^{19}F NMR Spectroscopy of [6-^{19}F]Tryptophan-Labeled *Escherichia coli* Dihydrofolate Reductase: Equilibrium Folding and Ligand Binding Studies†

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ABSTRACT: *Escherichia coli* dihydrofolate reductase contains five tryptophan residues distributed throughout its structure. In order to examine the regions of the protein surrounding these tryptophan residues, we have incorporated 6-fluorotryptophan into the protein. To assign the five resonances observed in the ^{19}F NMR spectrum, five site-directed mutants of the enzyme were made, each with one tryptophan replaced by a phenylalanine. The ^{19}F NMR spectra of the apoprotein, two binary complexes (with NADPH or methotrexate), and one ternary complex (with NADPH and methotrexate) were obtained. The chemical shifts of two of the tryptophan resonances (at positions 22 and 74) are particularly sensitive to ligand binding, while the remaining three (at positions 30, 47, and 133) change, but by less. Since several of the tryptophans are distant from the binding site, these results suggest that ^{19}F NMR can detect ligand-induced changes that are propagated throughout the structure. In the apoprotein, the resonances of the tryptophans at positions 22 and 30 are broadened. In the binary complex with NADPH, the resonances of tryptophans 30 and 74 are broadened while that of tryptophan 22 almost disappears. The line broadening of the tryptophan 22 resonance may reflect motion in that part of the protein, since it is near a region that is disordered in the crystal structure of the apoprotein and its NADP$^+$ complex. In contrast, in the ternary complex this region has a defined structure, and all resonances are of equal intensity and line width. The ^{19}F NMR spectra of the apoprotein and the three ligand complexes were also examined as a function of urea concentration. At urea concentrations well below the denaturation midpoint, the resonance assigned to tryptophan 22 in the apoprotein narrows and moves toward its denatured chemical shift. This behavior is predicted by a model that suggests that this region is undergoing rapid conformational changes between native-like and unfolded-like forms. The disappearance of the broadened tryptophan 22 resonance from its native chemical shift in the binary NADPH complex at very low urea concentrations may have a similar explanation. In the binary complex with methotrexate or the ternary complex with NADPH and methotrexate, all peaks decrease equally with increasing urea concentration, and all regions of the protein appear to be in slow exchange between the folded and unfolded forms.

Escherichia coli dihydrofolate reductase (DHFR[1]), a monomer of 159 amino acids and a molecular weight of 17 680, catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Its crystal structure is known to high resolution in the apo form (Bystroff & Kraut, 1991), as well as in several binary and ternary complexes (Bolin et al., 1982; Bystroff et al., 1990). Its small size, well characterized enzyme mechanism (Fierke et al., 1987; Penner & Frieden, 1987), and well-refined structure, as well as the reversibility of its folding reaction in the presence of chemical denaturants (Frieden, 1990; Touchette et al., 1986), make this protein a good model for protein folding studies.

The folding of *E. coli* DHFR has been studied using a number of experimental approaches. The rates of refolding of urea-denatured proteins have been measured by monitoring changes in fluorescence, absorbance, and circular dichroism as a function of the final denaturant concentration (Frieden, 1990; Jennings et al., 1993; Kuwajima et al., 1991; Touchette et al., 1986). These studies tell us that although the equilibrium process can be modeled by two states, intermediates exist and can be seen by kinetic methods. However, since fluorescence, absorbance, and circular dichroism spectra reflect global rather than specific properties, they do not provide information about specific regions of the protein. Rather, these results must be interpreted in terms of general concepts such as the formation of molten globule-like structures, secondary structure, tertiary interactions, or changes in the exposure of hydrophobic surfaces.

Folding studies of site-directed mutant proteins (Ahrweiler & Frieden, 1991; Garvey & Matthews, 1989; Garvey et al., 1989; Kuwajima, et al., 1991; Perry et al., 1987, 1989; Texter et al., 1992), while providing information on the role of specific amino acids and their effect upon rates of folding or unfolding, have also been carried out using techniques that measure global properties. Hence, such studies with mutant proteins are subject to the same limitations in interpretation as studies of the native protein: they do not necessarily provide information about specific regions of the protein, but rather must be interpreted in terms of the effect of a specific mutation upon global properties.

