A Reexamination of the Folding Mechanism of Dihydrofolate Reductase from *Escherichia coli*: Verification and Refinement of a Four-Channel Model†

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ABSTRACT: The mechanism of folding of dihydrofolate reductase from *Escherichia coli* was reinvestigated by studying the unfolding and refolding kinetics using absorbance and fluorescence spectroscopies. The original kinetic model proposed that folding involved a series of native, intermediate, and unfolded forms which interconverted through four independent channels linked by slow cis/trans isomerization reactions at Xaa–Pro peptide bonds [Touchette, N. A., Perry, K. M., & Matthews, C. R. (1986) *Biochemistry 25*, 5445]. Recently, alternative sequential models have been proposed [Frieden, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4413; Kuwajima et al. (1991) *Biochemistry 30*, 7693] which challenge the original proposal. Stopped-flow studies of the intrinsic tryptophan fluorescence demonstrated the presence of three (and tentatively four) kinetic phases in unfolding which correlated well with four phases previously observed in refolding experiments. By monitoring the binding of the inhibitor methotrexate during folding at varying relative concentrations of inhibitor to protein, it was found that the selective loss of the slow-folding phases at substoichiometric levels could only be explained by a four-channel folding model. Double-jump experiments (native → unfolded → native) showed that the four refolding channels are populated within 20 s at 15 °C and are not likely to be due to proline isomerization. Reverse double-jump experiments (unfolded → native → unfolded) demonstrated that interconversions between native conformers are more rapid than originally proposed. Interestingly, the majority of the protein folds through a channel to a native conformer that is minimally populated at equilibrium. This implies that although the folding of dihydrofolate reductase is ultimately under thermodynamic control, kinetic factors contribute to the transient populations of native species during folding.

A major approach toward the elucidation of the mechanism of folding of globular proteins is the detection of partially folded forms and the collection of detailed information on their structures and stabilities (Kim & Baldwin, 1990). Dihydrofolate reductase (DHFR)[1] from *Escherichia coli* is a useful target in this regard because its folding involves at least two transient intermediates which are detected by circular dichroism and/or fluorescence spectroscopies. The first of these intermediates appears within 5 ms and contains a significant component of secondary structure (Kuwajima et al., 1991; B. Jones and C. R. Matthews, unpublished results). No evidence for tertiary structure in this burst-phase species is apparent in tryptophan fluorescence (Garvey & Matthews, 1989). A subsequent intermediate appears in the several hundred millisecond time range and has a specific tertiary contact between two tryptophan residues which is present in the native conformation (Kuwajima et al., 1991). Neither of these intermediates can bind the active-site inhibitor methotrexate (MTX) nor the reduced cofactor NADPH (Frieden, 1990). Four subsequent slow reactions lead to species which are all capable of binding MTX (Touchette et al., 1986).

The original kinetic folding mechanism proposed for DHFR involved four parallel channels and a series of native, intermediate, and unfolded forms (Touchette et al., 1986). These channels were proposed to arise from kinetic heterogeneity in the populations of the unfolded protein, a heterogeneity which was ascribed to proline isomerization (Brandts et al., 1975). More recently, two alternative models were proposed to account for the observed data. Frieden (1990) postulated that folding follows a single channel which contains a series of intermediates whose folding culminates in a single native conformation. One of the intermediates is proposed to bind substrate (or MTX) and cofactor and is catalytically active; a second intermediate can bind MTX. Kuwajima et al. (1991) proposed that folding might involve two slowly interconverting parallel channels, each of which involves multiple native, intermediate, and unfolded forms of DHFR. This mechanism was based, in part, on the pairwise similarities of the far-UV CD kinetic difference spectra for the four slow-folding phases.

To sort out the correct folding model, a series of kinetic experiments which can differentiate among the current proposals were performed. Key among these was the ability to monitor directly the binding of methotrexate to the enzyme (Touchette et al., 1986). When these results were combined with those from a fluorescence study of the unfolding reaction and from a pair of double-jump experiments (Brandts et al., 1975; Nall, 1975; Kiefhaber et al., 1990), it was found that only a four-channel model was consistent with all of the data. The new results show that the interconversions between the channels are more rapid than first thought, suggesting that proline isomerization is unlikely to be the correct explanation. The four channels appear to arise as a late event in folding and reflect the development of tertiary structure.

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[1] Abbreviations: CD, circular dichroism; DHFR, dihydrofolate reductase; F31V, Phe-31 → Val; K_2EDTA, ethylenediaminetetraacetic acid, dipotassium salt; MTX, methotrexate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; UV, ultraviolet.

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MATERIALS AND METHODS

Reagents. Ultrapure urea was purchased from Schwarz/Mann and used without further purification; fresh solutions were prepared on the day of the experiment. Folic acid, MTX, and NADPH were purchased from Sigma. MTX affinity resin was purchased from either Pierce or Sigma. 7,8-Dihydrofolate was prepared by dithionite reduction of folic acid (Blakely, 1985) and stored at −70 °C as a suspension in 5 mM HCl. All other chemicals were reagent grade.

