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Stopped-flow NMR spectroscopy: Real-time unfolding studies of 6-^{19}F-tryptophan-labeled *Escherichia coli* dihydrofolate reductase

(unfolding intermediate/fluorescence/circular dichroism)

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ABSTRACT **_Escherichia coli_ dihydrofolate reductase (DHFR; EC 1.5.1.3) contains five tryptophan residues that have been replaced with 6-^{19}F-tryptophan. The ^{19}F NMR assignments are known in the native, unliganded form and the unfolded form. We have used these assignments with stopped-flow ^{19}F NMR spectroscopy to investigate the behavior of specific regions of the protein in real time during urea-induced unfolding. The NMR data show that within 1.5 sec most of the intensities of the native ^{19}F resonances of the protein are lost but only a fraction (≈20%) of the intensities of the unfolded resonances appears. We postulate that the early disappearance of the native resonances indicates that most of the protein rapidly forms an intermediate in which the side chains have considerable mobility. Stopped-flow far-UV circular dichroism measurements indicate that this intermediate retains native-like secondary structure. Eighty percent of the intensities of the NMR resonances assigned to the individual tryptophans in the unfolded state appear with similar rate constants ($k \approx 0.14$ sec^{-1}), consistent with the major phase of unfolding observed by stopped-flow circular dichroism (representing 80% of total amplitude). These data imply that after formation of the intermediate, which appears to represent an expanded structural form, all regions of the protein unfold at the same rate. Stopped-flow measurements of the fluorescence and circular dichroism changes associated with the urea-induced unfolding show a fast phase (half-time of about 1 sec) representing 20% of the total amplitude in addition to the slow phase mentioned above. The NMR data show that ≈20% of the total intensity for each of the unfolded tryptophan resonances is present at 1.5 sec, indicating that these two phases may represent the complete unfolding of the two different populations of the native protein.**

The mechanism by which a protein unfolds from or folds to its correct tertiary structure remains one of the key unanswered questions in biochemistry. Common experimental approaches measure rates of unfolding or refolding by monitoring changes in intrinsic fluorescence, absorbance, or circular dichroism. Unfolding that occurs in a single phase (1, 2) has been interpreted as indicating that the unfolding of a protein represents a single process and that observable unfolding intermediates do not exist. When multiple phases are observed, they may represent either separate pathways or the formation of intermediates on a single pathway. Fluorescence, absorbance, or circular dichroism measurements do not readily distinguish between these mechanisms; if intermediates exist, these techniques cannot provide specific information about the structure of such intermediates.

A number of studies have identified specific regions of secondary structure formed during the folding process by using hydrogen–deuterium exchange combined with multidimensional proton NMR spectroscopy (reviewed in refs. 3–5). This technique, by monitoring rates of amide-proton exchange, provides detailed and specific information about the behavior of the backbone of the protein and the formation of secondary structure but does not, in general, monitor side-chain environment. Thus, structural information about the formation of specific regions of tertiary structure during the folding process is still limited. In addition, few studies have addressed the structural changes which occur during unfolding.

Escherichia coli dihydrofolate reductase (DHFR; EC 1.5.1.3) is a monomer of 159 amino acids and molecular weight 17,680 which catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Its small size, well-characterized enzyme mechanism (6, 7), and well-refined structure (8–10), as well as the reversibility of its folding reaction in the presence of chemical denaturants (11, 12), make this protein a good model for protein-folding studies. *E. coli* DHFR contains five tryptophan residues distributed throughout its structure. We have previously prepared 6-^{19}F-tryptophan labeled *E. coli* DHFR, assigned the resonances observed in the ^{19}F spectrum of this protein to individual tryptophans, and studied its behavior at equilibrium in the presence of chemical denaturant (13).

In this paper, we monitor the real-time changes in the NMR spectrum of 6-^{19}F-tryptophan labeled *E. coli* DHFR to study the behavior of side chains during the urea-induced unfolding process. To accomplish this, we have used a stopped-flow device incorporated into the NMR spectrometer (14). To overcome the problem of spectral overlap, we have introduced a specific label and used ^{19}F rather than ^{1}H NMR spectroscopy.

