Refolding of [6-^{19}F]Tryptophan-Labeled *Escherichia coli* Dihydrofolate Reductase in the Presence of Ligand: A Stopped-Flow NMR Spectroscopy Study†

Sydney D. Hoeltzli and Carl Frieden*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Received August 8, 1997; Revised Manuscript Received October 30, 1997⊗

ABSTRACT: *Escherichia coli* dihydrofolate reductase contains five tryptophan residues that are spatially distributed throughout the protein and located in different secondary structural elements. When these tryptophans are replaced with [6-^{19}F]tryptophan, distinct native and unfolded resonances can be resolved in the 1-D ^{19}F NMR spectra. Using site-directed mutagenesis, these resonances have been assigned to individual tryptophans [Hoeltzli, S. D., and Frieden, C. (1994) *Biochemistry 33*, 5502−5509], allowing both the native and unfolded environments of each tryptophan to be monitored during the refolding process. We have previously used these assignments and stopped-flow NMR to investigate the behavior of specific regions of the protein during refolding of apo dihydrofolate reductase from urea in real time. These studies now have been extended to investigate the real time behavior of specific regions of the protein during refolding of dihydrofolate reductase in the presence of either NADP$^+$ or dihydrofolate. As observed for the apoprotein, in the presence of either ligand, unfolded resonance intensities present at the first observed time point (1.5 s) disappear in two phases similar to those monitored by either stopped-flow fluorescence or circular dichroism spectroscopy. The existence of unfolded resonances which disappear slowly indicates that an equilibrium exists between the unfolded side chain environment and one or more intermediates, and that formation of at least one intermediate is cooperative. The results of this study are consistent with previous fluorescence studies demonstrating that dihydrofolate binds at an earlier step in the folding process than does NADP$^+$ [Frieden, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4413−4416] and provide a structural interpretation of the previous results. In the apoprotein as well as in the presence of either ligand, the protein folds via at least one cooperatively formed, solvent-protected intermediate which contains secondary structure. In the presence of NADP$^+$, a stable native-like side chain environment forms in the regions around tryptophans 30, 133, and 47 in an intermediate which cannot bind NADP$^+$ tightly. Native side chain environment forms in the regions around tryptophans 22 and 74 only in the structure which is able to bind NADP$^+$ tightly. In the presence of dihydrofolate, stable native-like side chain environment forms cooperatively in the regions around each tryptophan in a non-native intermediate which must undergo a conformational change prior to binding NADP$^+$. The presence of ligands influences the processes which occur during the folding of dihydrofolate reductase, and the ligand may in effect serve as part of the hydrophobic core.

The information contained in the amino acid sequence of many proteins has long been known to be sufficient to direct the formation of the correct tertiary structure (*1*). Yet, the mechanism by which this occurs remains the subject of intense investigation today. Much experimental attention has been focused upon small, compact proteins and on folding events which occur rapidly. Processes such as proline isomerization or disulfide bond formation, which may be catalyzed in vivo, are sometimes, but not always, responsible for the late steps in protein folding. When recovery of biological activity has been monitored, it has been found to be recovered at a late stage in protein folding (*2*), suggesting that in order to gain a complete understanding of protein folding, these later steps must also be understood.

Structural information about events which occur during protein folding has been difficult to obtain. Information about formation of specific secondary structures has been successfully obtained by combining hydrogen/deuterium exchange with mass spectroscopy and multidimensional NMR spectroscopy (*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, information about the formation of specific regions of tertiary structure and about the formation of native side chain environment during protein folding is still quite limited.

To obtain such information, we have been using ^{19}F NMR spectroscopy to study unfolding (*6*) and refolding (*7*) of *E. coli* dihydrofolate reductase (DHFR)[1] in real time, following changes in the side chain environment rather than the formation of secondary structure. The technique we have used differs from the ^{1}H NMR technique used by several

† This work was supported by National Institutes of Health Grant DK13332.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S0006-2960(97)01962-4 CCC: $15.00
Published on Web 01/06/1998

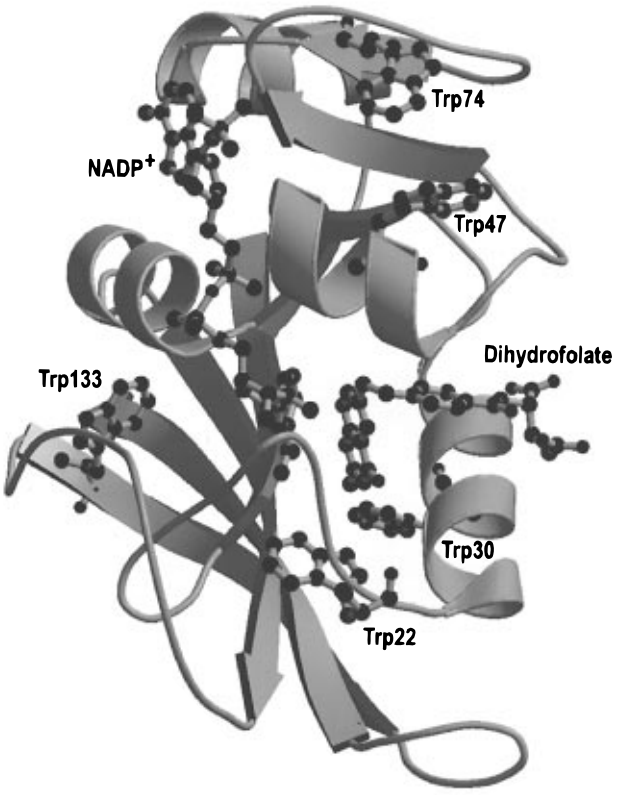


FIGURE 1: Ribbon diagram of the ternary complex (*13*) of *E. coli* DHFR with $NADP^+$ and folate. The side chains of the five tryptophan residues at positions 22, 30, 47, 74, and 133 are represented in ball-and-stick form. In the presence of dihydrofolate (shown in ball-and-stick form), all five tryptophans enter the native-like side chain environment at the same time. In the presence of $NADP^+$ (shown in ball-and-stick form), tryptophans 30, 47, and 133 enter the native-like side chain environment earlier than tryptophans 22 and 74. This figure was prepared using the programs Molscript (*33*) and Raster3D (*34, 35*).

other investigators (*8*) in that, using fluorine NMR, we can resolve and follow individual resonances in the unfolded as well as the native state. In our experiments, the stopped-flow NMR cell (*9*) allows injections to be repeated and the same time intervals averaged, permitting us to follow a process which is completed within several minutes.

E. coli DHFR 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 by a rather complex kinetic mechanism (*10, 11*). Its crystal structure is known in the apo form as well as in several ligand complexes (*12*−*14*), and it is known to undergo conformational change during catalysis. Its folding reaction in the presence of chemical denaturants (*15*) is reversible, and involves several phases whose structural nature has been unclear.

E. coli DHFR contains five tryptophan residues located in different elements of secondary structure and spatially distributed throughout the protein (Figure 1). 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; several lines of evidence indicate that the incorporation of this fluorine label into *E. coli* DHFR is not a structurally perturbing change (*16*).

From our previous stopped-flow NMR study of the changes in the ^{19}F spectrum during denaturant-induced unfolding (*6*), we concluded that the major phase of unfolding involves the formation of an intermediate that retains substantial secondary structure, but in which the side chains possess considerable mobility. Following the formation of this intermediate, all regions of the protein then unfold at equal rates. Recently, we applied stopped-flow ^{19}F NMR spectroscopy to study the behavior of side chains during the refolding of urea-denatured apo *E. coli* DHFR (*7*). We showed that the majority of the protein refolds via at least one intermediate which contains native-like secondary structure, but in which side chains retain considerable mobility until late in the folding process, when the side chain environment becomes stabilized.

In this paper, we show that, as observed for the apoprotein, substantial unfolded resonance intensity remains 1.5 s after initiation of refolding, and disappears in two phases, indicating that an equilibrium exists between the unfolded side chain environment and one or more intermediates. In the presence of $NADP^+$, the native-like apo side chain environment forms in the region surrounding three of the five tryptophans prior to tight $NADP^+$ binding and prior to the final phase observed by fluorescence, circular dichroism, and disappearance of unfolded resonance intensity. This result is similar to that observed for the same three tryptophans during refolding of the apoprotein. Distinct resonances for the remaining two tryptophans appear only for the $NADP^+$-bound form, showing that $NADP^+$ binds tightly during the final step in refolding. In contrast, in the presence of dihydrofolate, native resonance intensity for all five tryptophans appears earlier than the final phase observed by fluorescence, circular dichroism, or disappearance of unfolded resonance intensity, indicating that native-like side chain environment is significantly stabilized in a folding intermediate by the presence of dihydrofolate. These results indicate that the presence of ligands (and, presumably, other cofactors) may significantly alter the folding process.

