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A Hydrophobic Cluster Forms Early in the Folding of Dihydrofolate Reductase

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ABSTRACT **The rapid kinetic phase that leads from unfolded species to transient folding intermediates in dihydrofolate reductase from *Escherichia coli* was examined by site-directed mutagenesis and by physicochemical means. The absence of this fluorescence-detected phase in the refolding of the Trp-74Phe mutant protein strongly implies that this early phase in refolding can be assigned to just one of the five Trp residues in the protein, Trp-74. In addition, water-soluble fluorescence quenching agents, iodide and cesium, have a much less significant effect on this early step in refolding than on the slower phases that lead to native and native-like conformers. These and other data imply that an important early event in the folding of dihydrofolate reductase is the formation of a hydrophobic cluster which protects Trp-74 from solvent.**

Key words: protein folding, folding intermediate, hydrophobic effect, tryptophan fluorescence, site-directed mutagenesis

INTRODUCTION

One of the major goals in solving the protein folding problem is the identification of the initial events of this complex conformational change. Current speculation suggests that globular proteins fold to their native conformers by a sequential pathway,[1,2] and that the most rapid steps in folding are driven by the formation of secondary structure[3–6] or by the hydrophobic effect.[7–9] However, detailed evidence on the secondary and tertiary structures of folding intermediates has been lacking.

Three recent reports have made significant progress in the elucidation of structures of early folding intermediates. Two studies have used a tandem approach of hydrogen exchange kinetics and two-dimensional NMR spectroscopy to identify the rate at which protons of backbone amides are protected from solvent exchange. Hydrogen bond formation for the refolding of ribonuclease A[10] and cytochrome *c*,[11] both groups were able to demonstrate the early formation of *specific* elements of secondary structure, which also appear in the crystal structures of the native protein. The third report[12] provides an explanation for the appearance of an early disulfide intermediate in the refolding and reoxidation of unfolded, reduced bovine pancreatic trypsin inhibitor.[13] Two peptides, each containing a cysteine residue and adjoining sequences, display secondary structure similar to that of the native protein when covalently linked by the disulfide bond.

Our laboratory has previously proposed a model for the folding of dihydrofolate reductase (DHFR, EC 1.5.1.3) from *Escherichia coli* based upon in vitro studies using urea as a denaturant.[14] A striking observation from these earlier studies was that the fluorescence intensity of the fastest refolding phase (designated the τ_5 phase), which leads from a series of unfolded species to a collection of kinetic intermediates, *increases* as the reaction proceeds. This contrasts with the *decrease* in intensity expected from equilibrium studies on native and unfolded DHFR and what is observed for the four slower kinetic phases (τ_1–τ_4) that lead to native or native-like intermediate conformations. It was speculated that the τ_5 phase reflected the formation of a hydrophobic aggregate that protected one or more of the five tryptophan residues from quenching by solvent. These rapidly formed species were identified as intermediates and not native-like forms because they could not bind methotrexate, a stoichiometric inhibitor of the enzyme.

In a more recent study,[15] we noted that the amplitude of the τ_5 phase was significantly reduced by amino acid replacements of Val-75. The isopropyl side chain of Val-75 participates in a large hydrophobic core, as observed in the crystal structure of the binary complex of DHFR with methotrexate (MTX).[16] Because the disruption of the hydrophobic core by amino acid replacements of Val-75 might be transmitted through the peptide backbone and alter the environment of Trp-74, we hypothesized that changes in the environment of Trp-74 may be the major, if not sole, contributor to the fluorescence change in τ_5 phase.

To test this hypothesis, we have constructed and characterized the folding of a mutant DHFR in

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which the tryptophan at position 74 is replaced by phenylalanine, a nonfluorescent hydrophobic amino acid. We have also examined the effects of ionic quenching agents of fluorescence on the amplitudes of the various kinetic phases of refolding of the wild-type DHFR to test whether the increase in fluorescence of the τ_5 phase reflects the exclusion of one or more tryptophan side chains from solvent. Based on the results from these experiments, we report here the detection of another *specific* structure of an early folding intermediate: a compact hydrophobic cluster that surrounds Trp-74 in DHFR.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Site-directed mutagenesis was performed with the plasmid pTY1.V75A, the construction of which was described previously.[15] Briefly, pTY1.V75A contains the gene for DHFR with the substitution Val-75Ala; this mutation also introduced a unique *BstX*I restriction site. For construction of pTY1.W74F, we used the oligonucleotide 5′-GTAACG[TTC][GTC] AAGTCGGT-3′ (the mutagenic mismatches are underlined and the codons for Phe-74 and Val-75 are in brackets). This oligonucleotide was designed to (1) replace Trp with Phe at 74, (2) revert Ala to Val at 75, and (3) remove the unique *BstX*I restriction site to enhance the frequency of selecting bacterial cells with the mutagenized plasmid. The change of the three bases was read by sequencing the DNA in the area of interest in both directions, and the entire gene was sequenced to show that no other changes had occurred.