A standard buffer containing 10 mM potassium phosphate (pH 7.8), 0.2 mM K_2EDTA, and 1 mM β-mercaptoethanol, at a temperature of 15 °C, was used throughout the experiments. The refolding was greater than 95% reversible under these conditions.

Protein Source and Purification. Wild-type dihydrofolate reductase (DHFR, EC 1.5.1.3) was isolated from four sources: (1) *E. coli* strain W71-18 containing plasmid pTP64-1; (2) *E. coli* strain MV1190 containing plasmid pBF2; (3) *E. coli* strain MC294 which contains the plasmid pTY1; (4) *E. coli* strain AG-1 (Stratagene) containing the plasmid pWT1-3. Plasmids pTP64-1 and pWT1-3 contain the gene for wild-type DHFR under the control of an engineered consensus promoter and Shine–Dalgarno sequence (M. Iwakura, personal communication). The constructions of pBF2 and pTY1 were described previously (Kuwajima et al., 1991; Perry et al., 1986). DNA sequencing of all four plasmids confirmed the integrity of the wild-type DHFR genes. The results of protein folding experiments and the specific activities of the enzymes were independent of the source of the protein. The F31V mutant protein was isolated from *E. coli* strain MC294 containing plasmid pCK314 whose construction has been described elsewhere (Taira et al., 1987).

Protein purification was performed as described previously (Kuwajima et al., 1991). All preparations yielded material which migrated as a single band on both native and sodium dodecyl sulfate–polyacrylamide gels. Protein concentration was determined by using a molar extinction coefficient of 3.11×10^4 M^{-1} cm^{-1} at 280 nm for the wild-type protein (Touchette et al., 1986). Enzymatic activity was monitored by the method of Hillcoat (Hillcoat et al., 1967). The specific activity of the wild-type protein samples used in these studies ranged from 80 to 104 units mg^{-1} in 0.1 M imidazole chloride, pH 7.0 and 30 °C. The reported activity for the wild-type protein under these conditions is 85 units mg^{-1} (Hillcoat et al., 1967). The specific activity of the F31V mutant protein at pH 7.0 and 30 °C was 24 units mg^{-1}.

Spectroscopic Methods. The slow steps ($\tau > 10$ s) in folding were monitored by the time-dependent change in ϵ_{293} using manual mixing techniques on an AVIV 118CX absorbance spectrophotometer (Finn et al., 1992). Stopped-flow kinetic experiments were performed either on a Durrum 110 stopped-flow spectrophotometer in the fluorescence mode or on a Bio-Logic SFM-3 stopped-flow spectrophotometer in either the fluorescence or the absorbance mode. Experiments done on the Durrum used an excitation wavelength of 290 nm with a slit width of 5 mm and monitored emission at wavelengths greater than 325 nm with a Corning C.S. 0-54 ground-glass filter. To facilitate kinetic measurements, the spectrophotometer was interfaced to an AT&T PC6300 personal computer running the ASYST data collection software package (Asyst Software Technologies, Inc.). Fluorescence experiments done on the Bio-Logic instrument used an excitation wavelength of 290 nm and a bandwidth of 10 nm. The emission was monitored at wavelengths greater than 320 nm with a ground-glass filter provided by Bio-Logic. Data collection was done with the Bio-Kine software package running on a Swan Technologies 386/25 computer.

Double-Jump Experiments. The classic double-jump experiment (native → unfolded → native; Brandts et al., 1975) was performed on the F31V mutant DHFR by manual mixing of 100 μL of a concentrated stock solution of native protein (29 mg mL^{-1}) with 800 μL of 9.0 M urea solution at 15 °C so as to obtain a final urea concentration of 8 M. After a variable delay time, 150 μL of this protein/8 M urea solution was injected into a cuvette containing 750 μL of buffer at 15 °C. The solution was rapidly mixed and the refolding of the protein monitored by UV absorbance spectroscopy. The final urea concentration was 1.3 M, and the final protein concentration was 0.53 mg mL^{-1}.

The reverse double-jump experiment (unfolded → native → unfolded; Kiefhaber et al., 1990) was performed with the wild-type protein. In these experiments, 100 μL of a concentrated stock of protein (20 mg mL^{-1}) which contained 5.4 M urea was diluted with 800 μL of buffer to a final urea concentration of 0.6 M urea and allowed to fold for a variable delay time; 200 μL of the protein/0.6 M urea solution was then rapidly mixed in a cuvette containing 700 μL of 8.8 M urea, resulting in a final area concentration of 7 M and a final protein concentration of 0.50 mg mL^{-1}. The absorbance at 293 nm was then recorded as a function of time on the AVIV spectrometer.

Inhibitor Binding Studies. Refolding experiments in the presence of MTX were done by monitoring the change in absorbance at 380 nm using the Bio-Logic instrument. Upon binding to DHFR, the change in ϵ_{380} for MTX is −4942 M^{-1} cm^{-1} (Touchette et al., 1986). This wavelength is well outside the absorbance region of the protein and allows for the direct detection of the binding of inhibitor during folding.