Other studies have examined the formation of ligand binding sites, the recovery of enzymatic activity (Frieden, 1990; Jennings et al., 1993), or the effect of protein fragments upon folding (Hall & Frieden, 1989). Such studies have shown that the binding sites form in a time-dependent manner, with the dihydrofolate binding site forming prior to the NADP(H) binding site, the latter forming concurrently with the recovery of enzymatic activity (Frieden, 1990).

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[1] Abbreviations: DHFR, dihydrofolate reductase, EC 1.5.1.3; DHF, dihydrofolate; MTX, methotrexate; IFABP, intestinal fatty acid binding protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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In order to gain information about specific regions of the protein, we have employed the approach of incorporating fluorine-labeled tryptophan into proteins expressed in *E. coli* and have utilized ^{19}F nuclear magnetic resonance spectroscopy (NMR) to monitor the environment of the tryptophan side chain by observing changes in chemical shifts and line shapes. This approach, first used to study protein folding with the rat intestinal fatty acid binding protein (IFABP) (Ropson & Frieden, 1992), overcomes the problem of considerable spectral overlap in the one-dimensional proton spectrum of even the smallest proteins, while allowing specific regions of the protein to be monitored.

The effects of fluorine incorporation in proteins have been reviewed extensively by Gerig (1994). Fluorine is a spin $^1/_2$ nucleus that is present in 100% natural abundance and is similar in size and in NMR sensitivity to hydrogen (Gerig, 1989). Although fluorine is much more electronegative than hydrogen and the C–F bond has a high dipole moment, several lines of evidence suggest that the incorporation of fluorine-labeled amino acids is not, in general, a perturbing substitution (Gerig, 1989, 1994; Sykes & Hull, 1978). Furthermore, fluorine chemical shifts are spread over a larger range of frequencies than proton chemical shifts and are very sensitive to the environment of the fluorine nucleus. Thus, changes in the fluorine chemical shift may serve to report conformational changes in the protein. Specific fluorine chemical shifts can be assigned to specific amino acids by using site-directed mutagenesis to create mutant proteins.

We now apply these methods to *E. coli* DHFR labeled with [6-^{19}F]tryptophan. This protein contains five tryptophan residues distributed throughout the protein. In this report, we have assigned the resonances observed in the ^{19}F spectrum of native and denatured apo-DHFR and DHFR complexed with MTX, NADPH, or both ligands. These assignments were made by using site-directed mutagenesis to create mutant proteins, each with a single tryptophan to phenylalanine substitution. We have studied the behavior of the tryptophans as a function of urea concentration and of the presence or absence of ligand. Our results demonstrate that both native and unfolded forms are present at urea concentrations near the denaturation midpoint. Exchange between most regions in the native and unfolded forms of the protein appears to be very slow. In the apoprotein and the NADPH complex, but not in the MTX ligand complexes, one region of the protein appears to undergo conformational exchange between the native-like and unfolded-like forms at urea concentrations well below the denaturation midpoint, where little unfolded protein is present.

MATERIALS AND METHODS

Materials. Ultrapure urea was purchased from United States Biochemical (Cleveland, OH). Concentrated stocks were prepared, deionized with 1 g of mixed-bed resin (AG 501-X8) from Bio-Rad (Richmond, CA) per 150 g of urea, filtered through a 0.2-μm filter, and stored in aliquots at −70 °C until the day of use. The concentration of urea was determined by the refractive index at 25 °C using equations relating urea concentration to the refractive index (Pace, 1986). Methotrexate, NADP, and chemically reduced NADP were obtained from Sigma (St. Louis, MO). Dihydrofolate (DHF) was prepared from folic acid (Blakely, 1960) and stored at −70 °C in 5 mM HCl containing 50 mM 2-mercaptoethanol. Methotrexate agarose was from Sigma, and fast-flow DEAE-Sepharose was from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade.