MATERIALS AND METHODS

Materials. Ultrapure urea and isopropyl β-D-thiogalactopyranoside were purchased from United States Biochemical (Cleveland, OH). Urea stocks were either prepared the day of use or stored in aliquots at −70 °C until the day of use. The concentration of urea was determined by the refractive index at 25 °C using quantitative relationships between concentration and refractive index (*17*). [6-^{19}F]Tryptophan, methotrexate, folate, and methotrexate−agarose were obtained from Sigma (St. Louis, MO). Fast flow DEAE Sepharose was from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade.

Protein Labeling and Purification. DHFR containing [6-^{19}F]tryptophan was purified from 5 L fermentations of the *E. coli* auxotroph W3110TrpA33 containing the plasmid pTrc99DHFR as previously described (*7*). Cells were grown in a Biostat B fermentor (Braun Instruments, Allentown, PA) at 37 °C and pH 7 on M9 minimal medium supplemented with additional phosphate, amino acids, glucose, and vita-

[1] Abbreviations: DHFR, *Escherichia coli* dihydrofolate reductase, EC 1.5.1.3; $NADP^+$, nicotinamide adenine dinucleotide phosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

mins. The cells were harvested at $OD_{600} = 16$, resuspended in fresh medium containing [6-^{19}F]tryptophan in place of L-tryptophan, induced with 1 mM isopropyl β-D-thiogalactopyranoside for 2 h, and then harvested. The yield is approximately 1 mg of protein per 1 g wet weight of cells.

The protein purified from these fermentations was >90% labeled with [6-^{19}F]tryptophan as measured by comparison of the deconvoluted resonance intensity to the intensity of a known reference. The protein was purified and a substoichiometric amount of tightly bound folate removed by denaturation and renaturation as previously described (*16*). It should be noted that purified protein not subjected to denaturation and exhaustive diafiltration invariably contained a mixture of apo and folate-bound forms which could be clearly distinguished by ^{19}F NMR spectroscopy and detected as a discrepancy between the protein concentration determined by active site titration and that determined by absorbance. The absorbance spectrum of protein containing a substoichiometric amount of folate is very similar to that of the apoprotein unless the spectrum from 300 to 400 nm is examined at a protein concentration far higher than the linear range of most spectrometers at 280 nm.

The protein was stored in 80% ammonium sulfate 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 the intrinsic fluorescence using methotrexate, which forms a 1:1 complex, and was within 5% of that determined by absorbance (*18*) after the removal of residual folate.

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, 1 mM DTT, and KCl as indicated, and diluted to the desired enzyme concentration (2−10 μM). For NMR experiments, the protein was exchanged into buffer by diafiltration, concentrated through a YM10 membrane (Amicon, Beverly MA), and diluted with D_2O and 20 mM [4-^{19}F]phenylalanine to achieve a final buffer concentration of 50 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 15 mM DTT, 15% D_2O, 0.3 mM [4-^{19}F]phenylalanine, and KCl as indicated in the figure legends. Protein concentration was never higher than 2.6 mM. No correction to pH was made for D_2O content.

Stopped-Flow Fluorescence and Circular Dichroism Spectroscopy. Stopped-flow fluorescence studies were performed at 5 °C using an Applied Photophysics (Surrey, U.K.) spectrophotometer in the fluorescence mode with a path length of 0.2 cm. Equal volume drive syringes of 2.5 mL were used. Five hundred data points were collected in 5 s, and 500 additional points over the next 500 s. An excitation wavelength of 290 nm was used, and fluorescence was observed using a 305 nm cutoff filter. Five individual injections were averaged and analyzed. Averages of different injections collected on different days produced comparable rates.

The time course of circular dichroism changes was measured at 5 °C using an Applied Photophysics RX1000 rapid kinetics accessory equipped with a 0.2 cm path length stopped-flow cell, equal volume drive syringes of 2.5 mL, and fitted to a JASCO J600 circular dichroism spectrophotometer. Twelve hundred data points were collected at either 20 ms or 0.2 s intervals and combined. Five individual injections were averaged and analyzed. Averages 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 as previously described (*6, 9*). Details of the design of the cell and the mixer are available upon request. At 5 °C, 0.8 mL displaces approximately 90% of the old solution from the coil of the probe. The dead time of the apparatus was estimated at 100 ms.

NMR data were collected at 5 °C on a Varian Unity-Plus spectrometer operating at 470.3 MHz for ^{19}F using either a Nalorac Proton/Fluorine probe (for the dihydrofolate experiments) or a Varian Fluorine superconducting probe (for the $NADP^+$ experiments). All spectra were referenced to an internal standard of [4-^{19}F]phenylalanine. Chemical shifts are given relative to external trifluoroacetic acid. Data were acquired using a modification of the Varian VNMR s2pul pulse sequence (*9*) as previously described (*7*) that delays acquisition for a user-defined interval following an external trigger synchronized to sample injection and mixing. Data for each transient were acquired for 0.536 s using a 90° pulse and a spectral width of 5999 Hz. Periodically, an equilibrium spectrum of refolded protein was acquired following an additional 5 min delay (800 s after initiating refolding). Recycle times of less than 4 times the longest spin−lattice relaxation time (T_1), as previously determined (*16*), were corrected for T_1 assuming that $T_1 \gg T_2$.

The resulting arrayed free induction decays were summed to create a single arrayed FID file. Frequencies and areas were determined by joint analysis of the 100 FIDs using Bayesian analysis (*19*). First point correction was applied, and an initial model of base line and dc artifacts (but no other resonances) was used. Areas obtained by this method were comparable to those obtained by Lorentzian deconvolution of selected resonances in the Fourier-transformed spectrum, but contained less noise.

Protein samples at the concentration used in the NMR experiment were examined for aggregation as previously described (*7*). Samples of concentrated protein denatured at 1.3 mM and incubated in urea for less than 2 h in the presence of a 5-fold excess of DTT showed no evidence of aggregation. During the course of the stopped-flow NMR experiment, protein was denatured for less than 2 h. Active site titration of the refolded material used in the NMR experiment indicated that >85% of the methotrexate binding capacity was recovered.

Data Analysis. Exponential fits were obtained using the program Kaleidagraph (Synergy Software, Reading, PA). Kinetic analysis and fitting were performed using new versions of the programs Kinsim (*20*) and Fitsim (*21*) modified to run as 32-bit programs under DOS and to accept unequal data sampling intervals (*22*).

RESULTS

Stopped-Flow Fluorescence and CD Spectroscopies. The conditions used in these experiments (5 °C, refolding from 5.5 to 2.75 M urea) were chosen to slow refolding as much as possible. Under these conditions, only two of the four fluorescence phases observed at higher temperatures and/or lower final concentrations of urea can be resolved and there

is little (<5%) or no "burst" phase observed either by fluorescence or by CD spectroscopies, suggesting that we may be observing the early events in the refolding process. As previously discussed (*7*), the rate constants and amplitudes of the phases observed by fluorescence vary systematically with temperature, denaturant concentration, and KCl (S. D. Hoeltzli and C. Frieden, unpublished observations), suggesting that the equilibria between species have been affected by our choice of refolding temperature and final urea concentration, but that processes similar to those previously observed are occurring under these conditions. Similar rate constants and amplitudes are observed for refolding wild-type unlabeled and [6-^{19}F]tryptophan-labeled DHFR[2] (S. D. Hoeltzli and C. Frieden, unpublished observations). However, upon refolding in the presence of the ligands dihydrofolate or NADP$^+$, the intrinsic fluorescence of the protein is quenched, making analysis of rates and amplitudes problematic at some ligand concentrations. Changes in intrinsic fluorescence and in circular dichroism at 222 nm under conditions identical to those of the stopped-flow NMR experiment were determined.

NMR Spectroscopy. We have previously assigned the five resonances observed in the ^{19}F NMR spectrum of the native state and the four resonances observed in the ^{19}F NMR spectrum of the unfolded state of *E. coli* DHFR (*16*), and have described the effect of temperature on the chemical shifts of the five individual tryptophan residues in *E. coli* DHFR (*7*). Because five of five native and four of five unfolded tryptophan resonances have distinct chemical shifts, we can monitor changes in both the native and the unfolded environment in real time during refolding. Figure 2 shows the spectra of DHFR at equilibrium in the presence or absence of ligands under the final conditions of the stopped-flow experiments described below, as well as the spectrum of the urea-denatured protein. Several tryptophan resonances undergo significant chemical shift changes (Table 1) upon binding ligand, allowing us to monitor ligand binding at specific regions of the protein during refolding.