Data Fitting. The kinetic data were in general fit to a series of exponentials as described previously (Touchette et al., 1986); the nonlinear least-squares analysis yields amplitudes and relaxation times for each of the component phases. For the stopped-flow experiments, the slowest unfolding and refolding phases were too small in amplitude to fit accurately with an exponential term. In this case, the slow phase was approximated by the inclusion of a linear term in the fitting equation. This slow phase could, however, be accurately monitored when necessary by manual-mixing techniques using the double-beam AVIV 118CX spectrometer. The low signal-to-noise ratios observed with the native → unfolded → native double-jump experiment required that the relaxation times be fixed to values previously determined in standard refolding experiments. This was done to obtain useful estimates of the amplitudes as a function of delay time. The reverse double-jump experiment had sufficient signal to allow fitting of both relaxation times and amplitudes.

RESULTS

Previous studies of the reversible unfolding of DHFR have led to three different kinetic folding models:

(1) Touchette et al. (1986) proposed that folding proceeds through four independent channels and involves four unfolded forms, four intermediates which cannot bind MTX, and four native or native-like forms. The rates of interconversion between the channels were supposed to be slower than any of the folding reactions and reflect proline isomerization reactions.

(2) Frieden (1990) proposed a linear model on the basis of inhibitor, substrate, and cofactor binding, as well as the recovery of enzymatic activity during folding. This model involves a single unfolded form, three intermediates, and a

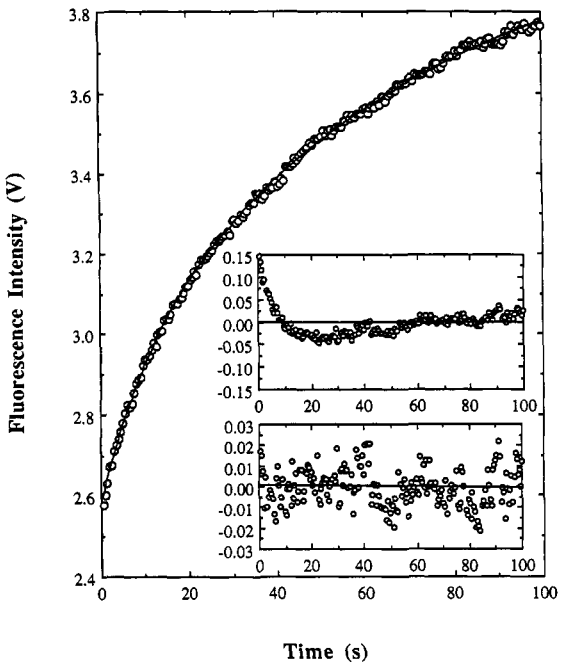

FIGURE 1: Fluorescence intensity of DHFR as a function of time for an unfolding jump from 0 to 7 M urea measured on the Bio-Logic SFM-3 stopped-flow spectrophotometer. Tryptophans were excited at 290 nm, and the emission was monitored at wavelengths greater than 325 nm. The solid line represents the calculated fit to the sum of two exponentials plus a linear term. Insets are plots of the residuals corresponding to fits of one (upper) and two (lower) exponentials plus a linear term, respectively. The solvent contained 10 mM potassium phosphate, 0.2 mM K_2EDTA, and 1 mM β-mercaptoethanol at pH 7.80; the temperature was 15 °C. The ordinate indicates photomultiplier tube voltage.

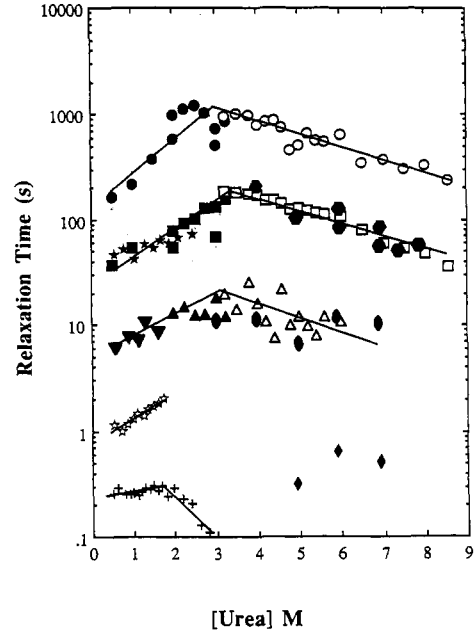

FIGURE 2: Dependence of the observed relaxation times for folding on the final concentration of urea. The τ_1 (●, ○), τ_2 (■, □), and τ_3 (▲, △) phases were measured on the Cary 118CX spectrophotometer. The closed symbols correspond to refolding reactions and the open symbols to unfolding reactions. The τ_2 (★), τ_3 (▼), τ_4 (☆), and τ_5 (+) refolding phases were observed by fluorescence techniques on the Bio-Logic SFM-3 stopped-flow spectrophotometer. The τ_2 (●), τ_3 (solid oval), and τ_4 (♦) unfolding phases were observed on the Durrum 110 stopped-flow spectrophotometer. Lines are drawn to aid the eye. Refolding experiments were accomplished by diluting a protein/5.4 M urea solution with buffer and urea to the desired final urea concentrations. The final protein concentration was typically 0.5 mg mL^{-1} for manual mixing experiments and 0.2 mg mL^{-1} for stopped-flow fluorescence experiments. Solvent conditions were identical to those described in Figure 1.

