9%) was obtained from Sigma.

Stopped-Flow CD Measurements. The refolding kinetics of lysozyme were monitored by stopped-flow CD at 289 nm and 20 °C using a JASCO J-720 spectropolarimeter equipped with a Biologic SFM3 stopped-flow unit. A 1-ms sampling rate and a path length of 1.5 mm were used throughout. The denatured protein (20 mg/mL in 6 M GdmCl or 90% (v/v) DMSO and 10% (v/v) H_2O) was diluted 11-fold with renaturation buffer containing the appropriate amount of denaturant to ensure that the final refolding conditions were identical in each experiment (545 mM GdmCl, 8% (v/v) DMSO, and 20 mM sodium acetate, pH 5.5). Data from 60 experiments were accumulated, averaged, and smoothed by a running average routine with a smooth width of 5 data points and fitted to a single-exponential function.

Stopped-Flow Fluorescence Measurements. The folding kinetics of lysozyme were monitored by fluorescence at 20 °C using an Applied Photophysics (SX-17MV) stopped-flow apparatus. An excitation wavelength of 280 nm was used, and total fluorescence intensity above 320 nm was monitored using a suitable filter. The excitation bandwidth was 2 mm. In these experiments denatured lysozyme (20 mg/mL in either 6 M GdmCl or 90% (v/v) DMSO) was diluted 11-fold into a suitable refolding buffer such that the final folding conditions were identical in each experiment (20 mM sodium acetate, 545 mM GdmCl, and 8% (v/v) DMSO, pH 5.5) unless otherwise stated. Refolding was monitored for 2 s using a 0.5-ms sampling time.

Determination of Dead Time in Refolding Experiments. Test reactions with dinitrophenylacetate at 20 °C (Barman & Gutfreund, 1964; Eccleston, 1987) were performed on the same instruments as were used for stopped-flow CD and fluorescence-detected refolding experiments. Dinitrophenylacetate (8 mM) in either 6 M GdmCl or 90% (v/v) DMSO at pH 2.5 was diluted 11-fold with NaOH (100 mM to 2 M), and base-catalyzed hydrolysis was monitored by measuring the change in absorbance at 325 nm. The kinetic traces of the reactions were fitted to a single exponential, and the amplitude of the reaction and the apparent rate of the pseudo-first-order reaction at each concentration of NaOH were noted. A plot of the amplitude versus the apparent rate gives a straight-line graph, the slope of which is a measure of the dead time of the instrument (Tonomura et al., 1978). Typical dead times of 3.5−4.0 ms were measured for experiments in the GdmCl and DMSO solutions, both with the Biologic SFM3 stopped-flow unit, which is equipped with a Berger ball mixer and stepping motors, and with the Applied Photophysics apparatus, which contains a T-mixer and is pneumatically driven.

Hydrogen Exchange Pulse Labeling Experiments. Lysozyme (20 mg/mL) was denatured in either 6 M deuterated GdmCl in D_2O or 90% (v/v) DMSO-d_6 and 10% (v/v) D_2O. By inclusion of D_2O in the denaturation solution significant back exchange of protein amides by residual H_2O in the highly hygroscopic DMSO-d_6 could be avoided. Hydrogen-exchange experiments were performed at various refolding times from 3.5 ms to 2 s at 20 °C as previously described

(Radford et al., 1992) using a Biologic QFM5 rapid-mixing quench-flow apparatus. The final refolding conditions were 545 mM GdmCl and 8% (v/v) DMSO in 20 mM sodium acetate, pH 5.5, in both refolding experiments. At each refolding time, amides which are not protected from exchange were protonated with an 8.4-ms labeling pulse at pH 9.6. At this pH the half-life of hydrogen exchange of a free amide is approximately 1 ms (Bai et al., 1993). Hydrogen exchange was then quenched by further dilution of the protein solution into 0.5 M acetic acid in H_2O, and the refolding reaction was then allowed to continue to completion. Samples were buffer exchanged into 40 mM deuterated sodium acetate in D_2O, pH 3.8, and concentrated by ultrafiltration at 4 °C. The time dependence of the proton occupancy of amides during the time course of folding was monitored by ^1H NMR. Data were fitted to the sum of two exponentials using the equation $y = Ae^{(-k_1t)} + Be^{(-k_2t)} + C$, where A and B are the fractional amplitudes of the two phases and k_1 and k_2 are their rate constants. For the zero time point (100% labeling) a solution of lysozyme in the same buffer with the same isotope composition as in the labeling pulse of the refolding experiments was heated to 80 °C for 10 min. This resulted in exchange of all amides to the correct final isotopic composition.

NMR Experiments. Phase-sensitive COSY spectra were recorded at 35 °C on a GE Ω500 spectrometer operating at 500 MHz and processed using FTNMR (Hare Research Inc.) as described elsewhere (Radford et al., 1992). For the pulsed hydrogen-exchange experiments proton occupancies of individual amides were derived from the normalized intensities of $C_\alpha H-NH$ cross peaks in each COSY spectrum and scaled relative to the zero time point sample (Radford et al., 1992). The protection kinetics of the indole NH proton of Trp-111 were measured using the intensity of the well-resolved peak in the 1-D spectrum of the pulse-labeled protein (Radford et al., 1992).