Stopped-Flow NMR Spectroscopy: Refolding in the Presence of NADP$^+$. Figure 3 shows the ^{19}F NMR spectra of [6-^{19}F]tryptophan-labeled DHFR at 50 of the 100 time points monitored in real time during refolding on dilution of the protein from 5.5 to 2.75 M urea at 5 °C in the presence of excess NADP$^+$. As noted under Materials and Methods and in the legend to Figure 3, these data were collected using a Varian superconducting fluorine probe. This probe substantially decreases the noise, thus increasing the signal-to-noise ratio by at least a factor of 4 (*23*).

Several observations are immediately apparent. First, as with the apoprotein, a substantial amount of all four unfolded resonances remains 1.5 s after initiation of refolding, and the remaining intensity disappears slowly. Second, little or no native resonance intensity is present at these early time points.[3] Third, several differences from the apo refolding experiment can be noted. Perhaps the most striking is the rapid appearance and disappearance of a resonance 0.2 ppm downfield of the chemical shift expected for Trp47 in the apo form and 0.4 ppm downfield of that expected for Trp47 in complex with NADP$^+$ (Figure 2, middle; Table 1), to be discussed below. It is also clear from examination of Figure 3 that in the presence of NADP$^+$, the native resonances appear at different rates. The areas of these resonances were determined by Bayesian analysis of the FID (as described under Materials and Methods) and further analyzed as described below. When the areas of the native and unfolded resonance for each individual tryptophan are summed for each time point, there is missing total resonance intensity early in the refolding process, indicating the formation of

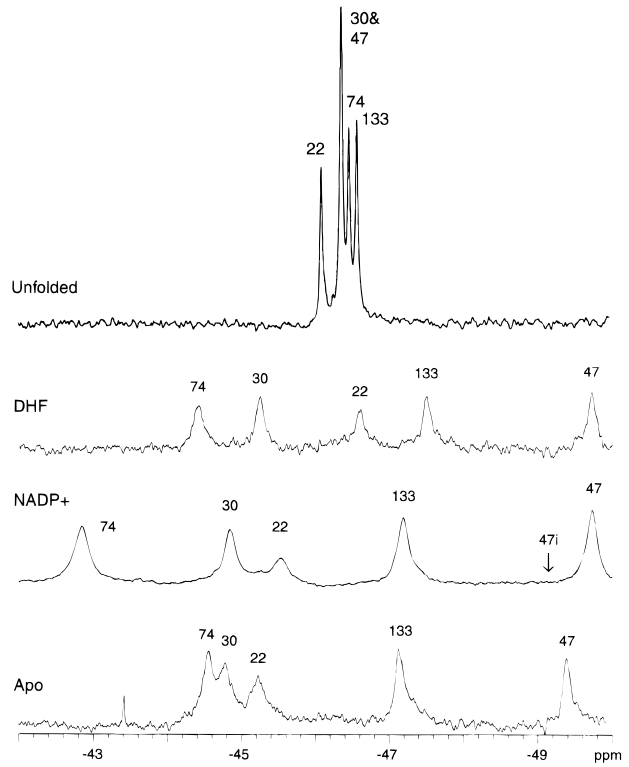


FIGURE 2: ^{19}F NMR spectra of [6-^{19}F]tryptophan-labeled DHFR at 5 °C in the presence of 2.75 M urea and the presence or absence of ligand, as indicated. The chemical shifts of the unfolded protein are indicated by lines. Bottom: apoprotein; 75 transients were collected using a Nalorac proton/fluorine probe with a recycle time of 3.2 s ($4 \times T_1$). Second from bottom: 3.8 mM NADP$^+$; 65 transients were collected using a Varian superconducting fluorine probe and a recycle time of 3.2 s. The chemical shift of a resonance observed upon refolding in the presence of NADP$^+$ is indicated by an arrow. Third from bottom: 1.0 mM DHF; 65 transients were collected using a Nalorac proton/fluorine probe and a recycle time of 3.2 s. Top: unfolded protein in the presence of 6.7 M urea; 65 transients were collected using a Nalorac proton/fluorine probe and a recycle time of 3.2 s. Other experimental conditions were as given under Materials and Methods.

Table 1: Chemical Shifts (ppm) of *E. coli* DHFR in 2.75 M Urea at 5 °C

	unfolded	apo	DHF	$\Delta\delta^a$	NADP$^+$	$\Delta\delta^b$
Trp22	−46.1	−45.4	−46.5	−1.1	−45.8	−0.4
Trp30	−46.3	−44.9	−45.1	−0.2	−44.9	0.0
Trp47	−46.3	−49.3	−49.6	−0.3	−49.6[c]	−0.3
Trp74	−46.4	−44.6	−44.3	0.3	−42.9	1.5
Trp133	−46.5	−47.2	−47.4	−0.2	−47.3	−0.1

[a] From apo form to DHF-bound form. [b] From apo form to NADP$^+$-bound form. [c] The chemical shift of the early appearing resonance (see text) is −49.17 ppm.

[2] In contrast to the wild-type protein, the fluorescence of the [6-^{19}F]-Trp-labeled protein increases for all phases observed upon refolding.

[3] This observation confirms our hypothesis that the native resonances observed at the earliest time points during the refolding of the apoprotein were probably folded material remaining from the previous injection (*7*).

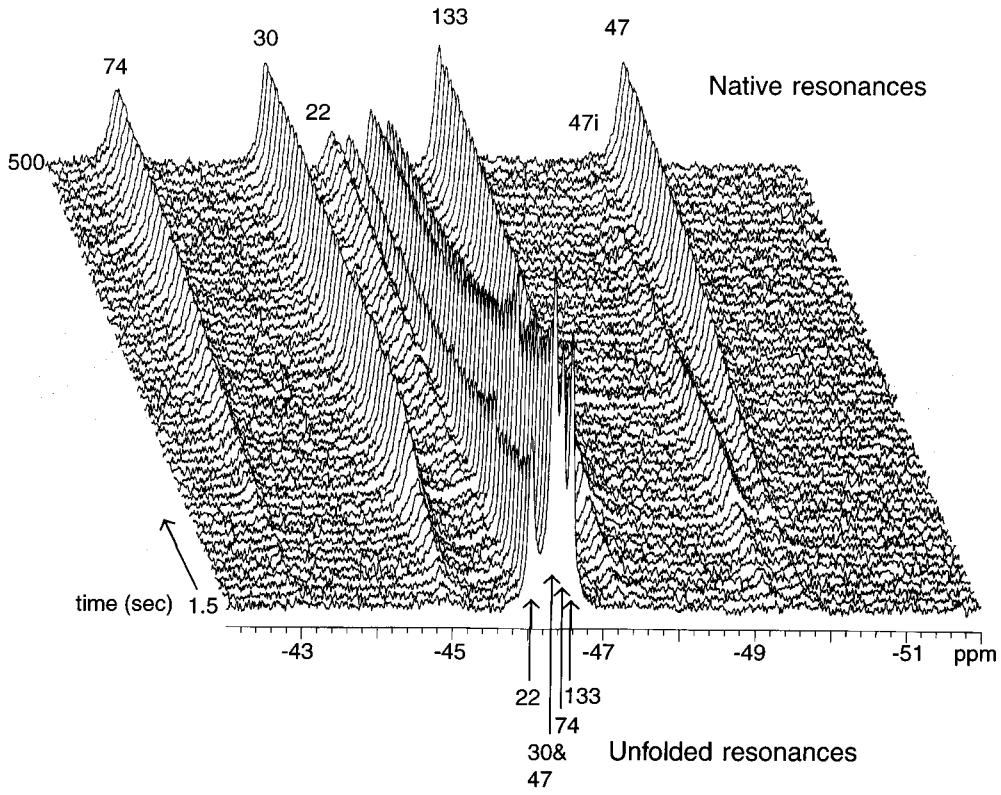


FIGURE 3: Stopped-flow ^{19}F NMR spectra of refolding of [6-^{19}F]tryptophan-labeled DHFR on dilution from 5.5 to 2.75 M urea at 5 °C in the presence of 3.8 mM NADP$^+$. The time of the data collection is shown on the *z*-axis. The final protein concentration was 0.61 mM. The buffer used contained 50 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 15 mM DTT, and no KCl. Data were collected using a Varian superconducting fluorine probe. Other experimental conditions are described under Materials and Methods. For each injection, 100 time points, each a single free induction decay, were obtained. Forty-one separate injections were summed for each time point and Fourier-transformed with a line broadening of 5 Hz. Of 100 time points, 50 are shown. The time points are not equally spaced; the first 10 spectra shown were collected 1.5, 3.6, 5.7, 9.8, 11.9, 14, 16, 18, 20, and 22 s after initiation of refolding. The next 10 spectra were collected at 3.2 s intervals and the remaining spectra at 6.9 s intervals. Because the first 20 spectra were collected with a recycle time $<4 \times T_1$, the resonance intensities between 4.6 and 21 s are expected (and appear) to decrease. Little or no native resonances are observed at early times (less than 20 s).