Protein Purification and Characterization

The W74F DHFR was purified by the procedure of Baccanari et al.[17,18] Purity was assayed by both SDS and native PAGE; when stained by Coomassie Blue, both gel types displayed single discrete bands that comigrated with wild-type DHFR. The extinction coefficient of W74F DHFR was determined by titrating the protein with the stoichiometric inhibitor MTX (a minimum occurs at 380 nm in the difference spectrum of the binary complex that allows for the quantitation of DHFR); the titration of the wild-type protein with MTX was used as a standard. The extinction coefficient of the native protein at 280 nm decreased from 3.11×10^4 to 2.66×10^4 M^{-1} cm^{-1}. In addition, the number of Trp residues in both wild-type and variant proteins was determined directly by comparing absorbances of both native and unfolded protein.[19] The estimated number of Trp residues decreased from 4.6 ± 0.1 in the wild-type protein to 3.6 ± 0.1 in the variant. Enzymatic activity was monitored as previously described[20] in 0.1 M imidazole chloride (pH 7.0) at 30°C. The specific activity of the purified W74F DHFR was 68 units/mg; for comparison, the reported specific activity for wild-type DHFR is 85 units/mg.[17]

Folding Studies

The equilibrium unfolding reaction was monitored by difference ultraviolet spectroscopy at 293 nm on a Cary 118X spectrophotometer, modified by AVIV Associates. The computer fitting of the equilibrium data has been described previously.[14] The dependence of the fluorescence intensity of unfolded DHFR on the concentration of urea when monitored on the Durrum 110 stopped-flow spectrophotometer was determined by manually pushing solutions of unfolded protein through the apparatus until a constant voltage was obtained. The dependence of intensity on urea concentration was linear from 4.0 to 8.0 M urea; this dependence was extrapolated to 1.0 M urea to determine the expected fluorescence amplitude at zero time in refolding studies. The unfolding transition was observed to be reversible by the recovery of both the native absorption spectrum and 95% of the enzymatic activity after 6 M urea was removed by dialysis.

Kinetic experiments were done on a Durrum 110 stopped-flow spectrophotometer in the fluorescence mode; samples were excited at 290 nm with a slit width of 5 mm and emission monitored at wavelengths greater than 325 nm with a Corning C.S.-54 ground-glass filter. The kinetic data were fit to a sum of exponentials:

$$A(t) = \sum A_i \exp(-t/\tau_i) + A \qquad (1)$$

where $A(t)$ is the total amplitude at the time t, A is the amplitude at infinite time, A_i is the amplitude corresponding to the individual phase, i, at zero time, and τ_i is the associated relaxation time. Data were fitted by using a nonlinear least-squares fitting program NLIN.[21]

The buffer used for all folding experiments was 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA, and 1 mM 2- mercaptoethanol. The temperature was maintained at 15°C. Final protein concentration was 0.4 mg/ml for equilibrium studies and 0.17 mg/ml for kinetic studies. Previous studies[14] have demonstrated that the thermodynamic and kinetic properties of folding are independent of the protein concentration.

Materials

The plasmid containing the gene for the H45R/W47Y/150F DHFR triple mutant was a generous gift of L. Luyuan and S.J. Benkovic. The purified proteins, W22H and W22F DHFRs, were equally generous gifts of L. Howell.

Ultrapure urea was purchased from Schwarz/Mann and used without further purification; fresh solutions were prepared on the day of experimentation. The methotrexate-agarose resin used in protein purification was obtained from Pierce. All other chemicals were reagent grade.