1-D ^1H NMR spectra and 2-D $^{15}N-^1H$ HSQC spectra of the denatured states were recorded at 20 °C and at 600 MHz ^1H frequency on a GE Ω600 spectrometer. The samples used for 1-D spectroscopy contained 20 mg/mL lysozyme pre-deuterated at all exchangeable sites and either 6 M deuterated GdmCl in D_2O or 90% (v/v) DMSO-d_6/10% (v/v) D_2O. Solution conditions for the HSQC spectra were identical to these except that H_2O was used instead of D_2O and the concentration of the fully protonated enzyme was 14 mg/mL. The pH of samples was adjusted to 5.2 by addition of small amounts of NaOD or DCl. Eight thousand one hundred ninety-two complex data points were acquired at a resolution of 0.86 Hz/point for the 1-D experiments, and the data were multiplied by a double-exponential window function and zero-filled once prior to Fourier transformation. 2-D $^{15}N-^1H$ HSQC experiments were recorded as 1024 complex data points at a resolution of 8.8 Hz/point in t_2 and as 256 complex points at a resolution of 9.5 Hz/point in t_1. Solvent was suppressed by presaturation. In the case of the sample in GdmCl, the spectrum was centered on the denaturant resonance and the spectrum was acquired by placing a 1:1 ^1H pulse before the final ^{15}N pulse of the HSQC sequence in order to suppress this peak. Residual solvent signals were removed by deconvolution during data processing prior to application of a Gaussian window function in t_2 and zero filling to give a data matrix of 2048 × 1024 real points. ^1H chemical shifts are referenced to TMS, and those of ^{15}N are referenced externally to $^{15}NH_3$.

Kinetic Analysis. Kinetic parameters were obtained by nonlinear least squares fitting to one or two exponential phases using the program Kaleidagraph (Abelbeck Software, Inc.). The amplitude of each phase in multiexponential processes was normalized to the total measured change between the native and denatured states.

RESULTS

NMR Spectra of Denatured Lysozyme. NMR spectra of denatured lysozyme were obtained under conditions identical to those used in the refolding experiments. The spectra in both 90% (v/v) DMSO and 6 M GdmCl at 20 °C (Figure 2a,b) indicate that in both cases the protein is globally denatured. The chemical shift dispersion of the peaks is substantially reduced relative to that observed in the spectrum of the native protein (Redfield & Dobson, 1988). There are, however, substantial differences in the spectra of the two denatured states reflecting differences in the residual structure present in each (Evans et al., 1991). The aromatic regions of the 1-D spectra of lysozyme in the different denaturants correlate closely with spectra obtained and analyzed previously for the protein in 100% (v/v) DMSO and in 8 M urea (Evans et al., 1991). In particular, the spectra in DMSO exhibit closer agreement with the spectra of the component amino acids (Wüthrich, 1986) than do the spectra in either urea or GdmCl. This is shown particularly clearly by the reduced dispersion of chemical shifts for the protons of the six tryptophan residues, suggesting that the residual nonrandom interactions associated with the involvement of these residues in hydrophobic clusters are significantly reduced in DMSO relative to those in the other denaturants (Dobson et al., 1984; Broadhurst et al., 1991; Evans et al., 1991). In accord with this, the residual chemical shift dispersion for other types of protons is also reduced in the DMSO-denatured state, particularly those of methyl groups whose net upfield shifts in the thermally denatured and urea-denatured states have been attributed to their interactions with aromatic residues (Evans et al., 1991).

Although the 1-D ^1H NMR spectra of the denatured states allow little information to be gained concerning the main chain of the protein, such information is available from observation of the $^1H-^{15}N$ correlations determined in 2-D HSQC experiments. This is exemplified in Figure 2 (c,d) by the well-resolved resonances of the 12 glycine residues of lysozyme. Although the complete assignments of the spectra of the two states are not yet available, it is clear that the pattern of chemical shifts is quite distinct in both the ^1H and ^{15}N dimensions. It is particularly interesting that the spread of ^1H chemical shifts is large (>0.5 ppm), as these shifts are known to be closely related to the conformational preferences of the main chain (Wüthrich, 1986; Wishart et al., 1992). It therefore appears that the extent and nature of residual nonrandom interactions involving the main chain as well as the side chains differ significantly in the two denaturants, in accord with the distinct chemical character of the solvents, and that the protein denatured in DMSO approximates more closely to a fully unstructured state than that present in 6 M GdmCl. Importantly, however, COSY spectra of lysozyme fully refolded from the GdmCl- and