single native form; the final intermediate was proposed to be enzymatically active. This mechanism does not account for the slowest folding reaction detected by both UV absorbance (Touchette et al., 1986) and far-UV CD spectroscopy (Kuwajima et al., 1991). This discrepancy presumably arises from the use of stopped-flow fluorescence by Frieden. Either the amplitude of this phase is too small to detect by fluorescence or the technique is not sufficiently stable to detect a folding reaction in the 10^2–10^3-s time range.

(3) Kuwajima et al. (1991) postulated a two-channel folding model based upon the results of stopped-flow CD experiments. Each channel has a pair of intermediates and a pair of native forms. As in the four-channel model, the interconversions between the channels were proposed to be slow compared to folding and to possibly reflect proline isomerization.

To eliminate the incorrect models, several tests were performed.

Single versus Multiple Native Conformers. Two unfolding phases, which had originally been observed by manual mixing UV absorbance techniques, account for ~90% of the absorbance change expected from equilibrium studies (Touchette et al., 1986). Although attempts to detect faster phases by stopped-flow absorbance were not successful, the presence of at least one additional faster phase was later observed by stopped-flow CD spectroscopy (Kuwajima et al., 1991).

Reasoning that the higher sensitivity of fluorescence spectroscopy might enhance the detection of minor unfolding phases, a series of unfolding reactions was performed on both the Durrum and Bio-Logic stopped-flow instruments. Figure 1 shows a representative kinetic progress curve for the unfolding reaction produced by jumping the urea concentration from 0 to 7 M on the Bio-Logic instrument. The small amplitude of the slowest phase, τ_1, and the instability of the fluorescence signal for time periods greater than 500 s made it impossible to obtain an exponential fit of this phase from data acquired by stopped-flow fluorescence techniques. Therefore, it was accounted for by including a linear time dependence in the fitting equation. The upper and lower insets in Figure 1 present the residuals of nonlinear fits for the sum of a single exponential and a linear term and for the sum of a two-exponential term and a linear term, respectively. The superior fit with two exponentials and a linear term shows that there are three kinetic phases, τ_1, τ_2, and τ_3, which occur in this time range. The linear change in fluorescence was shown not to be due to aggregation by the absence of any changes in the absorbance at 350 nm (data not shown).

Figure 2 shows the urea concentration dependence of the observed relaxation times for both unfolding and refolding reactions detected by fluorescence and/or absorbance spectroscopy. The relaxation time for the τ_1 unfolding reaction could only be reliably measured on a double-beam absorbance spectrometer. The relaxation time falls into the range from 200 to 1000 s and connects smoothly with the relaxation times for the slowest phase in refolding. The dependence of the τ_2 unfolding relaxation time on the final urea concentration measured by stopped-flow fluorescence is within experimental error of that previously determined by manual mixing experiments and absorbance spectroscopy (40–200 s; Touchette et al., 1986) and connects smoothly with the related phase in refolding. The τ_3 unfolding phase detected by fluorescence and absorbance spectroscopy occurs in the same time range as the τ_3 refolding reaction (5–25 s) and again connects smoothly. The inverted "V" or chevron shape for these three plots is that expected for a protein folding reaction (Tanford,

1968). Although the amplitude of the τ_3 phase in unfolding is small, comprising less than 10% of the total fluorescence change, its presence confirms the CD results (Kuwajima et al., 1991).

A fourth, faster phase, τ_4, in the 0.5–2-s time range was observed between 5 and 7 M urea by stopped-flow fluorescence on the Durrum instrument. The substantial scatter in the relaxation times and the inability to detect this phase in the unfolding transition zone reflect its very small amplitude. This phase comprises only a few percent of the total signal change and was not detected on the Bio-Logic instrument (due to mixing artifacts at high denaturant concentrations in the 100-ms time range). A fourth, faster phase was also observed at moderate urea concentrations by stopped-flow CD spectroscopy (Kuwajima et al., 1991). Considering the difficulty of detection, the assignment of this very fast phase in unfolding is currently tentative. In contrast, the τ_4 phase in refolding is the dominant phase in amplitude in both protein fluorescence and inhibitor binding studies (Touchette et al., 1986). The existence of at least three phases in unfolding (and probably a fourth) and their relationship to phases in refolding which lead to MTX binding (Touchette et al., 1986) provide strong evidence for the presence of multiple native conformers under equilibrium conditions.

Single versus Multiple Folding Channels. Schmid and Blaschek (1981) showed that ligand binding studies can differentiate between single and multiple folding channel models for ribonuclease A. The key experiment involved monitoring the amplitudes of the refolding phases as a function of the inhibitor concentration at *substoichiometric* ratios of inhibitor to protein.