We show that the rapid and slow phases observed by fluorescence and circular dichroism spectroscopy represent complete unfolding of 20% and 80% of the protein, indicating separate unfolding pathways for the different forms of the native protein. The slow phase of unfolding involves the formation of an intermediate which retains native-like secondary structure, but in which the side chains possess considerable mobility. Following the formation of this intermediate, all regions of the protein unfold at equal rates.

MATERIALS AND METHODS

Materials. Ultrapure urea was purchased from United States Biochemical. Concentrated stocks were prepared, deionized with 1 g of AG 501-X8 mixed bed resin (Bio-Rad) per 150 g of urea, filtered through a 0.2-μm filter, and stored in aliquots at −70°C until the day of use. The concentration of urea was determined by refractive index at 25°C by using quantitative relationships between concentration and refractive index (15). Methotrexate, 6-^{19}F-tryptophan, folate, and methotrexate agarose were obtained from Sigma. Fast-flow DEAE Sepharose was obtained from Pharmacia. All other chemicals were reagent grade.

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Abbreviations: DHFR, dihydrofolate reductase; DTT, dithiothreitol.
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Protein Labeling and Purification. DHFR used in stopped-flow NMR studies was purified from 500-ml cultures of *E. coli* strain W3110 *trpA33* containing the plasmid pMONDHFR as described (13). This protein was ≈40% labeled with 6-^{19}F-tryptophan. DHFR used in stopped-flow fluorescence and circular dichroism experiments was purified from 500-ml cultures of the same auxotroph containing the plasmid pTrc99DHFR, constructed by inserting the *folA* gene from plasmid pTY1 into the plasmid pTrc 99 A (Pharmacia). This protein was >90% labeled with 6-^{19}F-tryptophan. A substoichiometric amount of tightly bound folate remained after elution from the DEAE column and was removed by denaturing the enzyme in 5 M urea followed by diafiltration against 5 M urea in an Amicon stirred cell equipped with a YM3 membrane. The protein was renatured by a 4-fold dilution followed by diafiltration against buffer containing 62.5 mM potassium phosphate (pH 7.2), 125 mM KCl, 0.125 mM EDTA, 1.25 mM dithiothreitol (DTT), and 6.25 mM NaN_3. Protein was stored in this buffer until the day prior to use, then dialyzed or diafiltered into the buffer used in the experiment. The enzyme concentration was determined by active-site titration of intrinsic fluorescence by using methotrexate, which forms a 1:1 complex. The enzyme concentration determined by this procedure was within 5% of that determined by absorbance (16).

For fluorescence and circular dichroism experiments, the protein was dialyzed against a buffer containing 50 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, and 1 mM DTT and diluted to the desired enzyme concentration. For NMR experiments, the protein was exchanged into buffer containing 62.5 mM potassium phosphate (pH 7.2), 0.125 mM EDTA, 3.75 mM DTT, and 6.25 mM NaN_3 by diafiltration, concentrated through a YM3 membrane, and diluted with ^2H_2O and 20 mM 4-^{19}F-phenylalanine to achieve a final buffer concentration of 50 mM potassium phosphate/0.1 mM EDTA/3 mM DTT/5 mM NaN_3/15% ^2H_2O/1 mM 4-^{19}F-phenylalanine. No correction to pH was made for ^2H_2O content.

Unfolding Studies. Stopped-flow fluorescence studies were performed by using an Applied Photophysics (Surrey, U.K.) spectrophotometer in the fluorescence mode with a pathlength of 0.2 cm. Drive syringes of 1 ml and 2.5 ml were used, resulting in a 3.5-fold dilution of the protein solution. A total of 500 data points were collected in the first 5 sec, and 500 additional points were collected over the next 250 sec. An excitation wavelength of 290 nm was used, and fluorescence was observed by using a 305-nm cutoff filter. Data from three individual injections were averaged and analyzed. Averages of data from different injections collected on different days produced comparable rates.