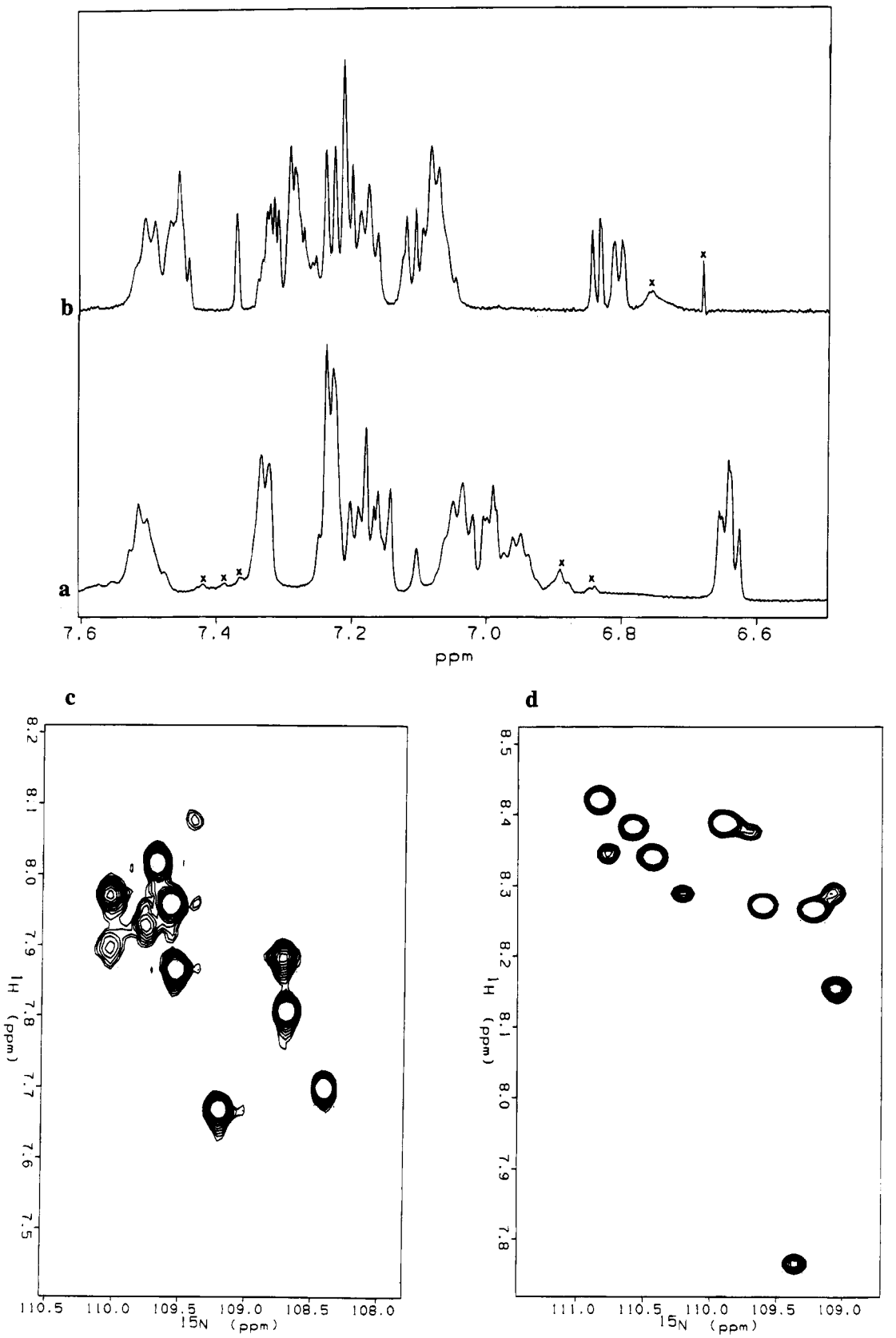

FIGURE 2: NMR spectra of lysozyme denatured in (a) 90% (v/v) DMSO-d_6, and 10% (v/v) D_2O or (b) 6 M deuterated GdmCl in D_2O at 20 °C. The aromatic regions of the 1-D ^{1}H NMR spectra are shown. Resonances labeled with an x arise from amide hydrogens which were not fully exchanged with the solvent in panel a and from residual GdmCl in panel b (see Materials and Methods). Regions of the ^{1}H−^{15}N HSQC spectra of lysozyme denatured in 90% (v/v) DMSO-d_6 and 10% (v/v) H_2O (c) or 6 M GdmCl in H_2O (d) at 20 °C are also shown to emphasize the chemical shift dispersion of the backbone resonances of the 12 glycine residues.

DMSO-denatured states were found to be identical to those of a sample which had not been refolded, confirming that under the experimental conditions used here refolding to the native structure is completely reversible.