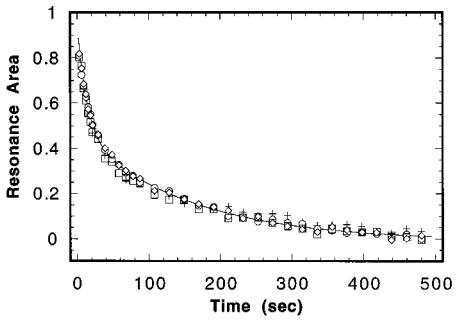


FIGURE 4: Time dependence of the changes in area of the four resonances assigned to Trp22 (+), tryptophans 30 and 47 (○), Trp74 (◇), and Trp133 (□) in the unfolded state during refolding in the presence of NADP$^+$. Data were collected and resonance areas determined as described under Materials and Methods and the legend for Figure 3. The data were fit to a two-exponential function (—) to yield the rates and amplitudes reported in Table 2. A three-exponential function did not improve the fit. Data shown in this plot were normalized to a total amplitude change of 1.

an intermediate which cannot be detected by ^{19}F NMR.

Unfolded Resonances in the Presence of NADP$^+$. As observed for refolding of the apoprotein, the disappearance of each of the four unfolded tryptophan resonances in the presence of NADP$^+$ (Figure 4) can be fit by a two exponential function (Table 2). The four unfolded resonances disappear with similar rate constants, indicating that these regions of the protein emerge from an unfolded environment at the same time. Thus, the formation of at least one intermediate is cooperative.

Table 2: Refolding in the Presence of NADP$^+$: Fluorescence, Far-UV CD (222 nm), and Disappearance of Unfolded ^{19}F NMR Resonances

	A_1	k_1	A_2	k_2
fluorescence (apo)[a]	−0.100	0.071	−0.049	0.006
50:1 NADP$^+$:DHFR	−0.125	0.087	0.041	0.003
circular dichroism (apo)[a]	3.44	0.082	3.05	0.006
^{19}F NMR[b]				
Trp22	1765	0.062	1400	0.006
Trp30&47	3411	0.074	3450	0.007
Trp74	2302	0.079	1948	0.006
Trp133	1988	0.073	2067	0.006

[a] All experiments were carried out in 50 mM phosphate, pH 7.2, 0.1 mM EDTA, no KCl, and 1 mM DTT at 5 °C. Fluorescence: 2 μM DHFR. Amplitudes for fluorescence are given as voltage. CD: 10 μM DHFR. Amplitudes for CD are given as millidegrees at 222 nm. Data were fit to a double exponential equation using the program Kaleidagraph (Synergy Software, Reading, PA). Estimated error of 10%. A small third phase may be present but cannot be resolved. [b] Data were collected using 610 μM DHFR in 50 mM phosphate, pH 7.2, 0.1 mM EDTA, 15 mM DTT, and no KCl at 5 °C. Amplitudes were determined by joint Bayesian analysis of the FIDs for all 100 time points. Estimated experimental and fitting error of about 10%.

Fluorescence data (Table 2) for refolding in the presence of a 50-fold excess of NADP$^+$ reveal that NADP$^+$ quenches the fluorescence of only the second phase observed. The

Table 3: Appearance of Native Resonances during Refolding

	with NADP$^+$ [a]				with dihydrofolate[d]	
	A	*k* (s^{-1})	*A*	*k* (s^{-1})	*A*[e]	*k* (s^{-1})
Trp22			2625	0.005	nd[f]	nd[f]
Trp30	7135[b]	0.037[b]	−3657[c]	0.009	5864	0.017
Trp47i	4659	0.037	−4129	0.008		
Trp47			4417	0.005	5640	0.014
Trp74			4144	0.005	6254	0.014
Trp133	2067	0.038	2344	0.004	6196	0.016

[a] Data were collected in 50 mM phosphate, pH 7.2, 0.1 mM EDTA, 15 mM DTT, and no KCl at 5 °C. Areas were determined by joint Bayesian analysis of the FIDs for all 100 time points. Estimated experimental and fitting error of about 10%. Data were fit to a double or single exponential equation using the program Kaleidagraph (Synergy Software, Reading, PA); where one amplitude and rate constant are given, a second exponential did not significantly improve the fit. [b] For this resonance only, the exponential fit to heights determined by Lorentzian deconvolution of each individual FID differed and could be fit by a single exponential function with a rate constant of 0.050 s^{-1}. [c] Negative amplitudes indicate a decrease in area. [d] Estimated experimental and fitting error of about 20%. Data were collected in 50 mM phosphate, pH 7.2, 100 mM KCl, 0.1 mM EDTA, and 15 mM DTT at 5 °C. [e] Data were fit to a single exponential equation using the program Kaleidagraph (Synergy Software, Reading, PA). Amplitudes were determined by joint Bayesian analysis of the FIDs for all 100 time points. [f] Because the resonance for native Trp22 in the binary dihydrofolate complex overlaps those for Trp74 and Trp133 in the unfolded state, this could not be determined.

second rate constant observed for the disappearance of unfolded resonances also corresponds very well to the second rate constant observed by fluorescence and circular dichroism spectroscopy (Table 2). The first rate constant observed by the latter techniques may be somewhat larger, possibly due to the contribution of a third, faster phase of amplitude too small to resolve under these experimental conditions and too rapid to be observed by the NMR experiment.

Native Resonances in the Presence of NADP$^+$. As indicated above, there are striking differences in the behavior of the five different native tryptophan resonances when refolded in the presence of NADP$^+$ (Table 3). Two examples are shown in Figure 5. The changes in the area of a resonance at −49.2 ppm, near, but not at, the chemical shift of Trp47 in the apo form during refolding (top, open circles), can be fit by a two-exponential function in which the amplitude of the first phase increases, followed by a decrease in the amplitude of the second phase. The changes in the area of the NADP$^+$-bound Trp47 resonance at −49.6 ppm during refolding (top, filled circles) are well fit by a single exponential function with a rate constant slightly smaller than that for the disappearance of the resonance at −49.2 ppm. Similarly, the changes in the areas of the native, NADP$^+$-bound resonances for tryptophans 22 and 74 during refolding are fit by a single exponential (Table 3). In contrast, the changes in the areas of the Trp133 resonance (bottom, open circles) and of the Trp30 resonance (Table 3) during refolding require a two-exponential function to obtain a good fit.

In order to interpret these differences, we must first consider the chemical shift changes observed upon NADP$^+$ binding (Figure 2; Table 1). Tryptophans 30 and 133 undergo little (less than 0.1 ppm) change upon binding NADP$^+$. The chemical shift of Trp74 changes dramatically (+1.5 ppm), Trp22 shifts −0.4 ppm, and Trp47 shifts −0.3 ppm upon NADP$^+$ binding. Thus, either the apo or the NADP$^+$-bound form of the protein could be responsible for

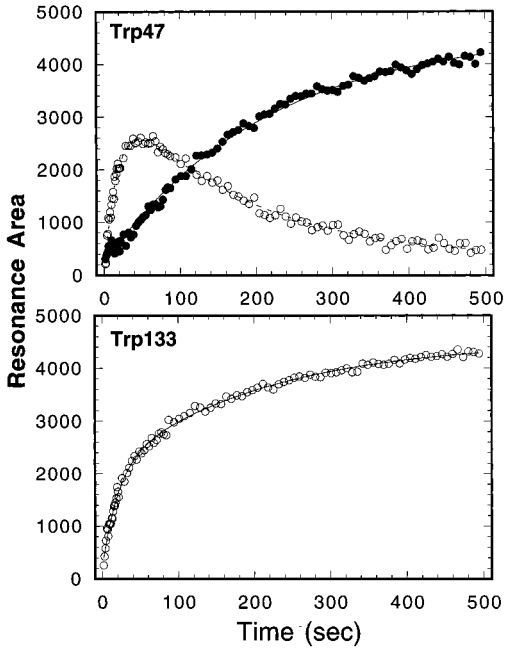


FIGURE 5: Time dependence of the area of the resonances assigned to Trp47 (top) and Trp133 (bottom) in a native-like intermediate or native environment during refolding in the presence of NADP$^+$. Data were collected and resonance areas determined as described under Materials and Methods and the legend to Figure 3. The data were fit to a single or a double exponential function (—) to yield the rates and amplitudes reported in Table 3. (Top) A resonance near, but not at, the native, apo chemical shift for Trp47 (○) appears rapidly, and then disappears with a rate constant slightly larger than the rate constant for the appearance of the native, NADP$^+$-bound resonance (●). A two-exponential function does not improve the fit to the latter. Bottom: The apo and NADP$^+$-bound resonances for Trp 133 (○) have similar chemical shifts. A two-exponential function is required to obtain a good fit.

the resonance intensity observed at the chemical shift of Trp30 or Trp133, while the resonances assigned to the apo and the NADP$^+$-bound form of the protein are distinct for tryptophans 22, 47, and 74.