The time course of circular dichroism changes was measured by using an Applied Photophysics RX1000 rapid-kinetics accessory equipped with a 0.2-cm pathlength stopped-flow cell and fitted to a Jasco (Easton, MD) J600 circular dichroism spectrophotometer. Drive syringes of 1 ml and 2.5 ml were used, resulting in a 3.5-fold dilution of the protein solution. A single time base was used with 1200 data points collected at 0.2-sec intervals. Data from five individual injections were averaged and analyzed. Averages of data from injections collected on different days produced comparable rates.

Stopped-Flow NMR Spectroscopy. An Applied Photophysics RX1000 rapid-kinetics spectrometer accessory was adapted for use essentially as described (14). The NMR tube previously described was modified to increase the linear velocity obtained with viscous solutions, thus maintaining turbulent flow at higher viscosities. This modification improved the mixing and minimized the effective volume necessary to clear reacted material from the cell. Details of this modification are available upon request. Approximately 0.7 ml must be delivered to completely displace the old solution from the coil of the probe. The dead time of the apparatus was estimated to be 100 msec.

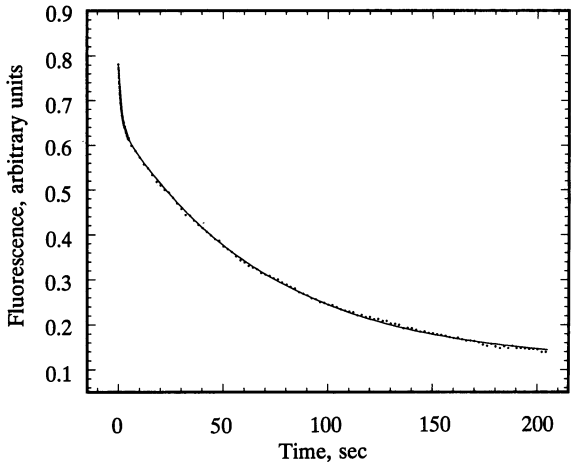

FIG. 1. Fluorescence changes observed on unfolding 6-^{19}F-tryptophan-labeled DHFR from 0 to 5 M urea at 22°C. The final protein concentration was 5 μM. The buffer contained 50 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, and 1 mM DTT. Other experimental conditions are described in *Materials and Methods.* The data (●) were fit to a two exponential function to yield the rates and amplitudes reported in Table 1. A three exponential function did not significantly improve the fit.

NMR data were collected on a Varian VXR-500 spectrometer operating at 470.3 MHz for ^{19}F with a Nalorac Proton/Fluorine probe. All spectra were referenced to an internal standard of 4-^{19}F-phenylalanine. All chemical shifts are given relative to external trifluoroacetic acid. Data were acquired by using a modification of the Varian VNMR s2pul pulse sequence. This modified sequence delays acquisition for a user-defined interval following an external trigger. After an initial delay of 1.5 sec, 30 transients were collected at 1.03-sec intervals; 20 transients were collected at 3.2-sec intervals; and 10 transients were collected at 15 sec intervals. Data were acquired for 0.503 sec by using a 90° pulse and a spectral width of 5999 Hz. Recycle times of less than four times the longest spin-lattice relaxation time (t_1), as previously determined (13), were corrected for t_1, assuming that $t_1 >> t_2$.

The resulting arrayed free-induction decays were summed by using a *C*-shell macro which increments through the number of injections for each time interval, making use of the VNMR addfid macro as described (14).

Data Analysis. Exponential fits were obtained by using the program KALEIDAGRAPH (Synergy Software, Reading, PA).

RESULTS

Fig. 1 shows a stopped-flow experiment measuring the change in intrinsic fluorescence upon denaturation of 6-^{19}F-tryptophan-labeled DHFR by 5 M urea. The rates obtained are similar to those observed for wild-type unlabeled DHFR under identical conditions (unpublished observation).† In the experiment shown, two phases with half-times of ≈1 and 50 sec were observed, indicating the presence of at least two processes.