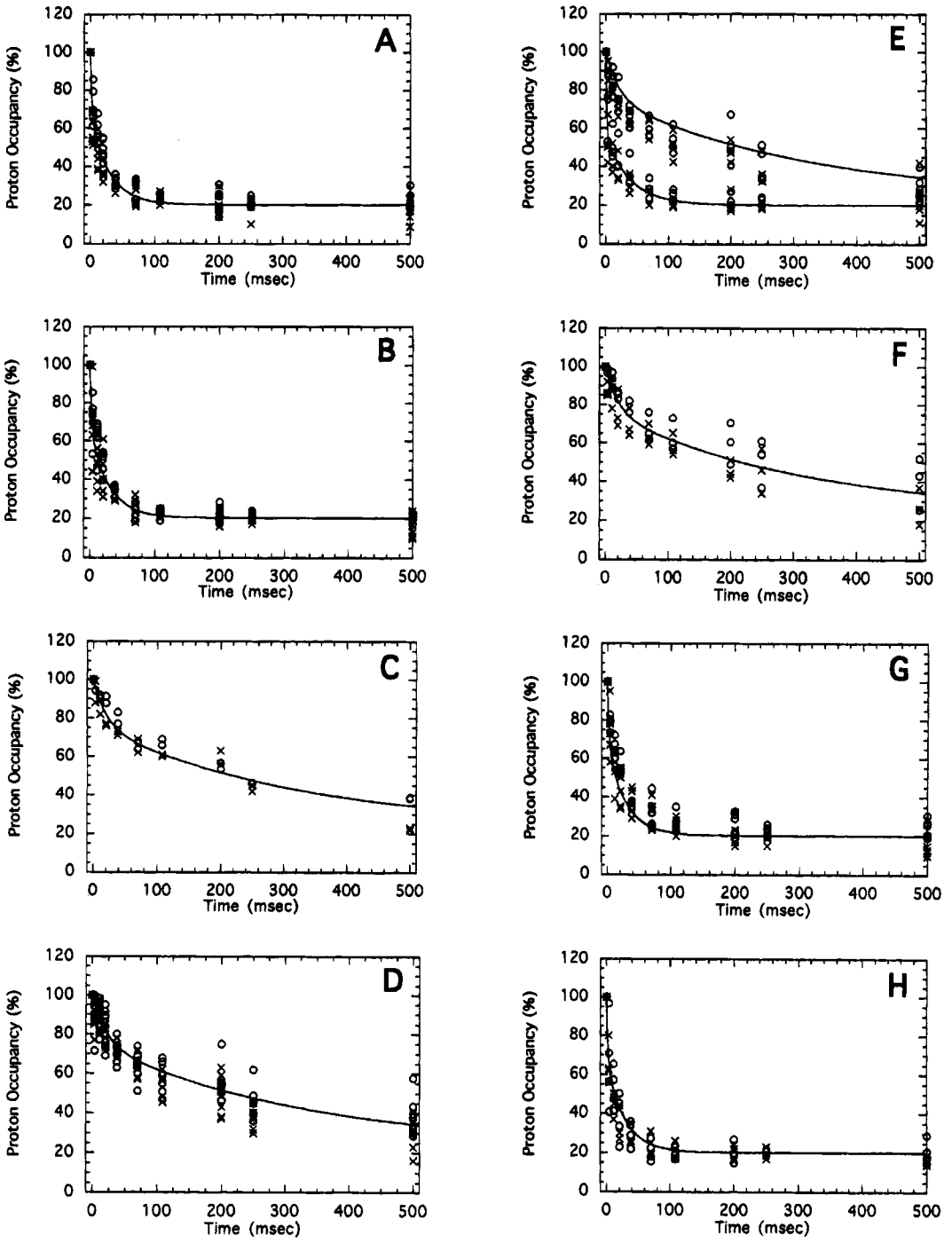

FIGURE 3: Time course for the protection of backbone amides from exchange: (×) refolding from 90% (v/v) DMSO; (○) refolding from 6 M GdmCl. In both experiments the final refolding conditions were identical (20 mM sodium acetate, pH 5.5, 545 mM GdmCl, 8% (v/v) DMSO, 20 °C). Data from the GdmCl- and DMSO-refolding experiments over each secondary structural element in the native protein were combined and fit to the sum of two exponentials. (A) Residues in helix A. (B) Residues in helix B. (C) Residues 38 and 39 in a small β-sheet in the β-domain. (D) Residues in the large β-sheet. (E) Residues within the long loop of the β-domain. One fast-decaying curve is drawn for residues Trp-63, Cys-64, and Ile-78; the other represents the time course of protection for the remainder of residues monitored in the loop region. (F) Residues within the 3_{10}-helix of the β-domain. (G) Residues in helix C. (H) Residues in helix D.

Pulse-Labeling Experiments. Refolding from the GdmCl- and DMSO-denatured states was examined by hydrogen-exchange pulse labeling. In these experiments care was taken to ensure that the final refolding conditions were identical (545 mM GdmCl, 8% (v/v) DMSO, pH 5.5, 20 °C). Labeling curves for 44 amides that are distributed throughout the native structure of lysozyme (Figure 1) were found to give reliable data over the entire time course studied. The protection profiles of these amides, grouped into their location in elements of secondary structure in the native protein, are shown in Figure 3. It is immediately apparent that the protection behaviors of all the amides studied in the two refolding experiments are very similar. The protection profiles of individual amides are also qualitatively very similar to those observed in previous experiments in which the refolding of the GdmCl-denatured state of lysozyme into 20 mM sodium acetate buffer lacking DMSO was studied (Radford et al., 1992). Thus, amides in the α-domain are effectively fully protected from exchange within 100 ms of the initiation of folding, while those in the β-domain are still

Table 1: Kinetics of Exchange Protection for the Refolding of Hen Lysozyme into 8% (v/v) DMSO and 545 mM GdmCl[a]

structural context	no. of probes[b]	fast phase		slow phase	
		amplitude (%)	time constant (ms)	amplitude (%)	time constant (ms)
helix A	6	38 ± 3	2 ± 1	41 ± 3	31 ± 3
helix B	7	28 ± 4	3 ± 1	51 ± 4	30 ± 3
small β-sheet (residues 38 and 39)	2	22 ± 6	22 ± 10	58 ± 6	336 ± 71
large β-sheet	8	22 ± 10	23 ± 8	46 ± 8	281 ± 59
loop	4[c]	27 ± 4	13 ± 4	51 ± 3	263 ± 39
residues 63, 64, and 78	3	44 ± 1	2 ± 1	36 ± 1	39 ± 1
3_{10}-helix in β-domain	2	24 ± 4	23 ± 7	55 ± 4	380 ± 57
helix C	6	31 ± 6	4 ± 1	47 ± 6	37 ± 7
helix D	4	35 ± 4	2 ± 1	45 ± 4	23 ± 3
3_{10}-helix in α-domain	2	39 ± 5	1 ± 1	44 ± 5	25 ± 3
Trp 111 indole	1	43 ±5	<1	38 ±5	30 ±4

[a] Final refolding conditions: 545 mM GdmCl, 8% (v/v) DMSO, and 20 mM sodium acetate, pH 5.5, 20 °C. [b] Measured by pulsed hydrogen–deuterium exchange coupled with NMR. [c] Without residues 63, 64, and 78.

Table 2: Summary of Kinetic Parameters for Refolding of Lysozyme from the DMSO- and GdmCl-Denatured States into Different Conditions[a]

	refolded into buffer lacking DMSO, pH 5.5[b]		refolded into buffer containing 545 mM GdmCl and 8% (v/v) DMSO, pH 5.5			
	GdmCl-denatured state		GdmCl-denatured state		DMSO-denatured state	
experiment	τ (ms)	A (%)	τ (ms)	A (%)	τ (ms)	A (%)
near-UV CD (289 nm)	330 ± 60	100	260 ± 40	100	250 ± 40	100
intrinsic Trp fluorescence						
fast phase	25 ± 3	48 ± 5	19 ± 2	66 ± 5	13 ± 2	67 ± 5
slow phase	340 ± 20	−52 ± 5	273 ± 10	−34 ± 5	192 ± 10	−33 ± 5
MeU-DiNAG	350 ± 50	100	280 ± 10	100	ND	ND
			average of both denatured states			
			A (%)	τ (ms)		
exchange protection						
α-domain (fast)	5 ± 3	48 ± 14	42 ± 6	2 ± 1		
α-domain (slow)	65 ± 25	52 ± 30	57 ± 6	29 ± 8		
β-domain (fast)	11 ± 5	29 ± 8	30 ± 10	20 ± 7		
β-domain (slow)	340 ± 180	71 ± 25	66 ± 9	315 ± 45		

[a] In all of these experiments, with the exception of the intrinsic tryptophan fluorescence intensity, no change in the dead time of the experiment was observed. In the former experiments the amplitudes quoted are normalized to the total change between the initiation of folding and 2 s, by which time folding is virtually complete. A very slow phase of small amplitude (<20%) possibly associated with proline isomerism (Kato et al., 1981) is not considered. In the intrinsic fluorescence experiment the amplitudes of the kinetic phases are expressed relative to the total measured fluorescence change; the magnitude of the dead time change was not quantified in these experiments. Hydrogen exchange labeling data from the GdmCl- and DMSO-denatured states refolded under identical conditions (545 mM GdmCl, 8% (v/v) DMSO) were not significantly different. The data, therefore, were combined, and the kinetic parameters shown are the fits to the combined data. ND, not determined. [b] Taken from Radford et al. (1992) and Itzhaki et al. (1994).