Given the chemical shift changes induced by NADP$^+$ binding, these results suggest the following interpretation: native-like side chain environment forms early in the regions of the protein around tryptophans 30, 47, and 133 in an intermediate which cannot bind NADP$^+$ tightly. The apo, native chemical shift is resolved from that of the native, NADP$^+$-bound form for Trp47; thus, a distinct resonance for Trp47 in this intermediate appears and disappears during the folding process. This resonance does not represent native DHFR since (1) its chemical differs from that of the apoprotein and (2) the rate constant for the disappearance of this resonance (0.008 s^{-1}) is much smaller than the rate constant for binding of NADP$^+$ (faster than 10^6 M^{-1} s^{-1}) to the apo, native protein under these experimental conditions (Hoeltzli and Frieden, unpublished observations). In contrast, since the native, apo-, and NADP$^+$-bound chemical shifts are not resolved for tryptophans 30 and 133, distinct resonances for the intermediate are not observed, and two processes are represented by the appearance of the areas of these resonances: (1) the formation of an intermediate containing native-like side chain environment; (2) the slow conversion of this intermediate to a form able to bind NADP$^+$ tightly.

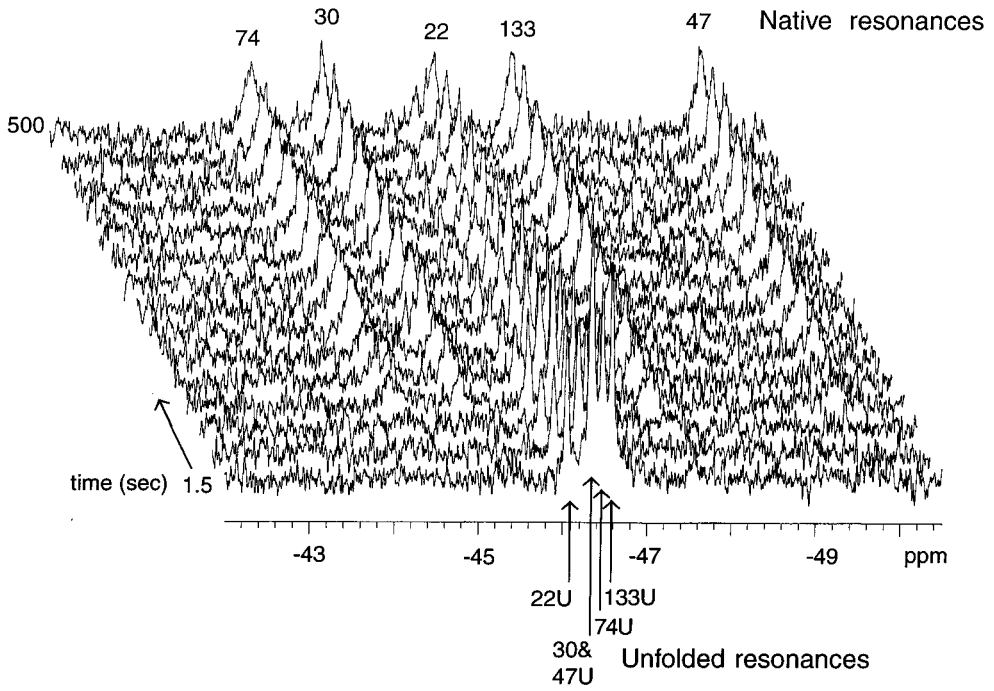


FIGURE 6: Stopped-flow ^{19}F NMR spectra of refolding of [6-^{19}F]tryptophan-labeled DHFR on dilution from 5.5 to 2.75 M urea at 5 °C in the presence of 1.0 mM dihydrofolate. The time of data collection is shown on the *z*-axis. The final protein concentration was 0.65 mM. The buffer used contained 50 mM potassium phosphate, pH 7.2, 100 mM KCl, 0.1 mM EDTA, and 15 mM DTT. Data were collected using a Nalorac proton/fluorine probe. Other experimental conditions are described under Materials and Methods. For each injection, 100 time points, each a single free induction decay, were obtained. Fifty-six separate injections were summed for each time point and Fourier-transformed with a line broadening of 5 Hz. Fifteen of 100 time points are shown. The time points are not equally spaced; the data shown were collected 1.5, 7.7, 15, 22, 45, 68, 94, 142, 191, 239, 287, 335, 383, 432, and 480 s after initiation of refolding. Because the first 20 spectra were collected with a recycle time $<4 \times T_1$, the resonance intensities between 4.6 and 21 s are expected (and appear) to decrease. At the current signal-to-noise ratio, it cannot be determined whether or not the intensities decrease to a greater extent than predicted for the recycle time used by the measured T_1. In this experiment, there are little or no native resonances at early times (less than 20 s).

This native-like intermediate contains little if any native side chain environment in the regions around tryptophans 22 and 74 (since distinct resonances are not observed near the apo chemical shifts of these tryptophans). Native-like side chain environment apparently forms in the region around tryptophans 22 and 74 at the same time the protein becomes able to bind $NADP^+$ tightly.

These results are similar to the results of our previous study of the refolding of apo DHFR, in which analysis of native resonance areas indicated that tryptophans 30, 47, and 133 sampled a native-like environment earlier than did tryptophans 22 and 74. However, in this previous study, the resonances in this native-like environment had non-native relaxation properties, as revealed by differences between rate constants obtained by fitting resonance heights and those obtained by fitting resonance areas[4] obtained from a least-squares fit of Lorentzian line shapes to the Fourier-transformed spectrum.

In contrast, in the presence of $NADP^+$, for tryptophans 22, 47, 74, and 133, the rate constants are the same regardless of whether heights or areas are analyzed (data not shown). This result indicates that the native structure which forms early has native-like relaxation properties and is presumably stabilized either by weak interactions with $NADP^+$ or by slow exchange with a small population of the native, $NADP^+$-bound form. However, for Trp30, the results obtained by analyzing areas differed from those obtained by analyzing heights (Table 3). The heights so obtained can be fit by a single exponential with a rate constant (0.050 s^{-1}) similar to the rate constant (0.037 s^{-1}) of the faster phase observed for Trp133. The areas require a two-exponential function with rate constants similar to those obtained by the Bayesian analysis. This result probably indicates the existence of chemical shift heterogeneity (as discussed below).

In the apo form of the protein at 5 °C, the resonances of Trp22 and Trp74 flank that of Trp30 (Figure 2); it is possible that some native, apo resonance intensity from either or both of these resonances is responsible for the excess amplitude observed in the first phase (Table 3), but (in contrast to Trp47) with a contribution too small to resolve from the apo resonance of Trp30. If native-like structure formed around Trp74 early in the folding process, as it does for Trp47, it should have been observable.

The total amplitude for Trp22 (Table 3) is less than that observed for the other resonances. In the ^{19}F NMR spectrum of the NADPH-bound form, the resonance for Trp22 is broadened almost into the base line (*16*). It is less broadened in the presence of $NADP^+$ (Figure 2, middle), but there is still missing intensity at equilibrium, probably as a consequence of molecular motion in this region of the protein.