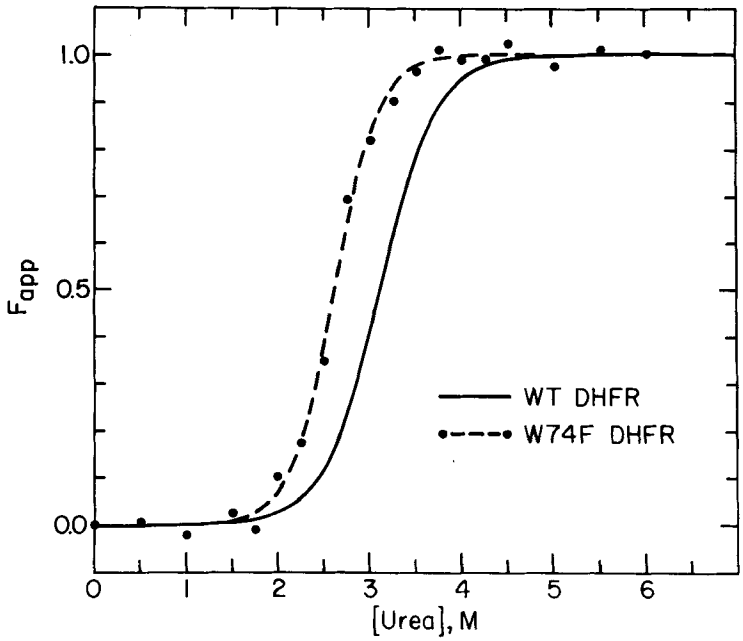

Fig. 1. Dependence of the apparent fraction of unfolded protein, F_{app}, on the urea concentration for wild-type and W74F DHFRs at pH 7.8, 15°C. Data collected by difference UV spectroscopy at 293 nm.

RESULTS

Equilibrium Studies

To both ascertain the effect of the W74F mutation on the stability and establish the appropriate concentrations of urea for kinetic experiments, we determined the urea-induced equilibrium unfolding transition for W74F DHFR (Fig. 1). The data for both wild-type[14] and mutant proteins are well fit by a two-state folding model involving significant populations of only the native, N, and unfolded, U, conformations. Assuming that the free energy difference between N and U, ΔG, depends linearly on the denaturant concentration,[22,23] we find that the mutation decreases the midpoint of the transition from 3.1 ± 0.1 to 2.6 ± 0.1 M urea. In contrast, the cooperativity (the dependence of ΔG on the urea concentration) increases from 1.9 ± 0.1 to 2.5 ± 0.1 kcal/mol/M,urea. When the stabilities are compared at the urea concentration corresponding to the midpoint for the wild-type DHFR, the mutant is found to be 1.2 kcal/mol less stable. This method of comparing stabilities appears to be more precise than comparisons in the absence of denaturant which require substantial extrapolation.[24,25]

Kinetic Studies

The role of Trp-74 in the early folding phase detected by fluorescence spectroscopy was tested by comparing the refolding reactions to 1.0 M urea for both wild-type and W74F DHFR. The results are shown in Figure 2, and the relaxation times and amplitudes are presented in Table I. The increase in fluorescence intensity observed in wild-type DHFR during the first second of the reaction is absent in the folding of the W74F mutant. In addition, the τ_5 phase was not observed for the mutant protein when the final concentration of urea was varied from 0.5 to 2.5 M, a range where this phase is observed in the wild-type protein. The simplest explanation for this result is that the change in fluorescence intensity in the τ_5 phase can be directly attributed to a change in environment surrounding Trp-74.

In contrast, the replacement of Trp-74 by Phe results in no observable effects on the amplitude of the two predominant slow folding phases detected by stopped-flow fluorescence studies (Table I). The relative amplitudes of the τ_2 and τ_4 phases are −38 ± 5 and −62 ± 3% for the wild-type protein and −39 ± 3 and −60 ± 3% for the W74F variant. We note that the τ_1 phase is too slow for accurate measurement with stopped-flow techniques and the τ_3 phase is of such small amplitude that changes are difficult to quantitate.