~60% unprotected after this time. At the longest time studied (2 s) about 20% of the proton occupancy at each amide site remains, presumably indicating the presence of a population of very slow folding molecules in both refolding experiments. A slow folding reaction of similar amplitude and rate constant has previously been observed in the kinetic refolding of hen lysozyme from the GdmCl-denatured state which, on the basis of double-jump experiments, was attributed to the presence of *cis* proline isomers in the denatured enzyme (Kato et al., 1981). This might also account, at least in part, for the very slow refolding process observed in the experiments described here.

The data in Figure 3 clearly show that in both refolding experiments residues within a particular element of secondary structure have very similar protection kinetics. Indeed, data for amides in each helix of the α-domain can be adequately fitted to the same function (Table 1). Similarly, a single function is sufficient to account for the protection kinetics of the majority of amides in the β-sheets, the long loop, and the 3_{10} helix in the β-domain. Exceptions to this are the residues Trp-63, Cys-64, and Ile-78, which lie in the long loop in the β-domain (Table 1). The amide hydrogens of these residues are more rapidly protected than the remaining amides in the β-domain, and it is interesting to note that this behavior is observed in both refolding experiments described here as well as in our previous refolding experiments from the GdmCl-denatured state in the absence of 8% (v/v) DMSO (Radford et al., 1992). All the labeling curves are found to fit well to a sum of two exponentials; a single-exponential decay does not describe the data adequately. There are no significant differences in the kinetic parameters for either the fast or slow phases in the two refolding experiments. The data from the two experiments for each secondary structural element, therefore, were combined and refitted. A summary of the resulting amplitudes and time constants for the protection kinetics of the individual secondary structural elements (ignoring the ~20% very slow phase, which is not observed by other spectroscopic techniques) is presented in Table 2.

The qualitative difference in the protection behavior of amides in the α- and β-domains in both refolding experiments manifests itself in their relative time constants and

amplitudes. The slow phase of protection of amides in the α-domain is characterized by a mean time constant and amplitude of 29 ± 8 ms and 57 ± 6%. By contrast, amides in the β-domain are protected much more slowly with a mean time constant for the slow phase of 315 ± 45 ms, the mean amplitude of this phase being 66 ± 9%. The difference in the rates of protection of the two domains is further exemplified in the fast phases of protection. In this case, protection of amides in the α-domain occurs with a mean time constant and amplitude of 2 ± 1 ms and 42 ± 6%, respectively. This is significantly faster than the corresponding mean time constant of protection of amides in the β-domain in this phase (time constant (τ) = 20 ± 7 ms), the mean amplitude of this phase being slightly smaller (30 ± 10%) than that of amides in the α-domain.

The occurrence of biphasic protection kinetics of amides in our previous refolding studies from the GdmCl-denatured state of lysozyme was shown to reflect the existence of parallel folding pathways (Radford et al., 1992). To determine whether this is also the case in the refolding studies described here, the pH of the labeling pulse was varied from 9.3 to 10.4 at two different refolding times (3.5 and 20 ms) after the initiation of folding. The proton occupancies of virtually all of the amides studied in the α- and β-domains show no significant dependence (<10%) on the pH of the labeling pulse at both refolding times studied and in both refolding experiments. This suggests that under these conditions, as in those studied previously (Radford et al., 1992), there are distinct populations of folding molecules and that parallel pathways exist in all of the refolding experiments. Moreover, these experiments reinforce the view that the refolding pathways from the GdmCl- and DMSO-denatured states are indistinguishable, at least as detected by hydrogen-exchange pulse-labeling methods, and that the origin of the multiplicity of folding pathways is unlikely to arise as a consequence of residual noncovalent interactions in the denatured state.

The protection from exchange of the indole hydrogen of Trp-111 serves as an additional probe for the detection of a solvent inaccessible and/or hydrogen-bonded environment around this side chain during refolding (Radford et al., 1992). The protection kinetics of Trp-111 are at least as fast as, and possibly faster than, those of amides in the α-domain (Table 1). In the native enzyme Trp-111 is buried within the α-domain. The backbone amide is located in the D-helix, and the indole hydrogen forms a hydrogen bond with the side-chain oxygen of Asn-27, which is located in the B-helix (Blake et al., 1967; Imoto et al., 1974; Grace, 1980). Thus, if protection of Trp-111 during refolding arises as a consequence of a native-like interaction with Asn-27, protection of Trp-111 may be used as a probe for the formation of a persistent interaction between the B- and D-helices early in folding. It is possible, however, that the protection of this residue arises, at least in part, from placement of the indole moiety in a hydrophobic environment. In accord with this, significant sequestering of tryptophan residues from solvent is seen on this time scale (Itzhaki et al., 1994). Whatever the origin of protection, however, the finding supports the notion that formation of persistent secondary structural elements in the α-domain is associated with the formation of a hydrophobic core and with the stabilization of at least some tertiary interactions.