Refolding in the Presence of Dihydrofolate. Figure 6 shows the ^{19}F NMR spectra of [6-^{19}F]tryptophan-labeled DHFR at 15 of the 100 time points monitored in real time

[4] The rate constants obtained by fitting resonance areas would be expected to be the same as those obtained by fitting resonance heights unless relaxation effects, chemical shift heterogeneity, or conformational exchange alter the line width, and thus the ratio of resonance height to resonance area during the folding reaction.

during refolding on dilution of the protein from 5.5 to 2.75 M urea at 5 °C in the presence of excess dihydrofolate. As noted under Materials and Methods, these data were collected using a conventional proton/fluorine probe, and the signal-to-noise ratio is thus lower than in the $NADP^+$ experiment.

As observed for the apoprotein (*7*) and for refolding in the presence of $NADP^+$, a substantial amount of all four unfolded resonances remains 1.5 s after initiation of refolding and the remaining unfolded resonance intensities disappear slowly. In contrast to the results obtained in the presence of $NADP^+$, the first readily observable native resonances (for tryptophans 30, 47, 74, and 133) appear at the chemical shifts of the dihydrofolate-bound form (given in Table 1).

In the presence of dihydrofolate, only four of the five native resonances are resolved during refolding (Figure 2). The resonance assigned to Trp22 in the native, dihydrofolate-bound form overlaps that assigned to Trp133 in the unfolded form (Table 1). Upon binding dihydrofolate, Trp22 undergoes the most significant change in chemical shift (−1.1 ppm); tryptophans 30, 47, 74, and 133 all change by about −0.2 ppm (∼100 Hz). Since the line widths of the native resonances are all around 50 Hz, if a significant population of native, apoprotein were present, this change in chemical shift should be discernible at least as broadening in the observed resonances. It is possible that a small population of native, apo resonances exists but cannot be detected due to limitations in the signal-to-noise ratio. This result suggests that, in contrast to the results obtained for refolding in the presence of $NADP^+$, the earliest native-like side chain environment is formed in structures able to bind dihydrofolate. As for the $NADP^+$ refolding experiment, the areas of these resonances were determined by Bayesian analysis of the FID (as described under Materials and Methods) and further analyzed as described below. When the areas of the native and unfolded resonance for each individual tryptophan are summed for each time point, there is more missing total resonance intensity early in the refolding process than was observed for refolding in the presence of $NADP^+$, probably because the dihydrofolate experiment contained salt, which slows refolding. As observed for refolding in the presence of $NADP^+$, the missing intensity suggests the formation of an intermediate which cannot be observed by ^{19}F NMR.

Unfolded Resonances in the Presence of Dihydrofolate. The disappearance of the resonance assigned to Trp47 in the unfolded environment is shown in Figure 7 (open circles). As observed for the apoprotein and for refolding in the presence of $NADP^+$, the areas of each of the four resonances assigned to the five individual tryptophans in the unfolded environment can be fit by a two-exponential function (Table 4) and disappear with similar rate constants, indicating that in all three cases these regions of the protein emerge from an unfolded environment at the same time and thus the formation of at least one intermediate is cooperative.

Native Resonances in the Presence of Dihydrofolate. The increase in the areas of the resonance assigned to Trp47 in the native dihydrofolate-bound environment is shown in Figure 7 (filled circles). Each of the four resonances assigned to the four resolved tryptophans can be fit by a single exponential function (Table 3). While the quality of the data does not permit the presence of a second, slow phase to be completely excluded, a two-exponential function does not significantly improve the fit. In contrast to the results

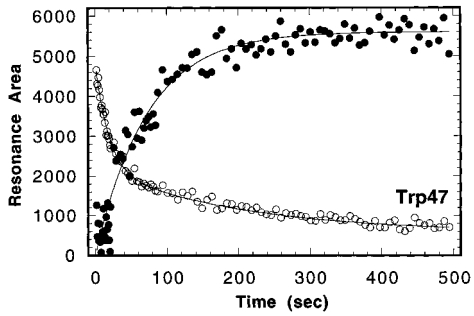


FIGURE 7: Time dependence of the area of the resonances assigned to Trp47 in the unfolded state (○) and in a native-like intermediate or in the native state (●) during refolding in the presence of dihydrofolate. Data were collected and resonance areas determined as described under Materials and Methods and the legend to Figure 6. The data were fit to double (unfolded) or single (native-like) exponential function (—) to yield the rates and amplitudes reported in Table 4 and Table 3, respectively.

Table 4: Refolding in the Presence of Dihydrofolate: Fluorescence, Far-UV CD (222 nm), and Disappearance of Unfolded Resonances

	A_1	k_1	A_2	k_2
fluorescence (apo)[a]	0.0060	0.064	0.0030	0.003
4:1 dihydrofolate:DHFR	−0.0006	0.021	−0.0097	0.007
circular dichroism (apo)[a]	9.14	0.074	5.30	0.008
10:1 dihydrofolate:DHFR	9.59	0.110	5.56	0.009
^{19}F NMR[b]				
Trp22	1571	0.025	1184	0.003
Trp30&47	2649	0.037	3194	0.004
Trp74[c]	1137	0.034	1295	0.005
Trp133[c]	2065	0.035	2076	0.008

[a] Fluorescence: 2 μM DHFR. Amplitudes for fluorescence are given as voltage. CD: 10 μM DHFR. Amplitudes for CD are given as millidegrees at 222 nm. Data were fit to a double exponential equation using the program Kaleidagraph (Synergy Software, Reading, PA). Estimated error of 10%. All experiments were carried out in 50 mM phosphate, pH 7.2, 100 mM KCl, 0.1 mM EDTA, and 1 mM DTT at 5 °C. [b] Amplitudes were determined by joint Bayesian analysis of the FIDs for all 100 time points, as described under Materials and Methods. Estimated experimental and fitting error of about 20%. Data were collected in 50 mM phosphate, pH 7.2, 100 mM KCl, 0.1 mM EDTA, and 15 mM DTT at 5 °C. [c] Corrected for the resonance of Trp22 in the binary dihydrofolate complex by subtracting an exponential function of rate constant 0.016 and amplitude equal to the mean value remaining at this chemical shift after refolding, at equilibrium.

obtained for refolding in the presence of $NADP^+$, the rate constants for the appearance of the native resonance intensity of all four resolved tryptophans are similar. This result indicates that, in the presence of dihydrofolate but not of $NADP^+$, all regions of the protein appear to form stable, native-like side chain environment at the same time, and in this sense the folding of the protein in the presence of dihydrofolate appears to be cooperative.

In the presence of dihydrofolate, the rate of appearance of the native resonances is significantly faster than the rate observed for the second phase of disappearance of unfolded resonance intensity. All of the expected amplitude is observed at the end of the single exponential function, indicating that there is no significant second phase. As with the $NADP^+$ data, the same rate constants were obtained whether heights or areas obtained from the Fourier-transformed dihydrofolate spectra were analyzed. This observation indicates that, as observed for refolding in the presence of $NADP^+$, dihydrofolate stabilizes the native-like sidechain environment at an earlier point in the folding

process than observed during refolding of the apoprotein.

Fluorescence data (Table 4) for refolding in the presence of a 4-fold excess of dihydrofolate reveal that dihydrofolate alters the amplitude of both phases observed. However, refolding in the presence of lower ratios of dihydrofolate to protein suggests that dihydrofolate binds more tightly to the structure formed during the second phase. It should be noted that the CD and fluorescence experiments were conducted at 10 and 2 μM DHFR, respectively, 60 or 300 times lower than the concentration (about 600 μM) used for the NMR experiments. Thus, a relatively weak binding interaction not observed by fluorescence could be detected at the protein concentration used in the NMR experiment.

DISCUSSION

In this work, we have extended our previous stopped-flow ^{19}F NMR studies of the folding of *E. coli* DHFR in real time (*6*, *7*) to examine its refolding in the presence of ligands. We are able to follow both loss of unfolded environment and appearance of native side chain environment, and to compare the results so obtained with the results of CD and fluorescence studies of refolding in the presence of ligands and with our previous results obtained for refolding of the apoprotein (*7*). As far as we are aware, this report presents the first structural information obtained for any protein by direct comparison of refolding in the presence and absence of ligands.