Bacterial Strains, Plasmid Construction, and Mutagenesis. DHFR was expressed from the *fol* A gene under control of the recA inducible promoter in plasmid pMONDHFR, which was created from plasmids pTY1 and pMON5840. The polymerase chain reaction (PCR) was used to amplify a 557 base pair fragment of plasmid pTY1 (Villafranca et al., 1983), which contained the DHFR coding region. The primers used, CGGGAAATCTTCATGATCAGTC and CGTAGAGG-TACCGGATAAGACG, contain a 2 base pair mismatch to the sequence flanking the *fol* A gene in pTY1 and introduce a *Bsp*HI site and a *Kpn*I site, respectively. The PCR-amplified fragment was digested with *Bsp*HI and *Kpn*I and ligated into plasmid pMON5840 (Olins & Rangwala, 1990) digested with *Kpn*I and *Nco*I (which creates a 5′ overhang compatible with *Bsp*HI). Ligation of the *Nco*I site with the compatible *Bsp*HI 5′ overhang destroys the *Nco*I site, but maintains the correct codon (Ile) in the second position of the DHFR open reading frame.

Site-directed mutagenesis to create five mutants, each with a single tryptophan to phenylalanine substitution, was carried out by the method of Kunkel et al. (1991). Single-stranded phagemid DNA was produced in *E. coli* strain CJ236 from plasmid pMONDHFR. The primers used were 24 bases long with a 2 base mismatch in the center (TGG to TTC). The ligated products of *in vitro* DNA synthesis were transformed into *E. coli* strain DH5, and each resultant plasmid was purified and used to transform *E. coli* tryptophan auxotroph W3110 *trp*A33 (Drapeau et al., 1968).

In all cases, the presence of the desired mutation and the absence of additional mutations were confirmed by double-stranded dideoxy-DNA sequencing using [^{35}S]dATP and Sequenase II from United States Biochemical (Cleveland, OH).

Protein Labeling and Purification. DHFR labeled with [6-^{19}F]tryptophan was purified from 500-mL cultures of *E. coli* strain W3110 *trp*A33 containing the plasmid pMONDHFR, which contains the *fol A* gene under control of the recA promoter. DHFR was labeled with [6-^{19}F]tryptophan essentially as described elsewhere (Li et al., 1990; Ropson & Frieden, 1992), except that M9 minimal medium was supplemented with 1 mM of all pyrimidine and purine bases and folate and 150 μM nicotinic acid, thiamine, and riboflavin, in addition to the 1% casein acid hydrolysate and 100 μM tryptophan previously used.

DHFR was purified essentially as previously described (Ahrweiler & Frieden, 1991), except that the crude cell lysate was precipitated with 0.25% poly(ethylene imine) prior to ammonium sulfate precipitation. The supernatant from a 40% ammonium sulfate precipitation was batch-bound to the MTX–agarose resin for 3 h to overnight and batch-washed with buffer containing 40 mM potassium phosphate (pH 6.0), 300 mM KCl, 2 mM EDTA, and 1 mM DTT prior to packing the resin into a column, washing, and eluting as described (Ahrweiler & Frieden, 1991). Fractions containing DHFR activity were pooled, exchanged into low-salt buffer by diafiltration through a YM3 membrane from Amicon (Beverly, MA), applied to a fast-flow DEAE-Sepharose column, and eluted with a linear gradient from 50 to 500 mM KCl in buffer containing 50 mM potassium phosphate (pH 7.2), 1 mM DTT, and 1 mM EDTA. These conditions completely resolved DHFR from unbound excess folate. A substoichiometric amount of tightly bound folate remained after elution from the DEAE column and was removed by denaturing the enzyme in 5 M urea followed by diafiltration in an Amicon stirred cell equipped with a YM3 membrane vs 5 M urea.