To characterize the extent of secondary structure present, we examined the unfolding of DHFR by stopped-flow circular dichroism at 222 nm under identical buffer and denaturant conditions (Fig. 2). The rates obtained are similar to those observed for wild-type unlabeled DHFR under identical conditions (unpublished observation) and comparable with previously published rates for unfolding (17) under slightly dif-

†In contrast to the wild-type, unlabeled protein, the fluorescence of 6-^{19}F-tryptophan-labeled protein decreases on unfolding.

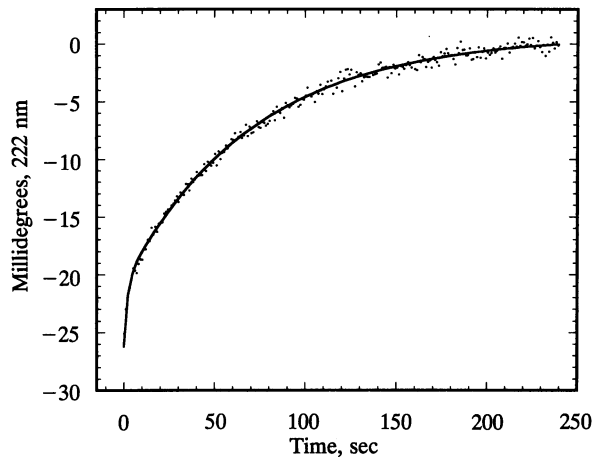

FIG. 2. Changes in circular dichroism at 222 nm observed on unfolding of 6-^{19}F-tryptophan-labeled DHFR from 0 to 5 M urea at 22°C. Final protein concentration was 13 μM. The buffer contained 50 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, and 1 mM DTT. Other experimental conditions are described in *Materials and Methods*. The data (●) were fit to a two exponential function to yield the rates and amplitudes reported in Table 1. A single exponential function did not fit the first 25 points.

ferent conditions, except that the slowest phase was not observed; unfolding was essentially complete at 240 sec. If additional phases are present, they represent a minor portion of the total amplitude. The data shown in Fig. 2 also indicate that ≈20% of secondary structure is lost with a half-time of 1.4 sec (similar to the fast fluorescence phase). The remaining 80% is lost with a half-time of about 50 sec (identical to the slow fluorescence phase). Since these rates reflect global properties, it is not possible to distinguish between loss of some secondary structure in all of the protein or loss of most secondary structure in part of the protein.

We have previously examined the ^{19}F NMR spectra of native and unfolded 6-^{19}F-tryptophan-labeled DHFR and assigned the five resonances observed in the native state and the four resonances observed in the unfolded state to the five tryptophan residues in *E. coli* DHFR (13). Because the native and unfolded resonances have distinct chemical shifts, we can use these assignments to investigate the structural basis of the rates observed by fluorescence and circular dichroism spectroscopy by examining the behavior of specific tryptophans (and thus, specific regions of the protein) during unfolding. The viscosity of the urea solution does not appear to have a significant effect on the rotational correlation time of the native protein since at 5 M urea in the ternary complex with methotrexate and NADPH, considerable native protein is present and the native resonances have linewidths similar to those observed in the absence of urea.

Fig. 3 shows the spectra obtained at 5 of the 60 time points obtained by using stopped-flow NMR spectroscopy to examine the unfolding of 6-^{19}F-tryptophan-labeled DHFR in 5 M urea. Three observations are immediately apparent. First, most of intensities of the native, apoDHFR resonances (shown in the bottom spectrum) are not observable in the first time point (at 1.5 sec). Second, only about 20% of the intensities of the unfolded resonances appear at this time. Third, the majority (≈80%) of the intensities of the unfolded resonances appear at a much slower rate. Small peaks may be present at the chemical shifts of tryptophans 74, 47, and 133 during the first minute of the unfolding time course, but the low signal-to-noise ratio (less than 2:1) makes their presence uncertain; accurate quantitation is not possible with the current data.