MTX binds at the active site of DHFR with a nanomolar dissociation constant (Blakely, 1985; Appleman et al., 1988). The binding gives rise to a maximum in the difference absorbance spectrum at 380 nm, a phenomenon which makes it a useful probe for following the formation of the substrate binding pocket (Touchette et al., 1986; Frieden, 1990). Under the experimental conditions adopted in these studies, the binding of MTX to the native enzyme occurs within the dead time of the stopped-flow instruments (<5 ms). Therefore, the observed changes in absorbance at 380 nm reflect protein folding rather than inhibitor binding reactions.

The refolding of DHFR as monitored by MTX (Figure 3) binding parallels the folding kinetics as measured by tryptophan fluorescence spectroscopy (Touchette et al., 1986), with one notable exception. Because an early intermediate in which Trp-47 and Trp-74 attain a nativelike interaction does not bind the inhibitor, a lag phase is observed in the hundreds of millisecond time frame when monitoring the absorbance at 380 nm (Figure 3, inset). The relaxation times of the four subsequent phases detected by MTX binding are within experimental error of those determined by tryptophan fluorescence. The amplitudes and relaxation times of these four phases do not depend upon the MTX/protein ratio in the range of 1/1 to 4/1 (Touchette et al., 1986; Frieden, 1990).

Refolding of DHFR in the presence of *substoichiometric* amounts of MTX shows that at progressively decreasing ratios of MTX/DHFR there is a selective loss of the amplitudes of the slow-folding phases (Figure 3). These data are most easily explained by proposing that species capable of binding the inhibitor are being formed by parallel pathways. At substoichiometric concentrations of inhibitor, the rapidly formed species would bind all of the available MTX and deplete the supply available to the slower folding species. It is important to note that even at substoichiometric concentrations of inhibitor, the concentration of MTX was well above the dissociation constant (Blakely, 1985; Appleman et al., 1988; Frieden, 1990).

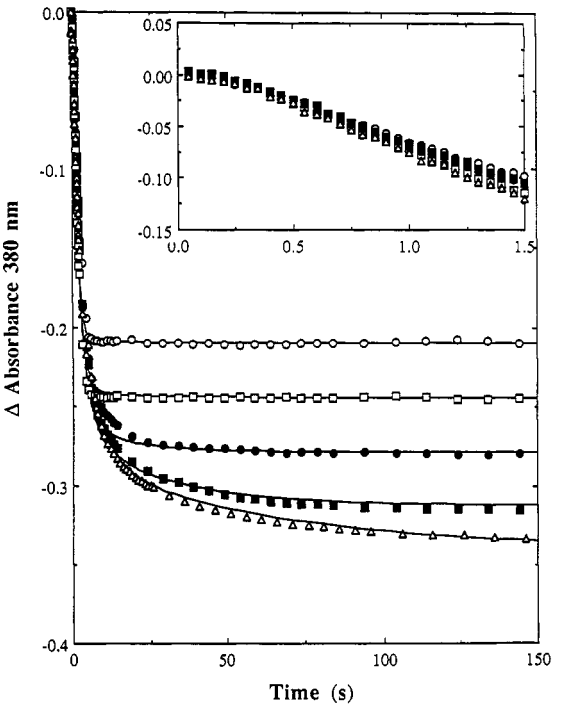

FIGURE 3: Plot of the change in absorbance at 380 nm as function of time for the refolding reaction from 5.4 to 0.54 M urea at various MTX/DHFR ratios, measured on the Bio-Logic SFM-3 stopped-flow spectrophotometer in the absorbance mode. The inset shows the first 1.5 s of the reaction. The MTX/DHFR ratios are 1.1/1 (△), 0.9/1 (■), 0.8/1 (●), 0.7/1 (□), and 0.6/1 (○). The solid lines are those obtained from simulations using KINSIM for a four-channel model as described in the text. The solvent conditions were identical to those described in Figure 1.

Kinetic simulations were done using KINSIM (Barshop et al., 1983) to demonstrate that the four-channel model is the only one consistent with this behavior. The results for the one-, two-, and four-channel models at 0.7 and 1.1 MTX/DHFR ratios are compared with the experimental data in Figure 4A–C. At and above stoichiometric levels of inhibitor, all of the models predict the observed behavior. The one- and two-channel models require absorbance changes for the DHFR/MTX complexes during the folding process to achieve a successful simulation while the four-channel model requires an absorbance change only upon binding of MTX to DHFR. With these same restrictions at substoichiometric levels of MTX, only the four-channel model correctly predicts the selective loss of the slow-folding phases (Figure 4A–C). Therefore, the one- and two-channel models can be eliminated; the four-channel model remains as the best explanation. This conclusion is also supported by the agreement between the simulations and the data over the range of MTX/DHFR ratios from 0.6/1.0 to 1.1/1.0 (Figure 3, solid lines).