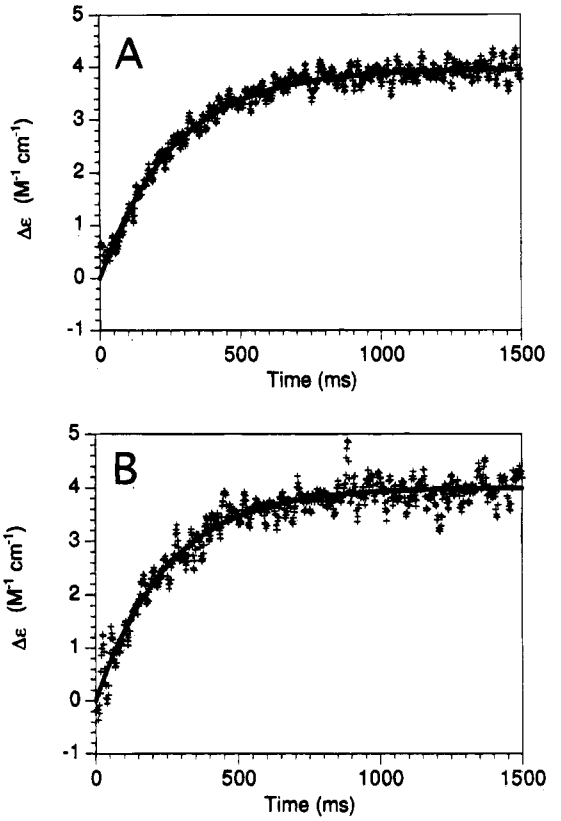

FIGURE 4: Kinetic progress curves of folding as monitored by stopped-flow CD at 289 nm. (A) Refolding from 6 M GdmCl. (B) Refolding from 90% (v/v) DMSO. The data have been fitted to a single-exponential function. The refolding conditions were 8% (v/v) DMSO, 545 mM GdmCl, and 20 mM sodium acetate, pH 5.5, 20 °C.

CD- and Fluorescence-Detected Refolding Kinetics. Stopped-flow CD experiments in the near-UV region at 289 nm were carried out to complement the pulse-labeling data as an independent means of monitoring the rate of formation of tertiary interactions during the folding process. In each experiment the initial and final folding conditions were identical to those used in the pulse-labeling experiments. The kinetics of folding from the two denatured states measured by near UV-CD are shown in Figure 4. In agreement with the pulse-labeling data, the rates of folding indicated by this technique are also indistinguishable. The curves were fitted to single-exponential functions which yielded time constants of 260 ± 40 and 250 ± 40 ms for the refolding experiments from the GdmCl- and DMSO-denatured states, respectively (Table 2). In each case, the time constants resemble those of the slow phase of protection of amides in the β-domain and there is no evidence for a fast phase or a change in the ellipticity in the dead time of the experiment. As shown in our previous studies (Itzhaki et al., 1994), this phase corresponds to the formation of the fully native enzyme and provides further evidence that the folding processes from the GdmCl- and DMSO-denatured states are indistinguishable.

Due to the high absorbance of DMSO it was not possible to monitor the development of secondary structure during folding directly by kinetic CD experiments in the far-UV. As a means of following very early folding events, therefore, the refolding process from the two denatured states was monitored under conditions identical to those used above (545 mM GdmCl, 8% (v/v) DMSO, pH 5.5, 20 °C) by

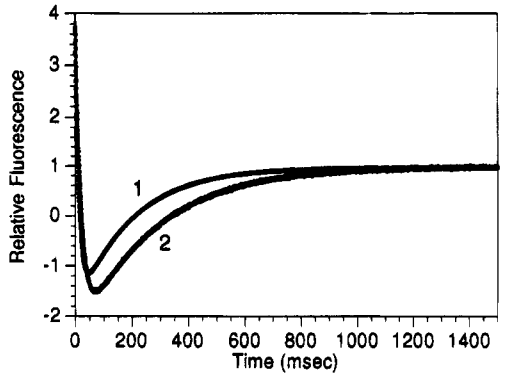

FIGURE 5: Time course of refolding of lysozyme in 545 mM GdmCl, 8% (v/v) DMSO, and 20 mM sodium acetate, pH 5.5, 20 °C monitored by intrinsic tryptophan fluorescence. (1) Refolding from the denatured state in 90% (v/v) DMSO. (2) Refolding from the denatured state in 6 M GdmCl. The fluorescence intensity is normalized to that of the native protein under the same conditions, which is given a value of 1.0.

measuring the change in intrinsic fluorescence of tryptophan residues in stopped-flow fluorescence experiments. The profile for each refolding experiment is shown in Figure 5. The overall fluorescence changes observed during folding are very similar; in each case two measurable kinetic phases are observed. First, a substantial decrease in fluorescence intensity occurs so that by *ca.* 100 ms the intensity reaches a minimum at a value substantially less than that of the native enzyme. In the second phase the fluorescence intensity increases again to the value of the native protein. These two kinetic phases have previously been characterized in the refolding of lysozyme from 6 M GdmCl into the same buffer, but lacking DMSO (Itzhaki et al., 1994). The fast kinetic event has been attributed to the development of non-native intramolecular quenching mechanisms in at least one of the folding intermediates populated at that time (Itzhaki et al., 1994). It is intriguing to note, however, that although the overall fluorescence profiles in the refolding experiments from the GdmCl- and DMSO-denatured states are very similar, small differences in the rate constants of the measurable kinetic phases are apparent (Figure 5). The fluorescence profiles were fitted to the sum of two exponentials, and the resulting time constants and amplitudes are shown in Table 2. By this analysis the rates of both the fast and slow phases appear to be significantly faster in the refolding experiment from the DMSO-denatured state. In order to establish that these differences were significant a detailed series of experiments using the model compound dinitrophenylacetate (DNPA) were carried out (see Materials and Methods). These experiments clearly showed that the dead times of the two experiments are indistinguishable (~4 ms) and that the different kinetic profiles cannot be attributed to mixing artifacts. The complexity of the behavior of the signal arising from six tryptophan residues means, however, that it is difficult to interpret the origin of the kinetic phases at a molecular level.