An alternative explanation for the absence of the τ_5 phase in the W74F protein is that the mutation had accelerated the relaxation time of the τ_5 phase such that the reaction had gone to completion during the mixing time of the stopped-flow instrument (20 msec). If such an acceleration had occurred, the initial fluorescence intensity of the refolding reaction for the mutant protein would be considerably higher than that expected from equilibrium mea-

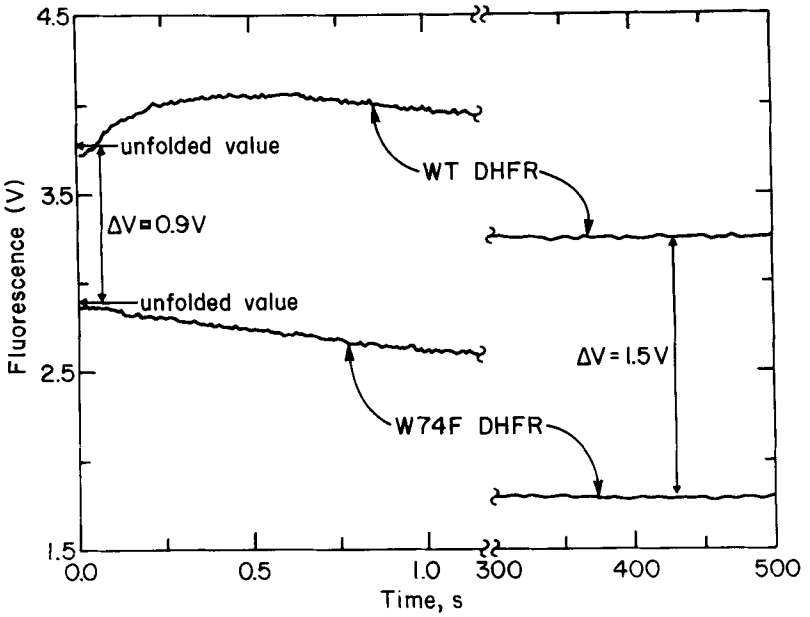

Fig. 2. The first second and the last 200 seconds of the refolding reactions of wild-type (top) and W74F (bottom) DHFRs as monitored by stopped-flow fluorescence spectroscopy. The arrows on the *y* axis indicate the fluorescence values expected for the unfolded proteins in 1.0 M urea, which were obtained as described in the Methods section. Reaction was initiated by dilution of protein, which had been unfolded in buffer containing 5.4 M urea, into buffer containing sufficient urea to make a final concentration of 1.0 M (native conditions). The transmittance was arbitrarily set and then kept constant for both wild-type and mutant proteins.

TABLE I. The Effects of Substitutions of Tryptophans in DHFR on the Relaxation Times and Amplitudes of the Major Phases Observed in Refolding From 5.4 to 1.0 M Urea*

DHFR	τ_5[†]	A_5[‡] (%)	τ_4	A_4 (%)	τ_2	A_2 (%)
WT	0.23 ± 0.02	66 ± 4	1.3 ± 0.1	−62 ± 2	32 ± 4	−38 ± 5
W74F	n.d.[§]	n.d.	1.8 ± 0.1	−60 ± 3	20 ± 3	−39 ± 3
H45R/W47Y/I50F	0.60 ± 0.02	62 ± 6	3.7 ± 0.1	−69 ± 5	29 ± 2	−31 ± 4
W22F**	0.26 ± 0.03	69 ± 6	1.4 ± 0.1	−34 ± 5	54 ± 3	−66 ± 5

*Equilibrium studies have demonstrated that each DHFR is fully unfolded in 5.4 M urea and that 1.0 M urea was well within the native baseline (data not shown). We note that the τ_3 phase is of low amplitude and is not always observed in refolding; therefore, changes in amplitude are difficult to quantitate and are not reported. The τ_1 phase is too slow to measure by stopped-flow technique.

[†]Relaxation times for the given kinetic phase in refolding. The units are seconds. Values are averages from three consecutive refolding jumps; errors represent standard deviations.

[‡]Relative amplitudes of a given kinetic phase in refolding. The absolute amplitudes of the two major slower phases, τ_4 and τ_2, were added and this value was used as a reference to obtain percentages. The negative signs for the A_4 and A_2 values indicate that the fluorescence intensity decreases, which is opposite in direction to the increase in intensity of the τ_5 phase. Errors are calculated by propagation of standard deviations.

[§]n.d. indicates that the τ_5 phase was not detected in the refolding of W74F DHFR.