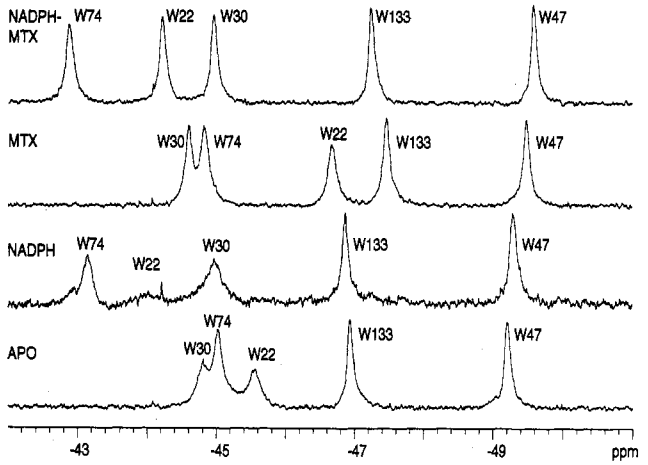

FIGURE 1: ^{19}F NMR spectra of native apo *E. coli* DHFR and the DHFR–MTX, DHFR–NADPH, and DHFR–MTX–NADPH complexes. All spectra were collected at 22 °C on a Varian VXR500 spectrometer equipped with a Nalorac indirect detection probe. Spectra were collected without proton decoupling using a recycle time sufficient to allow the recovery of equilibrium intensity between transients and referenced to an internal standard of 1 mM [4-^{19}F]-phenylalanine, as described in Materials and Methods. The apo, MTX, and MTX–NADPH complexes represent 500 transients of 0.8 mM DHFR in 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2, collected with a spectral width of 6547 Hz and 5248 complex data points. The NADPH complex represents 2752 transients of 0.3 mM DHFR in the same buffer, collected with a spectral width of 5704 Hz and 4544 complex data points and adjusted to the same intensity using the internal standard as a reference. All spectra were processed with 5-Hz line broadening and zero-filling to 8192 points prior to Fourier transformation.

Urea was removed and the protein renatured by a 4-fold dilution followed by diafiltration vs buffer.

NMR Spectroscopy. Unless otherwise indicated, NMR data were collected on a Varian VXR-500 spectrometer operating at 470.3 MHz for ^{19}F using a Nalorac indirect detection or a Nalorac hydrogen/fluorine probe. When indicated, additional NMR data were collected on a Varian XL300 spectrometer operating at 282.2 MHz or a Varian Unity 600 spectrometer operating at 564.4 MHz using a Varian indirect detection probe. All solutions contained a standard buffer consisting of 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2. No correction to pH was made for the D_2O content. All experiments were carried out at 22 °C. All spectra were referenced to an internal standard of 4-fluorophenylalanine, which was referenced to external trifluoroacetic acid for each experimental condition. The ^{19}F spin–lattice relaxation times (T_1) were determined by the inversion recovery method. Data were collected using a 90° pulse and a 3.4-s recycle time (>4× the longest T_1 of 0.8 s).

The program DNMR5 (LeMaster et al., 1989) was used to assess the effects of chemical exchange upon line shape and chemical shift as a function of urea concentration. Because this program requires that all of the resonances in a single state have the same line width, the data could not be quantitatively fit. Instead, a spectrum with the appropriate line width and chemical shift was simulated using DNMR5 by varying the population in each state and the sum of the exchange rates until a simulated spectrum superimposable on the peak being modeled in the observed spectrum was obtained.

RESULTS

Ligand Binding. Figure 1 shows the ^{19}F NMR spectra of native apo-DHFR and holo-DHFR complexed with either MTX, NADPH, or both MTX and NADPH. The assignments of the five resonances in each spectrum to the five tryptophan residues in DHFR were made by creating five mutant DHFRs, each containing a single tryptophan to phenylalanine substitution, purifying the mutant proteins, and examining their ^{19}F NMR spectra under the same conditions. Although most of the chemical shifts of the remaining tryptophans in the mutant proteins were altered by only tenths of a ppm, the chemical shift of Trp47 in the Trp74Phe mutant and of Trp74 in the Trp47Phe mutant was changed by almost 1 ppm (data not shown). Trp47 and Trp74 are in close proximity in the native structure, and thus the substitution of phenylalanine for one might be expected to affect the local environment and hence the chemical shift of the other. Although the side chains of Trp47 and Trp74 approach within several angstroms, the six positions on each ring are >6 Å apart, sufficiently distant that fluorine–fluorine coupling would not be expected to occur. Several of the resonances, notably Trp133 and Trp47, have a small shoulder (approximately 10% of the area of the main peak), which may represent a second, less populated, environment for the fluorine on that tryptophan residue. The [6-^{19}F]tryptophan-substituted wild-type enzyme appears to have enzyme activity and stability nearly identical to those of the unlabeled wild-type protein, indicating that the introduction of the fluorine label is probably not a structurally perturbing change.