In our previous studies (Itzhaki et al., 1994) binding of a fluorescent inhibitor, 4-methylumbelliferyl *N,N'*-diacetyl-β-chitobiose (MeU-diNAG), was used as a probe of the formation of the native enzyme through its binding to the active site cleft which lies between the α- and β-domains. Binding results in an increase in the fluorescence of MeU-diNAG that can easily be monitored in a stopped-flow experiment in the absence of interference from the intrinsic fluorescence of the protein (Yang & Hamaguchi, 1980). The rate of formation of the native protein, therefore, was measured by this approach under refolding conditions identical to those used in the experiments described above (545 mM GdmCl, 8% (v/v) DMSO, 20 mM sodium acetate, pH 5.5, 20 °C). In the refolding experiment from the GdmCl-denatured protein a curve which fitted well to a single exponential with time constant 280 ± 10 ms was obtained. This is similar to the rate of development of the native-like near-UV CD signal and to the rate of protection of amides in the β-domain in the majority of molecules, and it is consistent with the view that formation of the fully native protein occurs on this time scale. It proved not to be possible, however, to perform this experiment to monitor refolding from the DMSO-denatured state, possibly because the high concentration of DMSO in the denatured protein interferes with the binding of the inhibitor to the active site cleft.

Refolding under Different Final Conditions. The kinetic parameters described above are compared in Table 2 with those obtained previously (Radford et al., 1992; Itzhaki et al., 1994) from experiments in which the GdmCl-denatured state of lysozyme was refolded at pH 5.5 and 20 °C into 20 mM sodium acetate, lacking DMSO. In this manner it is possible to examine the influence of the 8% (v/v) DMSO in the refolding buffer on the characteristic features and kinetic parameters of the folding process. As judged by the kinetics of binding of the fluorescent inhibitor MeU-diNAG during folding, the presence of 8% (v/v) DMSO has little effect on the rate of formation of the enzyme active site and presumably also, therefore, of the native enzyme. This is consistent with the observation that such low concentrations of DMSO have only a minor effect on the structure and stability of the native protein (Dobson et al., 1984). In accord with this, the formation of fixed tertiary interactions involving tryptophan residues as measured by kinetic CD experiments in the near-UV, the slow phase of protection of amides in the β-domain, and possibly also the slow phase in the burial of tryptophan residues as monitored by their intrinsic fluorescence are completed on this time scale. It is intriguing to note, however, that the kinetics of folding as judged by these techniques are consistently slightly faster in the refolding experiments in the presence of DMSO. Given the errors involved in these experiments, however, the significance of this observation is difficult to judge. By contrast with the small increase in overall folding kinetics, the rate of protection of amides in the α-domain is clearly substantially faster when the protein is refolded into buffer containing 8% (v/v) DMSO. In this experiment complete protection of all amides in the α-domain occurs within about 100 ms. By contrast, this process takes about 200 ms when refolding occurs into buffer lacking DMSO. Despite this, the amplitudes of the different kinetic phases are not changed in presence of 8% (v/v) DMSO.

DISCUSSION

It has become increasingly apparent in recent years that significant hydrophobic interactions persist in the denatured states of many proteins (Evans et al., 1991; Neri et al., 1992; Shortle, 1993; Wüthrich, 1994). An important question, therefore, is the role that such interactions play in determining pathways of protein folding. One of the most striking results presented in this paper is the observation that the lysozyme

folding process is not influenced significantly by the initial denaturation conditions, even though the two denatured states studied, formed in 90% (v/v) DMSO and 6 M GdmCl, differ significantly in their residual structure (Figure 2). This indicates that the independent nature of the two folding domains, including the observation that the α-domain may fold to a protected state in the absence of a stable structured β-domain, does not arise as a consequence of residual interactions in the denatured state since the extent of these interactions is very different in the two denatured states studied here. In addition, the observed double-exponential protection kinetics of all amides in both folding experiments, coupled with the lack of a pH dependence in the extent of labeling, suggests that parallel routes occur in the folding pathways from both denatured states. This is also an important result in that it suggests that the origin of multiple pathways does not arise from the conformational properties of the denatured protein, although a contribution from the presence of the four disulfide bonds cannot be ruled out. Finally, given the very different chemical and physical properties of DMSO and GdmCl, the similar folding kinetics from the different denaturants indicates that diffusion of the denaturant from the collapsing polypeptide chain appears not to play an important role in determining the early events in the folding pathway.

If the presence of residual interactions within the denatured state is not the origin of the multiple pathways and other complexities observed in the refolding behavior of lysozyme, then the explanation presumably must lie in the events that occur after the initiation of the folding process. One possibility is that the rapid collapse of the protein in the first few milliseconds of folding leads to a range of states which, on average, have a native-like content of secondary structure (Radford et al., 1992; Chaffotte et al., 1992), but only some of which are readily amenable to folding (Radford et al., 1992). Direct evidence in support of this notion was obtained in experiments in which the folding pathway of lysozyme was monitored by hydrogen-exchange labeling detected by mass spectrometry (Miranker et al., 1993). These experiments demonstrated that folding branches into parallel paths before amides are significantly protected from hydrogen exchange. In other words, within the ensemble of conformations resulting from the collapse there are some molecules that are able to proceed with folding more rapidly than others. One explanation of particular interest is that this is associated with different topological features of the collapsed state, a population of which may be able to fold efficiently without rearrangement, while others may need first to rearrange, potentially a slow process in a partially folded state, particularly in the presence of disulfide bonds (Dobson et al., 1994). On the basis of this model, and consistent with previous data (Radford et al., 1992; Miranker et al., 1993; Itzhaki et al., 1994), about 30% of molecules (the population observed to fold rapidly to a native-like state) would form the correct topology in the collapsed state in both folding domains, while the remainder require rearrangement in one or both domains prior to formation of the native state (Radford & Dobson, 1994).