**Jennings and Matthews, unpublished data.

surements on the unfolded form. The values expected at 1 M urea for both wild-type and variant DHFRs are shown in Figure 2 (arrows). The close agreement between the initial fluorescence intensity (2.8 ± 0.1 V) and the value that is predicted from equilibrium measurements of the unfolded protein (2.9 ± 0.1) rules out such an acceleration of the τ_5 phase in the W74F variant.

A second alternative explanation for the disappearance of the τ_5 phase in the W74F variant is that the mutation at position 74 significantly altered the environment of another tryptophan residue during the early stages of folding, and that the τ_5 phase is actually due to that other tryptophan. The most likely candidate for an alternative tryptophan is Trp-47 whose distance of closest approach to Trp 74 is 4.5 Å. The relative positions of Trp-74 and Trp-47 are shown in Figure 3. However, when the refolding kinetics of a triple mutant (H45R/W47Y/150F; Luyuan and Benkovic, unpublished data) containing the Trp-47Tyr mutation was studied, the τ_5 phase was detected and its relative amplitude was

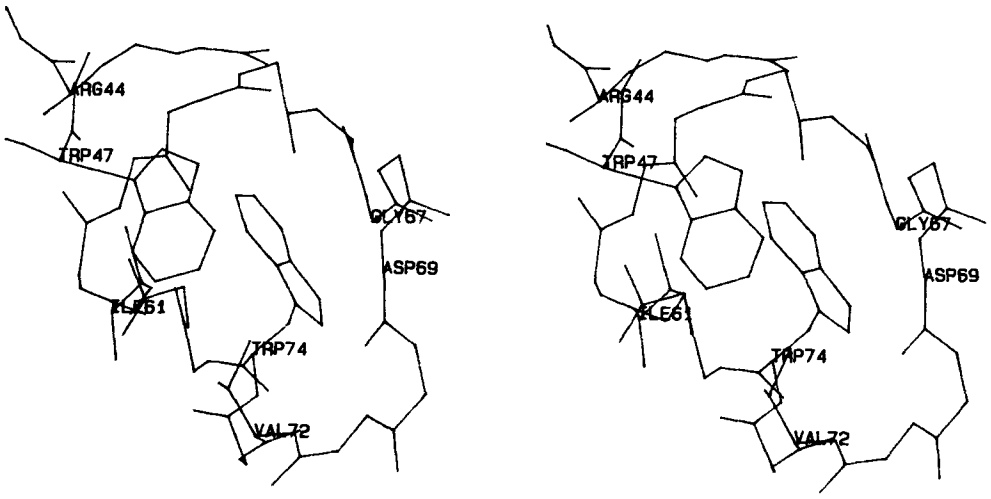

Fig. 3. Immediate environment of Trp-74. Trp-74 is the central residue of the three-residue edge strand, βD, and is bordered by residues 58–63 in the βC strand and by a loop, from residues 64–72. The indole ring of Trp-74 is 85% buried within this hydrophobic pocket, with only the δ1 carbon accessible to solvent. Specifically, the aromatic carbons of the indole ring make direct van der Waals contacts with atoms of Ile-61, Gly-67, and Val-72, and the indole nitrogen makes a hydrogen bond with the backbone carbonyl oxygen of Asp-69. In addition, the shortest distances between Trp-74 and the aliphatic arm of Arg-44 and the indole ring of Trp-47 are approximately 4.5 Å, which is slightly outside the recognized distance for direct van der Waals interaction.[31]

found to be unchanged: 62 ± 7 vs. 66 ± 4% for wild-type (Table I). Therefore, the τ_5 phase is not due to Trp-47. We note that the relaxation time for the τ_5 phase increased from 0.23 ± 0.02 second in the wild-type protein to 0.60 ± 0.02 second in the triple mutant (Table I), demonstrating that at least one of the three replacements has affected the rate at which Trp-74 is protected from solvent by the hypothetical hydrophobic cluster. Studies on single amino acid replacements that will pinpoint the cause of this effect are in progress.