This comparison reveals several similarities. In the presence of either dihydrofolate or $NADP^+$, as well as in our previous study (*7*) of the apoprotein, the unfolded protein does not all collapse to an intermediate as an early event in folding, but, rather, substantial unfolded environment remains 1.5 s after initiation of refolding, as reflected by the unfolded resonance intensities assigned to all five tryptophans. Those resonances disappear in two phases, revealing that at least two processes are involved. In the presence of either ligand as well as in the apoprotein, formation of native side chain environment occurs more slowly than does the fast phase of loss of unfolded environment (with missing intensity). This result suggests two conclusions. First, the unfolded protein must be in equilibrium with an early intermediate. Second, the formation of this early intermediate is cooperative, since the unfolded resonances for at least four of the five tryptophans disappear at the same rate. As previously observed for the apoprotein, major fluorescence and CD changes appear to correspond more closely to loss of unfolded environment rather than to formation of native side chain environment. This observation suggests that, for *E. coli* DHFR, secondary structure formation (as measured by CD changes) and altered solvation (as measured by fluorescence) correspond more closely to formation of an early, collapsed intermediate (not observable by ^{19}F NMR) rather than to formation of native tertiary structure. This observation may prove general, but so far there is little information about the rate of disappearance of unfolded environment at specific residues or in specific regions available for other proteins.

The appearance of native resonances reveals striking differences between the refolding of the apo form, the dihydrofolate-bound form, and the $NADP^+$-bound form. In the apoprotein, three of the five tryptophans (tryptophans 30, 47, and 133) showed evidence of native-like side chain environment appearing prior to the final step, but this native-like environment had non-native relaxation properties or non-native chemical shift heterogeneity, or both. During refolding of apo DHFR, formation of stable, native side-chain environment for all five tryptophans is a late step in the folding process (*7*).

In the presence of dihydrofolate, formation of stable native-like side chain environment occurs in a single phase, more rapidly than the second phase of loss of unfolded environment, and at the same rate for tryptophans 30, 47, 74, and 133 (and probably for Trp22 as well). Essentially all the expected amplitude appears in this phase. It is not possible for two separate populations of unfolded protein, refolding at different rates, to form native-like side chain environment in a single phase faster than one of the rates at which the unfolded environment disappears, but this result would be expected if the second phase observed for disappearance of unfolded environment results from an equilibrium between unfolded protein, one or more intermediates, and the final native form. The simplest explanation of these results is that dihydrofolate binds to and stabilizes the side chain structure in an intermediate, prior to the final step in protein folding. This observation agrees with fluorescence results showing that dihydrofolate is able to quench the intrinsic protein fluorescence of both phases observed under the conditions of the NMR experiment (Table 4). Again, in the sense that native resonances appear at the same rate in all examined regions of the protein, formation of native side chain environment in the presence of dihydrofolate is cooperative.

The behavior of the protein refolded in the presence of $NADP^+$ is more complex. Formation of an intermediate containing stable, native side chain environment in the region near tryptophans 30, 47, and 133 occurs prior to the final step in protein folding, and prior to the binding of $NADP^+$. The intermediate then undergoes a conformational change to a structure able to bind $NADP^+$ tightly. Because the apo and $NADP^+$ -bound chemical shifts are well-resolved for Trp47, a distinct resonance can be observed Trp47 in this intermediate; for tryptophans 30 and 133, the apo and $NADP^+$-bound chemical shifts are not resolved, and the formation of this intermediate is evident as a rapid phase. Native-like environment forms in the regions near tryptophans 22 and 74 in this latter structure, as evidenced by the lack of significant resonances at the apo chemical shift for these tryptophans. This observation agrees with fluorescence results showing that $NADP^+$ is able to quench the intrinsic protein fluorescence of the second phase observed under the conditions of the NMR experiment (Table 2).

Previous fluorescence studies of the refolding of DHFR in the presence of ligands (*2*) revealed that dihydrofolate binds earlier in the folding process than does $NADP^+$. This stopped-flow NMR study confirms this result, but provides for the first time a structural interpretation. In the presence of $NADP^+$, native-like side chain environment forms in some regions prior to formation of a structure able to bind $NADP^+$ tightly. In contrast, dihydrofolate is able to bind to the earliest intermediate containing native-like side chain environment, and alters the structure of this intermediate by stabilizing native-like side chain structure in all regions of the protein probed by the five tryptophans. However, this

structure which binds dihydrofolate and which possesses native-like side chain environment is not the native protein, but must undergo a further, slow, conformational change. Three lines of evidence demonstrate that the structure which binds dihydrofolate is not the native structure: (1) the results of the earlier fluorescence study which demonstrated that during refolding in the presence of dihydrofolate and $NADP^+$, $NADP^+$ affects only the amplitude of the final phase (*2*); (2) enzymatic activity is recovered only during the final phase of the refolding process; (3) in this study, the observation of a slow phase for the disappearance of unfolded protein after the formation of this intermediate. The structural basis of this conformational change is as yet unclear. While the results of this study are consistent with previous observations, this study reveals that the presence of different ligands may affect the stability of structures formed during the refolding of the protein; in effect, the ligand may influence the folding pathway.

These interpretations do not depend upon specific knowledge of the structure, but they are consistent with the crystal structures of the ligand complexes (*13*). A diagram of the ternary complex with $NADP^+$ and folate is shown in Figure 1. All regions of the protein are well-ordered in the ternary and the binary folate complex. Folate binds in a hydrophobic cleft which roughly bisects the molecule. Our results demonstrate that in the presence of DHF, native side chain environment is stabilized in the regions of the protein surrounding all five tryptophans during an early point in the folding process. In the presence of $NADP^+$, native side chain environment is stabilized first in the regions of the protein surrounding tryptophans 30, 47, and 133. Tryptophans 30 and 133 flank a region of the β-strand core of the protein in which amide protons show the earliest protection from exchange with deuterium (*24*). Trp47 is located in an α-helix which does not itself show early protection from exchange, but which is adjacent to two β-strands (βB and βE) which do. In the binary complex with $NADP^+$, the loop between residues 16−20 is disordered (*13*). Trp22 is adjacent to this loop. Previous results (*16*) as well as results from this study (Figure 2) indicate that in the native binary complex with $NADP^+$ at equilibrium, this region of the protein has unusual motional properties. Thus, it is not surprising that the native structure in this region of the $NADP^+$·DHFR complex should be stabilized late. In the folate complex, Trp22 is adjacent to the pyrimidine ring, and its structure would be expected to be stabilized by the presence of ligand.

It is somewhat surprising that the region around Trp74 is slow to form native-like structure in the presence of $NADP^+$. Trp47 and Trp74 (although not the 6- position of each) are within 4 Å of each other. Although none of the tryptophans make direct contact with either ligand, Trp74 is close to the 2′-phosphate of $NADP^+$ and undergoes a pronounced change in chemical shift upon $NADP^+$ binding. Hence, it might be expected to be stabilized by the presence of even weak interactions with $NADP^+$. However, Trp74 is located in a "coil" region at the surface of the protein and thus may have fewer stabilizing contacts. It has been proposed (*25, 26*) that hydrophobic collapse of Trp47 and Trp74 into a native-like environment (an "exciton pair") is an early step in the folding of DHFR. Our data do not support this conclusion. It should be noted that no amides in this region demonstrate early protection from exchange with deuterium (*24*).

Many previous studies of DHFR folding (*15, 25, 27, 28*) have focused upon proposed folding mechanisms, many quite complex and involving multiple pathways as well as multiple intermediates (*26, 29, 30*). The results presented in this study, and in our previous stopped-flow NMR studies (*6, 7*), can be adequately modeled by a simple sequential series of events. There is seldom a unique mechanism able to model a set of kinetic processes, especially when the structural interpretation of the amplitude changes observed (e.g., changes in fluorescence) is uncertain. However, it should be emphasized that the existence of multiple processes does not demonstrate the existence of multiple pathways, even when the structural basis of an observed amplitude change is known with certainty (as with the several phases observed for the disappearance of the unfolded environment). This is especially true of processes which possess some degree of reversibility.

Four critical observations emerging from this study are the following: (1) dihydrofolate binds to an earlier stage in the folding process than does $NADP^+$ and stabilizes the side chain environment in the regions of the protein near all five tryptophans in this non-native structure; (2) at least two processes (one slower than the formation of some native side chain environment) affect the disappearance of the unfolded environment; (3) some of the total resonance intensity for each tryptophan is missing during the first seconds of the folding process; and (4) solvent protection and secondary structure formation correspond to loss of unfolded environment, not appearance of native side chain environment. Whatever the mechanism, these results indicate the presence of at least two intermediates with distinct structural properties. One intermediate is not observable by ^{19}F NMR, but is solvent-protected and contains secondary structure. The other can bind dihydrofolate, but not $NADP^+$.