These results are illustrated by examining a plot of the intensity of the unfolded resonance specifically assigned to

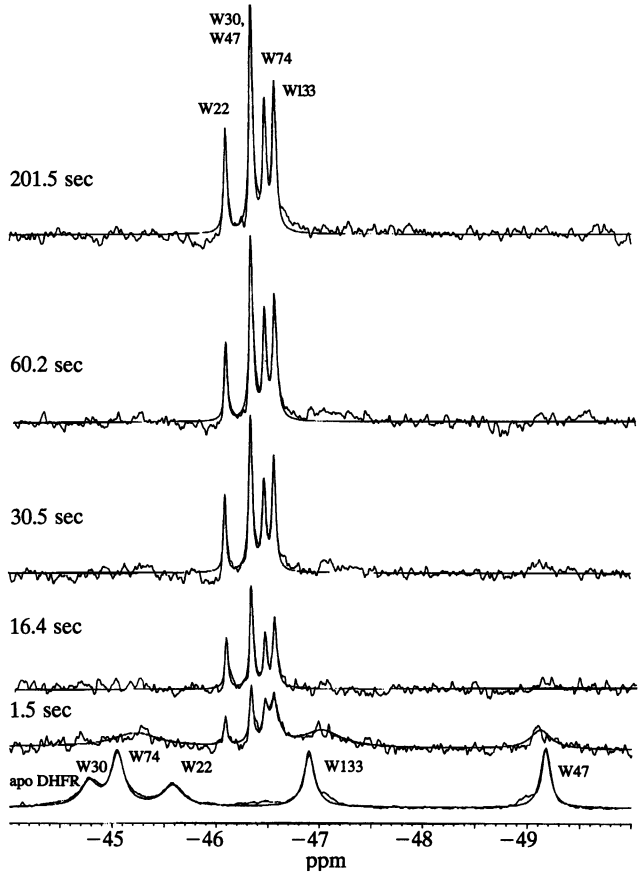

FIG. 3. Stopped-flow ^{19}F NMR spectra of unfolding of 6-^{19}F-tryptophan-labeled DHFR from 0 to 5 M urea at 22°C. Final protein concentration was 0.89 mM DHFR. The buffer contained 50 mM potassium phosphate (pH 7.2), 1 mM EDTA, 5 mM NaN_3, and 3 mM DTT. Other experimental conditions are described in *Materials and Methods*. For each injection, 60 time points, each a single free-induction decay, were obtained. A total of 75 separate injections were summed for each time point and Fourier transformed with a line broadening of 10 Hz. The intensity of each resonance was determined by using a Lorenzian curve fitting routine (VNMR software; Varian). The bottom spectrum represents 256 transients of 0.89 mM native apoDHFR in buffer.

tryptophans 30 and 47 (Fig. 4). Approximately 20% of the total intensity is already present at first time point (1.5 sec). The majority (≈80%) of the unfolded intensity appears at a rate

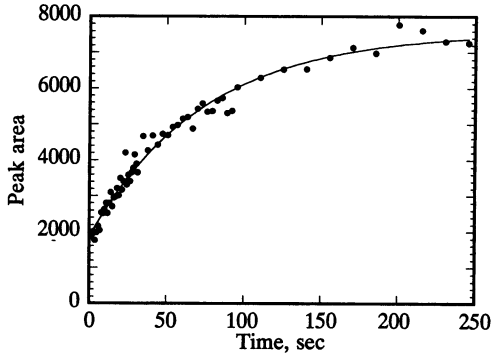

FIG. 4. Peak intensity for the resonance assigned to W30 and W47 in the unfolded protein. Data were collected and peak intensities determined as described in *Materials and Methods* and the legend for Fig. 3. The data (●) were fit to a single exponential function to yield the rates and amplitudes reported in Table 1. An initial phase representing ≈20% of the total intensity occurred too rapidly to be observed by this method.