In conjunction with another study in our laboratory, the folding of W22H and W22F variant DHFRs (E.E. Howell, unpublished data) has also been investigated (Jennings and Matthews, unpublished data). Trp-22, the next closest Trp residue, is approximately 22 Å from position 74. Both the relaxation times and relative amplitudes for the τ_5 phase are the same within experimental error as the values for the wild-type protein in these two variant DHFRs (data for W22F presented in Table I). Therefore, the τ_5 phase does not reflect a change in environment of this tryptophan. We note that the relative amplitudes of the two major slow refolding phases, τ_2 and τ_4, have switched so that the τ_2 phase is dominant in the W22F variant. Further studies will determine if this is due to the loss of fluorescence from Trp-22 or to an effect of the W22F mutation on local secondary of tertiary structure. Although the replacements of Trp-30 and Trp-133 have not yet been constructed, these positions are even further from Trp-74 and, therefore, would seem less likely to be involved. Taken altogether, these results strongly imply that Trp-74 is the sole source of the increase in fluorescence intensity in the early folding phase detected for wildtype DHFR.

To test the previous hypothesis[14] that the increase in fluorescence is due to the burying of Trp-74 in a hydrophobic cluster, we posed the following question: Do ionic quenchers of fluorescence, which are not expected to penetrate into a hydrophobic cluster, selectively affect the amplitude of either the τ_5 phase or the two major slower phases in refolding? The results of adding potassium iodide or cesium chloride to the refolding reaction are shown in Table II. As a control experiment for the effect of the increase in ionic strength caused by 0.2 M KI and CsCl, refolding was also performed in the presence of 0.2 M KCl. Whereas the presence of KI has a small effect on the amplitude of the τ_5 phase (value is 74% of the value in the presence of KCl), it greatly reduces the amplitude of the τ_2 phase (to 15% of the KCl value), and eliminates the τ_4 phase altogether. The cationic quencher cesium has similiar effects. The amplitude of the τ_5 phase in the presence of CsCl is 88% of the value in the presence of KCl; however, cesium significantly reduces the fluorescence of both the τ_2 and τ_4 phases (49 and 35%, respectively, of the values in the presence of KCl).

DISCUSSION

The results presented in this paper strongly support and extend the proposed hypothesis[14] that the τ_5 phase in the refolding of DHFR corresponds to the burying of one or more of the five tryptophans in DHFR in a hydrophobic pocket large enough to pro-

TABLE II. The Effect of 0.2 M Potassium Chloride, Potassium Iodide, and Cesium Chloride on the Amplitudes of the Major Phases Observed in the Refolding of Wild-Type DHFR From 5.4 to 1.0 M Urea*

Wild-type DHFR	A_5	A_4	A_2
—	+0.74 ± 0.05	−0.70 ± 0.05	−0.42 ± 0.04
+0.2 M KCl	+0.87 ± 0.06	−0.54 ± 0.03	−0.49 ± 0.02
+0.2 M KI	+0.64 ± 0.04	Not detected	−0.07 ± 0.03
+0.2 M CsCl	+0.77 ± 0.03	−0.19 ± 0.03	−0.24 ± 0.02

*The units for the values of the various amplitudes are volts. A_5, A_4, and A_2 represent the amplitudes of τ_5, τ_4, and τ_2 phases, respectively. As stated in Table I, the τ_3 phase is of low amplitude and is not always observed in refolding; therefore, changes in amplitude are difficult to quantitate and are not reported. The τ_1 phase is too slow to measure by stopped-flow technique. Errors represent standard deviations of values that are averages of three separate refolding reactions.

tect the indole ring(s) from solvent. The new evidence for the hydrophobic cluster model is the failure of ionic quenching agents to significantly diminish the amplitude of the τ_5 phase in comparison with their effects on the amplitudes of major slow refolding phases, τ_2 and τ_4. In addition, results from site-directed mutagenesis studies strongly suggest that the increase in fluorescence of the τ_5 phase can be assigned to a change in environment of one unique tryptophan, Trp-74.

It is not clear at this stage why Trp-74 behaves in such a fashion. Trp-133 has a lower solvent exposure in the crystal structure of DHFR and Trp-47 is involved in the same hydrophobic cluster as Trp-74. A possible explanation is that Trp-74 is the only tryptophan contained in one of the eight strands of the β-sheet that forms the structural core of this protein. Therefore, the τ_5 phase may reflect the concerted development of the β-sheet and this hydrophobic cluster. Such a concerted construction of core and sheet addresses the dual problem of removing a large number of hydrophobic side chains from the unfavorable interaction with solvent (formation of core) and the requirement to satisfy the large number of hydrogen bonds between strands (formation of sheet). An alternative possibility is that the τ_5 phase reflects the rate at which the indole ring diffuses into a preformed hydrophobic cluster. Further biophysical and mutagenic studies will be required to explore these possibilities.