Support for such a suggestion comes from a number of sources. Conformational restraints in the denatured state have been suggested to account for the complexity of the folding of other proteins such as ribonuclease T_1 (Kiefhaber et al., 1992), ribonuclease A (Udgaonkar & Baldwin 1988, 1990), and cytochrome *c* (Roder et al., 1988). In these cases, however, slow steps in folding can be associated with the existence of specific non-native interactions within the denatured protein associated with incorrect proline isomers or non-native ligation of the covalently bound heme. In the latter case, however, very fast folding of the protein ($\tau < 1$ ms) has been found to occur under conditions where denaturation results in a stable molten globule with the correct ligands to the heme (Sosnick et al., 1994). It is likely that under these conditions the protein has the appropriate topological state to fold efficiently and is analogous to the fast-folding population of molecules within the early stages of lysozyme folding. Additional evidence that non-native topologies can indeed form during refolding has come from the detection of a wrongly folded but globular state of insulin-like growth factor trapped during oxidative refolding (Miller et al., 1993).

The fundamental properties of the lysozyme folding pathway do not appear to depend significantly on the details of the protein sequence. Thus, for example, human lysozyme, which differs from hen lysozyme in 40% of its sequence, also shows fast and slow folding events which arise from multiple folding pathways (Hooke et al., 1994). In addition, the presence of disulfide bonds does not seem to be a fundamentally important factor in the folding behavior, at least as judged by a derivative of hen lysozyme lacking a single disulfide bond linking the N- and C-terminal regions of the polypeptide chain (Eyles et al., 1994; Denton et al., 1994). Both of these proteins, however, differ from wild-type hen lysozyme in the nature and kinetics of subsequent steps in folding, indicating that these interactions are most important in the later stages of folding which involve the formation of persistent structure.

In view of the overwhelming similarities in the folding pathways of lysozyme from the GdmCl- and DMSO-denatured states, small but surprising differences in the rate of burial of tryptophan residues, presumably in hydrophobic structure, monitored by the change in intrinsic tryptophan fluorescence, are observed during the two folding processes. As judged by this technique, the burial of tryptophan residues from bulk solvent appears to be more rapid in the refolding experiments from the DMSO-denatured state, although the overall fluorescence profiles are similar in accord with the view that the pathways are not fundamentally different. The molecular origin of this result is difficult to decipher, however, due to the large number of factors that can contribute to the observed fluorescence intensity and the fact that the identity of the fluorophores in the partially folded molecules is not known. It is consistent, however, with the view that an ensemble of conformations forms when the molecule collapses within the first 2 ms of folding and that there could be subtle differences in the distribution of early intermediates obtained when the two denatured states collapse. This could give rise to the differences in the intrinsic fluorescence kinetics observed and possibly also to minor changes in the overall folding kinetics that are at, or beyond, the limits of detection.

By contrast with the small effects of changing the initial denaturant, we have found, in agreement with earlier data (Kato et al., 1981), that the properties of the folding pathway are more highly dependent on the final folding conditions. Thus, the presence of 8% (v/v) DMSO in the refolding buffer appears to accelerate *ca.* 2-fold the rate of protection of

amides in the α-domain, in comparison with folding experiments into the same buffer but lacking DMSO (Table 2). Despite this, the rate of formation of the native enzyme as judged by the binding of MeU-diNAG, the immobilization of aromatic residues in the hydrophobic core, and the rate of protection of amides in the β-domain in the slow phaseare not substantially perturbed by the presence of 8% (v/v) DMSO in the refolding buffer.

If the slow step in the formation of the fully protected α-domain is associated with the need to reorganize incorrectly folded members of the collapsed ensemble, the enhancement of the rate of this process in the presence of DMSO could be associated with the reduction in stability of such species or lowering of the barrier to their interconversion, facilitating a more rapid rearrangement to an appropriate conformation for the next step in folding. It could also result from a change in the distribution of species in the collapsed state, if this increased the number of molecules populating the fast folding pathways. Interestingly, these experiments emphasize the independence of the steps forming the α- and β-folding domains of lysozyme, since the overall rate of acquisition of the native structure is not influenced substantially by 8% (v/v) DMSO despite the more rapid formation of the α-domain in low concentrations of the organic solvent. The acquisition of the native state appears, therefore, to be limited by the formation of the β-domain and not the α-domain. It is tempting to speculate that this is associated, at least in part, with the relative complexity of forming correct interactions within β-sheet regions, which has been observed experimentally in the folding of interleukin 1-β (Varley et al., 1993) in a 20-residue synthetic peptide from the β-sheet region of lysozyme (Yang et al., 1994) as well as in predications of early events in folding (Dill, 1990; Yapa et al., 1992; Chelvanayagam et al., 1992; Rooman & Wodak, 1993). It is also the case, however, that there are two disulfides in this region of the structure. Further experiments to examine the effect of a range of refolding conditions may reveal details of the identity, stability, and kinetic barriers of interconversion of individual partially folded states in the lysozyme folding pathway.

Taken together, the studies described here have demonstrated that the folding pathways of lysozyme from the DMSO- and GdmCl-denatured states are virtually indistinguishable. The complexity of folding, including the existence of distinct partially folded states and alternate folding routes, therefore, does not appear to result solely from the residual noncovalent interactions in the initial denatured state. The results have shown that the detailed kinetics of the folding process are, however, intimately related to the conditions under which refolding takes place. This adds a cautionary note when the results of experiments performed under different conditions are compared but also provides an opportunity to dissect further the nature and stability of transient folding intermediates. In the light of the present results we have speculated on possible molecular details of the very early events in the folding pathway of lysozyme and on the nature of the dominant forces that drive the folding molecules to their native three-dimensional structures. In particular, the results are consistent with the proposition that the nature and stability of the different populations in the collapsed state of a protein can have a profound influence on the kinetics and the molecular details of the events occurring during folding.

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