Table 1. Rate constants and amplitudes for unfolding of 6-^{19}F-tryptophan-labeled DHFR in 5 M urea

Method	A_1*	k_1, sec^{-1}	A_2*	k_2, sec^{-1}
Fluorescence	0.205	0.727 ± 0.006	0.795	0.014 ± 0.0001
Circular dichroism	0.199	0.499 ± 0.036	0.801	0.014 ± 0.0001
NMR: Trp-22	0.237†	ND‡	0.763	0.010 ± 0.002
NMR: Trp-30 + Trp-47	0.225†	ND‡	0.775	0.015 ± 0.001
NMR: Trp-74	0.252†	ND‡	0.748	0.020 ± 0.003
NMR: Trp-133	0.438†§	ND‡	0.562	0.015 ± 0.004

*Amplitudes are given as the ratio of the amplitude of the first (A_1) or second (A_2) phase to the total amplitude. Data were fit to a single or double exponential equation by using the program KALEIDAGRAPH (Synergy Software).
†The amplitude of the first phase (A_1) was estimated by extrapolating the exponential fit of the slow phase to zero time.
‡ND, not determined; the rate constant associated with this phase was too fast to be determined by stopped-flow NMR.
§This value is probably an overestimate due to scatter in the initial time points.

comparable with the slow rate observed by fluorescence and far-UV circular dichroism spectroscopies, with a half-time of ≈50 sec.

The rates at which the intensities of the unfolded resonances assigned to tryptophans 22, 74, and 133 appear are similar to the rate at which the intensity of the unfolded resonance assigned to both tryptophans 30 and 47 appears (Table 1). In examining these data, we noted that, because of the low signal-to-noise ratio, there is considerable scatter in the early data points. Thus, we might not be able to detect small differences in the behavior of different tryptophans. The spectrum of an equivalent concentration of unfolded protein at equilibrium showed that little if any (<5 percent) of the intensity of the unfolded resonances was missing in the spectrum of the final time point collected (201 sec).

DISCUSSION

In this and previous studies (12, 17, 18), fluorescence and circular dichroism studies of the unfolding of *E. coli* DHFR indicate the presence of a biphasic process. However, from these studies, specific structural correlations could not be made. For example, it could not be determined whether the fluorescence change associated with the first, rapid phase represented the partial unfolding of most of the protein or the complete unfolding of part of the protein. Likewise, it could not be determined whether the circular dichroism change associated with the rapid phase represented a decrease in the secondary structure of all of the protein or the loss of all of the secondary structure from part of the protein.

In the present study, we have utilized stopped-flow ^{19}F NMR to monitor the behavior of different regions of the protein during the unfolding process. The striking result is that most of the intensities of the native resonances are lost within 1.5 sec, while only 20% of the intensities of the unfolded resonances appear. The missing intensities may be explained by either heterogeneity of tryptophan environments or conformational exchange between two or more environments at an intermediate rate on the NMR time scale. Either explanation indicates the presence of an intermediate in which the side chains are in a nonnative environment. Far-UV circular dichroism results indicate that the majority of the ellipticity of the native protein is still present at 1.5 sec. Thus, this intermediate must possess native-like secondary structure. Secondary structure is lost with the same rate at which the intensity of the unfolded resonance assigned to each individual tryptophan appears. Once this intermediate forms, the unfolding process appears to be highly cooperative, with different regions of the protein unfolding at the same rate.

The formation of this intermediate does not explain the two phases observed by stopped-flow circular dichroism and fluorescence spectroscopies because there is only a small change (≈20%) in both the fluorescence signal and the ellipticity observed at 222 nm in the first seconds of the unfolding reaction in which this intermediate forms. Thus, this intermediate is apparently not detectable by fluorescence or far-UV circular dichroism spectroscopies. The two phases observed by these methods could represent either a sequential unfolding mechanism involving a different intermediate or two unfolding pathways. Stopped-flow NMR observations of the unfolded peak intensity of each individual tryptophan indicate that the phases observed by circular dichroism and fluorescence correspond to the complete unfolding of 20% and 80% of the protein, respectively, suggesting that two separate unfolding pathways are involved.