The destabilization of DHFR ($\Delta\Delta G$ = −1.2 kcal/mol) by the substitution of a well-packed tryptophan (Fig. 3) by phenylalanine is not surprising. Phe is almost 40 Å^3 smaller in volume than Trp and would not be able to maintain the same van der Waals interactions as Trp. Furthermore, the absence of a hydrogen bond that exists in the wild-type protein between the indole nitrogen and the backbone carbonyl oxygen at position 69 might be expected to decrease the stability.* The importance of both van der Waals interactions and hydrogen bonds to protein stability has been emphasized by Alber et al.[26] in their crystallographic and thermodynamic studies of multiple replacements of Thr-157 in the phage T4 lysozyme. The decreases in stability for the alanine, valine, and leucine substitutions, which all lose a specific hydrogen bond and perturb the van der Waals interactions, range from −1.3 to −1.6 kcal/mol. In addition, Fersht and colleagues[27] have found that hydrogen bond interactions between substrate and enzyme can account for 0.5 to 1.5 kcal/mol in binding energy.

Given the hypothesis that the τ_5 phase represents the burying of Trp-74 and the observation that Trp-74 is virtually buried in the native conformation (Fig. 3), it is possible that the structure surrounding Trp-74 in this early formed folding intermediate may be similar to that found in the native structure. Such a structural model is indirectly supported by the following observations:

1. The differences in fluorescence between wild-type and W74F DHFR observed throughout the refolding reaction (Fig. 2) suggest that the environment about Trp-74 does not significantly change subsequent to the τ_5 phase. The difference at zero time (0.9 ± 0.2 V) reflects the difference in fluorescence intensity of DHFRs containing four (W74F) and five (wild-type) tryptophans when unfolded in high concentrations of denaturant. The fitted amplitude of the τ_5 phase (0.74 ± 0.05 V) measures the increase in emission of Trp-74 due to an environmental change during the early step of folding. The sum of these values (1.7 ± 0.2 V) is equal within experimental error to the difference at infinite time (1.5 ± 0.2 V) which measures the difference in flu-

*It should be noted that this structural interpretation of the stability change for the Trp-74Phe mutant assumes that the native conformation is unaffected by the presence of 3.1 M urea, the standard state used in this analysis. The validity of this assumption is supported by the previous observation[14] that the urea-induced unfolding of DHFR follows a two-state model. If the native conformation in 3.1 M urea were to differ energetically (and, therefore, structurally) from the conformation in the absence of urea, the two-state model would not have been adequate.

orescence intensities of the native proteins. Therefore, no significant changes in the environment about Trp-74 are detected by fluorescence spectroscopy subsequent to the τ_5 phase in refolding.

2. The W74F mutation produces no observable effects on the amplitudes of the two predominant refolding phases, τ_4 and τ_2, that lead to native or native-like conformations. Therefore, Trp-74 makes no observable contributions to these slow refolding phases, again implying that no large changes in environment near Trp-74 occur during the late steps of folding.

A recent NMR study[28] of a partially folded state of α-lactalbumin (the molten globule state induced by low pH, elevated temperature, or low concentrations of denaturant[29]) revealed that certain aromatic residues found in the hydrophobic core of the native protein displayed defined structure within the molten globule, i.e., their resonances were shifted when compared with the spectrum of the unfolded protein. In addition, the chemical shifts of these side chains were displaced from those found in the native protein, indicating that the molten globule state is intermediate between unfolded and native conformations. These results are similiar to those of the present study and are evidence for the importance of the hydrophobic effect in stabilizing not only native but also partially folded forms of proteins.

The development of the hydrophobic cluster in the folding intermediate in DHFR and its similarlity to the hydrophobic core that is observed in the native conformation can now be examined through site-directed mutagenesis. Systematic mutations can be made throughout the core found in the native conformation (Fig. 3) and effects upon the amplitude and relaxation time of the τ_5 phase can be determined. If effects are observed at the same positions as those that participate in the native cluster, it would support the proposal that the kinetic intermediate has a structural core similiar to the native conformer. We have previously used mutagenesis to probe the slower steps of the folding of DHFR which occur late in the process.[15,30] It is now clear that this important tool can be used to examine the fast phase in refolding and thus help illuminate the structure of the initial kinetic intermediate.

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