Interconversions between Unfolded Forms of DHFR. The previous explanation for the slow interconversions between folding channels invoked rate-limiting cis/trans isomerization at Xaa–Pro peptide bonds (Touchette et al., 1986). This proposal can be tested by a double-jump unfolding/refolding experiment first described by Brandts et al. (1975). The procedure involves a double jump of 0 → 8.0 → 1.3 M urea with the time spent at 8.0 M urea recorded as the delay time. An increase in the amplitude of a slow-folding phase as a function of delay time is characteristic of a proline isomerization reaction occurring in the unfolded protein.

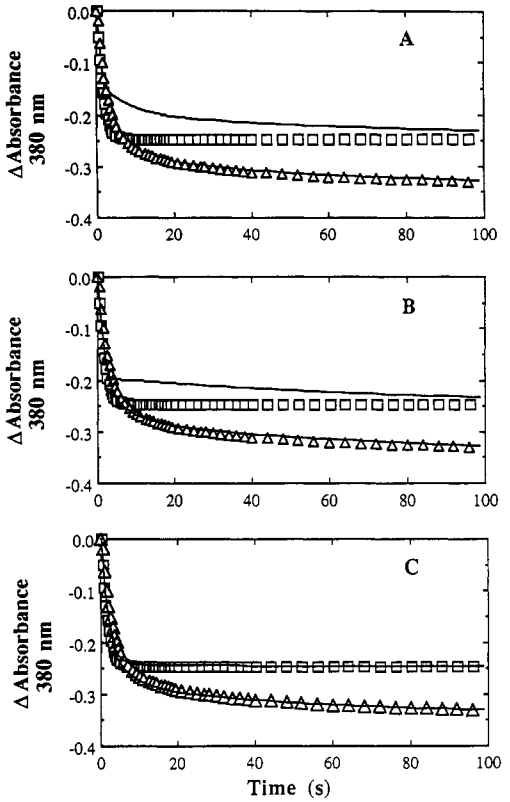

FIGURE 4: Plots of the change in absorbance at 380 nm predicted by KINSIM for the (A) single-channel, (B) two-channel, and (C) four-channel models compared to the data observed at two different MTX/DHFR ratios: 0.7/1 (□) and 1.1/1 (△).

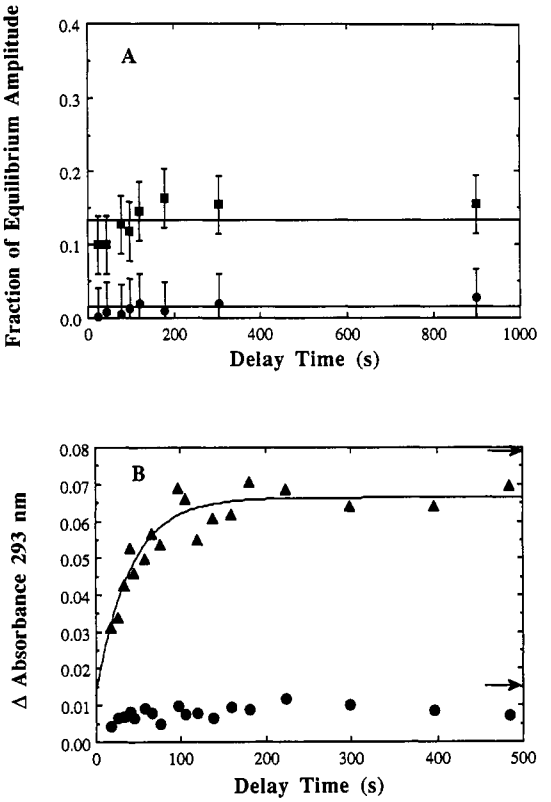

FIGURE 5: Plots of amplitude recovery for the double-jump experiment (native → unfolded → native) for the F31V mutant (A) and the reverse double-jump experiment (unfolded → native → unfolded) for the wild-type protein (B). (A) Normalized amplitudes for the τ_1 (●) and τ_2 (■) refolding phases for the F31V mutant as a function of delay time (time spent at 8 M urea) in the double-jump experiment. The solid lines indicate the average values for the amplitudes. (B) Amplitudes of the τ_1 (●) and τ_2 (▲) unfolded phases of the wild-type protein as a function of delay time (time spent at 0.6 M urea) in the reverse double-jump experiment. The solid line represents a calculated fit to a single-exponential equation as described in the text. Arrows indicate the amplitudes expected from unfolding experiments beginning with fully native DHFR. Solvent conditions were identical to those described in Figure 1.

Conditions for the double-jump experiment must be chosen such that the protein is unfolded rapidly relative to proline isomerization reactions (10–1000 s at room temperature; Grathwohl & Wuthrich, 1981). Because the wild-type DHFR does not meet this criterion (Touchette et al., 1986), the mutant protein in which Phe-31 is replaced with Val was investigated. This mutant unfolds within a few seconds at 8 M urea but exhibits the same refolding kinetics as the wild-type protein (Perry et al., 1987; J. Onuffer, unpublished results). Thus, the folding mechanism is not altered by this mutation.