Previous observations made by ligand binding (6, 19) show that in native *E. coli* DHFR, ligand binding occurs in two steps. The first step is a diffusion controlled process, and the second is a conformational change from a nonbinding to a binding form. Similarly, enzyme kinetic data show a lag in the velocity as a function of time, which is interpreted as the conversion of an inactive to an active form (7). Both results suggest that two forms of the apoprotein exist. The ratio of these forms differs for different isozymes and is also affected by experimental conditions, such as pH. At pH 7, Cayley *et al.* (19) observed that ≈75% of *E. coli* RT500 DHFR Form II binds NADPH rapidly. Penner and Frieden (7) found that 80% of the enzyme purified from plasmid pTY1 is in the form able to bind NADPH. The latter is the form of the enzyme used in this study.

The simplest explanation of the two phases observed by circular dichroism, by fluorescence, and by the appearance of unfolded peak intensities by stopped-flow NMR is that these two phases represent the complete unfolding of two different native forms of the enzyme. NADPH-binding studies gave an apparent rate constant of about 0.025 sec^{-1} for the slow phase of binding (which presumably represents conversion from the nonbinding to the binding form of enzyme). Kinetic simulation by using the program KINSIM (20) indicates that this rate of interconversion between different native forms of the protein would permit unfolding to occur by separate pathways at the two unfolding rates we observed. The first, rapid phase may represent the unfolding of the nonbinding form and the second, slower phase may represent the unfolding of the form of the enzyme which is able to bind ligand. If this suggestion is correct, it implies that apparently subtle changes in the native structure may have a profound effect upon the stability of a protein.

Our results indicate that unfolding of the majority of *E. coli* apoDHFR involves the formation of a nonnative intermediate which contains native-like secondary structure but in which many of the tertiary contacts must be disrupted (allowing considerable side-chain mobility). Interpretation of these results in terms of the structure of the intermediate is more difficult. A molten globule state has been characterized as a state containing the near-native amount of secondary structure and an absence of specific side-chain interactions (reviewed in ref. 21). Therefore, this intermediate would appear to have the properties of a "molten globule." This suggestion is consistent with studies of molten-globule states of guinea pig

α-lactalbumin (22) and lysozyme (23, 24) in which partial hydrogen-exchange protection suggests the presence of secondary structure in a protein that lacks the rigidity of the native tertiary structure. This suggestion is also consistent with the results of recent studies of hydrogen-bond breakage during unfolding of ribonuclease A (25), which indicate that the hydrogen-bond network that maintains the secondary structure of this protein is broken at the same (slow) rate. In ribonuclease A, only 30% of the intensity of valine 63 is present at a time when circular dichroism spectroscopy indicates that 85% of the native secondary structure is present (26), indicating the presence of an intermediate containing a high degree of secondary structure, but in which the side chains have considerable mobility.

Presumably, in such a molten-globule state, the side chains are solvated. If so, it is somewhat puzzling that the formation of this intermediate produces no apparent change in the fluorescence of the protein, as might be expected upon solvation. One possible explanation might be the manner in which the fluorescence was observed. Unfolding of 6-^{19}F-tryptophan-labeled DHFR involves both a red shift of the emission maximum and fluorescence quenching (unpublished results); since we observed the entire emission spectrum above 305 nm, we were not able to monitor for a red shift in emission maximum during the unfolding process. Another explanation is that the structure has become somewhat expanded but not all regions of the protein are solvent accessible. Such a state has been postulated by Shakhnovich and Finkelstein (27) and characterized as a "dry molten globule." This state has been recently proposed by Baldwin and coworkers (26) to describe the unfolding of ribonuclease A because the intermediate formed during unfolding of this protein retains its near-UV circular dichroism spectrum and is strongly protected from hydrogen exchange. It is possible that the major pathway for the unfolding of *E. coli* DHFR involves the formation of a dry-molten-globule intermediate; hydrogen-exchange studies of the unfolding of this protein will be necessary to investigate this point.

Only a few studies of the denaturation-induced unfolding of a protein have been reported in the literature. No such studies have been performed using stopped-flow NMR to examine the unfolding process in real time. The current experiments, although technically difficult, allow a direct, real-time measurement of the environment of side chains in different regions of the protein to be examined as a function of time. As technology improves, this type of experiment should become less demanding and provide useful information for both unfolding and folding processes.

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