On the basis of the previous fluorescence study (*2*), we proposed Scheme 1 as the simplest mechanism able to explain the four observed fluorescence phases:

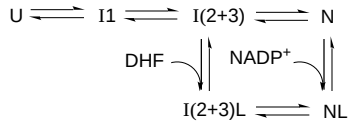
Scheme 1

$$U \xrightarrow{k_1} I1 \xrightarrow{k_2} I2 \xrightarrow{k_3} I3 \xrightarrow{k_4} N$$

Under the conditions used in the previous study (refolding from 4.5 to 1.3 M urea, 10 °C), refolding was essentially irreversible; dihydrofolate bound to I2, while $NADP^+$ bound only to N. In the presence of both dihydrofolate and $NADP^+$, $NADP^+$ affects only the amplitude of the final phase.

The conditions used in this study (refolding from 5.5 to 2.75 M urea, 5 °C) favor reversibility, and only two of the four previously observed phases can be resolved. On the basis of these results, we modify Scheme 1 to suggest the simplest mechanism able to fit the unfolded resonance intensity, the fluorescence and CD data, and the native resonance intensities of all resolvable tryptophans in the presence of both dihydrofolate and $NADP^+$:

Scheme 2



In Scheme 2, L represents a ligand-bound form of the protein; the formation of NL is essentially irreversible. As was the case for refolding of the apoprotein, the formation of I1, I(2+3), and I(2+3)L must be reversible in order to adequately model the data. I1 cannot be observed by ^{19}F NMR spectroscopy and is detectable under these conditions only as missing resonance intensity during the first seconds of the refolding process. Likewise, under these conditions, I2 and I3 cannot be resolved and are referred to jointly as I(2+3). The I(2+3) forms possess native-like side chain environment in the regions around tryptophans 30, 47, and 133. This native-like environment is observed in the apoprotein but is not stabilized, as demonstrated by its non-native relaxation properties. Dihydrofolate binds to the I(2+3) form and alters its structure by stabilizing the side chain environment in the regions around Trp74 (and probably Trp22). The dihydrofolate·I(2+3) form must then undergo a slow conformational change (whose structural basis is uncertain) to a structure (presumably the native protein) able to bind $NADP^+$ tightly. We suggest this mechanism as a tool for conceptualizing the average properties of the molecules at different stages of the folding process, rather than as a description of a set of structurally uniform states.

In a previous study of the unfolding of DHFR by stopped-flow ^{19}F NMR spectroscopy (*6*), we observed the rapid formation of an intermediate containing substantial secondary structure and native-like fluorescence, but possessing altered side chain mobility. A similar observation of an unfolding intermediate containing secondary structure and protection from hydrogen exchange, but possessing altered side chain mobility, was made for ribonuclease A (*8, 31*). However, a contrasting observation was made for a recent study of the unfolding of hen egg white lysozyme (*32*); although the unfolding is very slow, both exchange of indole protons and loss of native proton resonances for three of the six tryptophans take place in a single phase, and no intensity is missing. This phase coincides with the major phase observed by CD and fluorescence, suggesting that the hydrophobic core retains native-like side chain environment until it unfolds cooperatively. While one possible explanation of this difference in results may lie in the different sensitivities of ^1H and ^{19}F NMR to motional differences, Laurents and Baldwin (*32*) suggest that another explanation may lie in the relative solvent exposure of the residues in question. In the case of RNase A, the residue monitored is partially solvent-exposed; the lysozyme residues which remain ordered are deeply buried in the hydrophobic core.

While the tryptophans monitored in our studies are distributed throughout the structure and are not all solvent-exposed and several (notably Trp30 and Trp133) are in hydrophobic clusters, the chemical shifts of all five tryptophans in the apoprotein are affected in the same fashion by D_2O (S. D. Hoeltzli and C. Frieden, unpublished observation), suggesting that all of the fluorines are solvent-accessible. One possibility suggested by the differences in the side chain behavior during refolding of apo DHFR and its refolding in the presence of two different ligands observed in this study is that the hydrophobic core of some apoproteins may be intrinsically more flexible, and hence more accessible to solvent. In the case of DHFR (and presumably, thus, of other enzymes), the ligand may in effect serve as part of the hydrophobic core.

Refolding studies are often carried out in the absence of structural cofactors or structural ions, as well as in the absence of ligands. Our results suggest that the presence of ligands may influence the dynamic properties of side chain behavior during protein folding. These results imply that the presence of ligands or cofactors may influence the rate at which different processes occur and thus the relative importance of different driving forces during protein folding.

ACKNOWLEDGMENT

We thank Dr. D. A. D'Avignon for assistance with the NMR work at the Washington University Chemistry Department High Resolution NMR Facility, Drs. D. P. Cistola and K. B. Hall for the use of the proton/fluorine probe, Varian Associates for the use of the superconducting fluorine probe, Drs. P. A. Keifer and H. D. W. Hill for assistance with the NMR work at Varian Associates, Ms. Sara Heisel for excellent technical assistance with protein purification, and Dr. A. C. Clark for helpful discussions.

REFERENCES

1. Anfinsen, C. B., Haber, M., Sela, M., and White, F. H., Jr. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1309−1314.
2. Frieden, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4413−4416.
3. Englander, S. W., and Mayne, L. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 243−65.
4. Baldwin, R. L. (1993) *Curr. Opin. Struct. Biol. 3*, 84−91.
5. Roder, H. (1989) *Methods Enzymol. 176*, 446−473.
6. Hoeltzli, S. D., and Frieden, C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9318−9322.
7. Hoeltzli, S. D., and Frieden, C. (1996) *Biochemistry 35*, 16843−16851.
8. Kiefhaber, T., Labhardt, A. M., and Baldwin, R. L. (1995) *Nature 375*, 513−515.
9. Hoeltzli, S. D., Ropson, I. J., and Frieden, C. (1994) *Tech. Protein Chem. 5*, 455−466.
10. Fierke, C. A., Johnson, K. A., and Benkovic, S. J. (1987) *Biochemistry 26*, 4085−4092.
11. Penner, M. H., and Frieden, C. (1987) *J. Biol. Chem. 262*, 15908−15914.
12. Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., and Kraut, J. (1982) *J. Biol. Chem. 257*, 13650−13662.
13. Bystroff, C., Oatley, S. J., and Kraut, J. (1990) *Biochemistry 29*, 3263−3277.
14. Bystroff, C., and Kraut, J. (1991) *Biochemistry 30*, 2227−2239.
15. Touchette, N. A., Perry, K. M., and Matthews, C. R. (1986) *Biochemistry 25*, 5445−5452.
16. Hoeltzli, S. D., and Frieden, C. (1994) *Biochemistry 33*, 5502−5509.
17. Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
18. Baccanari, D., Phillips, A., Smith, S., Sinski, D., and Burchall, J. (1975) *Biochemistry 14*, 5267−5273.
19. Bretthorst, G. L. (1990) *J. Magn. Reson. 88*, 533−551.
20. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.
21. Zimmerle, C. T., and Frieden, C. (1989) *Biochem. J. 258*, 381−387.
22. Dong, Q., and Frieden, C. (1997) *Trends Biol. Sci. 22*, 317.
23. Anderson, W. A., Brey, W. W., Brooke, A. L., Cole, B., Delin, K. A., Fuks, L. F., Hill, H. D. W., Johanson, M. E., Kotsubo, V. Y., Nast, R., Withers, R. S., and Wong, W. H. (1995) *Bull. Magn. Reson. 17*, 98−102.
24. Jones, B. E., and Matthews, C. R. (1995) *Protein Sci. 4*, 167−177.

25. Garvey, E. P., and Matthews, C. R. (1989) *Biochemistry 28*, 2083−2093.
26. Kuwajima, K., Garvey, E. P., Finn, B. E., Matthews, C. R., and Sugai, S. (1991) *Biochemistry 30*, 7693−7703.
27. Perry, K. M., Onuffer, J. J., Gittelman, M. S., Barmat, L., and Matthews, C. R. (1989) *Biochemistry 28*, 7961−7968.
28. Garvey, E. P., Swank, J., and Matthews, C. R. (1989) *Proteins: Struct., Funct., Genet. 6*, 259−266.
29. Iwakura, M., Jones, B. E., Falzone, C. J., and Matthews, C. R. (1993) *Biochemistry 32*, 13566−13574.
30. Jennings, P. A., Finn, B. E., Jones, B. E., and Matthews, C. R. (1993) *Biochemistry 32*, 3783−3789.
31. Kiefhaber, T., and Baldwin, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2657−2661.
32. Laurents, D. V., and Baldwin, R. L. (1997) *Biochemistry 36*, 1496−1504.
33. Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946−950.
34. Merritt, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. D50*, 869−873.
35. Bacon, D. J., and Anderson, W. F. (1988) *J. Mol. Graphics 6*, 219−220.

BI971962U