Figure 5A shows the fractions of the amplitudes recovered in the slow τ_1 and τ_2 phases as a function of the delay time for the 0 → 8.0 → 1.3 M urea double jump. Given that these two reactions comprise the bulk of the unfolding signal detected by any technique, it was surprising to discover that the amplitude associated with the τ_1 and τ_2 refolding phases represented only 2% and 13%, respectively, of the total change in amplitude. Furthermore, these amplitudes do not change with increasing delay time when the experimental error is considered. If proline isomerization in the unfolded protein was to play an important role in defining the channels, the amplitudes of both phases should decrease with increasing delay time as other unfolded conformers become populated. These results are contrary to the predictions of the original folding model and require an alternative explanation (see Discussion).

Interconversions between Native Forms of DHFR. Reverse double-jump experiments were performed to test for interconversions that might occur between native conformers. This procedure involves a double jump from 5.4 → 0.6 → 7.0 M urea, with the time spent at 0.6 M urea recorded as the delay time. Unfortunately, the wild-type protein does not refold rapidly compared to proline isomerization, and there is no appropriate mutant which can resolve this problem. Even with this limitation, some useful information can be obtained from a study of the wild-type protein.

The amplitudes of the τ_1 and τ_2 unfolding phases are plotted as a function of delay time in Figure 5B. The amplitude for the τ_2 reaction is regained with a relaxation time of 40 ± 9 s. This value is within experimental error of the corresponding τ_2 value for refolding at 0.6 M urea (Figure 2), probably reflecting, in part, the limitation of the rate-limiting folding reaction noted above. However, the τ_1 amplitude is recovered within the dead time of manual mixing and transfer (~20 s). This suggests that the species responsible for the τ_1 unfolding reaction can be populated at least in part by an alternative, rapid pathway to the slow, τ_1, refolding reaction. The incomplete recovery of the equilibrium amplitude (Figure 5B, arrows) for both phases may reflect error in the multiple mixing techniques and some irreversibility due to refolding at relatively high protein concentrations (P. Jennings, unpublished data). This discrepancy was shown not to reflect slower folding or redistribution reactions by the fact that no further amplitude was recovered for delay times up to 4 h (data not shown).

DISCUSSION

Parallel versus Sequential Folding Mechanisms. The substoichiometric MTX/DHFR binding study provides a

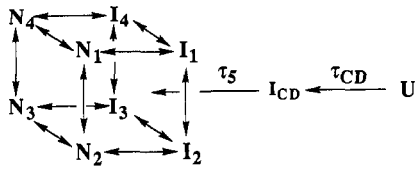

FIGURE 6: Proposed kinetic mechanism for the folding of DHFR. Single-headed arrows in the τ_{CD} and τ_5 phases indicate that these phases are only detected in refolding.

definitive test for choosing between parallel and sequential folding pathways. The selective loss of the slow kinetic phases at 380 nm as the MTX/DHFR ratio falls below 1/1 can only be explained by the four-channel, parallel mechanism. This behavior indicates that all of the available MTX at substoichiometric levels is absorbed by the fast-folding forms. Although the slow-folding reactions certainly occur, there is no corresponding change in the absorbance at 380 nm because there is no free MTX to bind to the slow-folding species. The sequential folding model predicts that all four phases should be detected at all ratios of MTX/DHFR; the amplitudes should diminish in concert, proportional to the ratio of inhibitor to protein. The two-channel model is also not capable of explaining the results of the substoichiometric MTX binding experiments based on similar arguments.

If one makes the assumption that binding of MTX to any of the four native conformers of DHFR results in an identical extinction coefficient change, then the amplitudes of the four binding phases can be used to quantitate the amount of protein folding through each channel. The percentages of the total signal change for the τ_1, τ_2, τ_3, and τ_4 channels are 6%, 13%, 23%, and 58%, respectively, at and above a 1/1 molar ratio of MTX/DHFR [this work and Touchette et al. (1986)]. Thus, the bulk of the protein folds through the faster channels which lead to minor native conformers.

Kinetic Mechanism for the Folding of DHFR. Figure 6 shows a folding mechanism which is consistent with all of the available data. The first step of folding involves a very rapid collapse of the unfolded form to the first intermediate which has a significant content of secondary structure, I_{CD}. This process occurs in the dead time of the stopped-flow CD instrument (18 ms; Kuwajima et al., 1991; 5 ms, B. Jones and C. R. Matthews, unpublished results). The I_{CD} intermediate is then processed to a set of intermediates, I_1–I_4, first detected by fluorescence spectroscopy (Touchette et al., 1986), in the hundreds of milliseconds time range. These I_1–I_4 intermediates then fold to the native forms N_1–N_4 via the four slow-folding reactions τ_1–τ_4. Because only the N_2 species can bind either MTX or NADPH independently, it is presumed to be the catalytically active form. Another form, N_3, may bind the cofactor in the presence of MTX (Frieden, 1990).

Having demonstrated the existence of four folding channels, the issue arises as to when these channels first appear during the folding reaction. Double-jump experiments with the F31V mutant protein at 15 °C demonstrate that the unfolded forms equilibrate much more rapidly than would be expected if proline isomerization controlled interconversion. Within 20 s, the species corresponding to the slow τ_1 and τ_2 phases are fully equilibrated. This result implies that the separation between channels must arise during the folding reaction.

Although a single phase is detected for the τ_5 reaction over the range of urea concentrations from 0.5 to 3.0 M (Figure 2), four distinct intermediates must be produced at this stage to explain the observation of four phases in the MTX binding experiment. If the separation into the four channels were to occur after formation of the intermediate in the τ_5 reaction, i.e., at the point where the native conformers appear, only a single phase would have been detected for MTX binding (Amdur & Hammes, 1966). Therefore, the separation into channels must occur during the τ_5 reaction or the preceding burst-phase reaction which forms I_{CD}. As noted above, the observation of only a single kinetic phase for the τ_5 reaction over a *range of urea concentrations* strongly suggests that the four channels most likely arise during this phase. This set of intermediates appears relatively late in the folding reaction, just prior to the rate-limiting formation of native conformers, and contains both secondary and tertiary structure. Potential structural explanations for the different channels and the formation of the four native conformers will be considered in a future communication (Jennings et al., manuscript in preparation).

The observation of four unfolding phases by both fluorescence and CD spectroscopies supports the existence of multiple native conformers. The smooth connectivity of these relaxation times in unfolding and refolding demonstrates the kinetic reversibility of the corresponding reactions. This observation is also consistent with the two- and four-channel mechanisms but contradicts the expectations of the sequential model containing only a single native conformation (Frieden, 1990). If the rate-limiting step in unfolding is the reverse of the rate-limiting step in refolding, then only a single slow-unfolding phase should have been observed for the one-channel model.

The very rapid recovery of the τ_1 unfolding amplitude in the reverse double-jump experiment (Figure 5B) can only be explained if the N_1 species forms from another native species on a time scale that is short compared to the τ_1 and τ_2 refolding reactions. A direct conversion from either N_3 or N_4 to N_1 under native conditions is proposed because the τ_3 and τ_4 phases are the only refolding reactions sufficiently rapid to account for this behavior. Although the data presented in this paper do not permit a definitive choice, the fact that the τ_1 phase is observed in unfolding (Figure 2) means that the back-reaction from N_1 to its source in the reverse double-jump experiment must be very slow. This implies that the associated equilibrium constant must greatly favor the N_1 form over its precursor. The more logical choice is the N_4 conformer which represents at most a few percent of the native ensemble. Further experiments are required to test this proposal.

The complete recovery of the τ_2 amplitude within 250 s in the reverse double-jump experiment (Figure 5B) indicates that the shuffling of native conformers following refolding must occur on a time scale similar to that of folding itself. Because more than 80% of the protein folds through the τ_3 and τ_4 channels, the amplitude of the major, τ_2, unfolding phase depends critically upon the redistribution of the N_3 and N_4 conformers. The data in Figure 5B show that the equilibrium distribution of native conformers can be attained within 250 s.

Multiple folding channels have also been invoked to explain the folding behavior of cytochrome *c* (Elove & Roder, 1991) and hen lysozyme (Radford et al., 1992). As with DHFR, it appears that the multiple folding pathways observed in lysozyme are *not* related to proline isomerization but to some other heterogeneity. The proposal that the channels in the folding of DHFR arise as the tertiary structure appears suggests that differences in domain/domain orientations may provide an explanation. Another possibility is that peptide bond isomerization at non-proline residues may be sufficiently slow after the tertiary structure has appeared to allow separation into folding channels. A refined X-ray structure

of DHFR (Bystroff & Kraut, 1991) in the absence of inhibitor and/or cofactor shows that the Gly-95–Gly-96 peptide bond is a mixture of the cis and trans isomers, unlike the binary and ternary complexes which show that the cis isomer is preferred (Bystroff et al., 1990; Bolin et al., 1982).

Kinetic Control of Folding. Although protein folding has long been assumed to be under thermodynamic control, recent results on the folding of serpins (Carrel et al., 1991) and the α-lytic protease (Baker et al., 1992) show that kinetic factors can play a dominant role. The folding of DHFR offers a variation on this theme, namely, that kinetic control can occur transiently before being ultimately overridden by thermodynamic factors. The τ_4 phase is the major phase in the development of the native manifold of conformers, as indicated by the binding of 58% of the MTX [this work and Touchette et al. (1986)]. However, over the time course of folding, the native manifold redistributes so that this species diminishes to become a very minor conformer.

The same argument could be made for a slow-folding reaction in ribonuclease A in which a nativelike form with enzymatic activity is the dominant species after 50 s (Schmid, 1983). At the completion of folding, this conformer diminishes to an undetectable level. The transient control of folding by kinetic factors is an alternative to the absolute control seen with serpins (Carrel et al., 1991) or the α-lytic protease (Baker et al., 1992).

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