In the apoenzyme, the resonances assigned to both Trp22 and Trp30 are broadened. In the binary complex with NADPH, the resonances assigned to Trp30 and Trp74 are broadened, and that of Trp22 has almost disappeared. In the ternary complex with MTX and NADPH, as well as in the binary complex with MTX, however, all five of the resonances have approximately the same intensity and the same line width. Thus, differences in line width or intensity cannot be a consequence of unequal incorporation of [6-^{19}F]tryptophan into the protein, but rather must reflect some characteristic of the liganded or unliganded protein itself. Although the spectra in Figure 1 are not proton-decoupled, proton decoupling during acquisition did not significantly affect the line width of the broadened peaks.

To examine whether the observed line broadening in the apoprotein or the NADPH complex was temperature-dependent, as might be expected of line shape changes caused by conformational exchange, spectra were collected from 5 to 45 °C. To better observe the resonances of Trp22 and Trp30, spectra of the apo-Trp74Phe mutant were also collected over this temperature range. The observed chemical shifts changed with temperature, but significant temperature-dependent changes in line shape were not observed (data not shown). This result suggests that if the broadening of a specific resonance is a consequence of exchange between different conformers, the rate for that exchange is not very temperature-sensitive.

To examine whether the observed line broadening was field-dependent, the line width of the apoprotein was examined at three spectrometer frequencies. Field dependence might be expected if line broadening is caused by conformational exchange or if chemical shift anisotropy (as will be discussed later) is a significant relaxation mechanism. Table 1 lists the line widths of the apoprotein measured at 282.2, 470.3, and 564.4 MHz. The line widths of all of the resonances show some field dependence, although that of Trp133 and Trp47 is slight. Trp30, Trp74, and Trp22 are not well-resolved at the two higher fields; hence, their deconvoluted line widths may not be as accurate. Both Trp30 and Trp74 approximately

Table 1: Line Widths (Hz) at Half-Height for 0.7 mM apo-DHFR in 50 mM Potassium Phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2[a]

frequency (MHz)	tryptophan resonance				
	Trp30	Trp74	Trp22	Trp133	Trp47
282.2	50.4	35.8	54.7	35.4	33.2
470.3	88.2	67.4	93.5	44.4	40.9
564.4	100.3	88.2	140.3	46.9	50.3

[a] Data were collected at 22 °C and referenced to an internal standard of 4-fluorophenylalanine. All spectra were collected without proton decoupling using a 90° pulse and a recycle time of 4× the longest T_1. The 282.2-MHz spectrum was collected on a Varian XL300 instrument using a spectral width of 4344 Hz and collecting 12 992 complex data points. The 470-MHz spectrum was collected on a Varian VXR 500 instrument using a spectral width of 6000 Hz and collecting 6016 complex data points. The 564.4-MHz spectrum was collected on a Varian Unity 600 instrument using a spectral width of 7874 Hz and collecting 5952 data points. The line widths were determined from spectra deconvoluted using a Lorenzian curve-fitting routine (VNMR software, Varian Associates, Palo Alto, CA). Spectra were processed with 2-Hz line broadening and zero-filling to 16 384 points prior to Fourier transformation and deconvolution.

Table 2: Chemical Shifts (ppm) of DHFR in the Indicated Ligand Complex[a]

ligand	Trp22	Trp30	Trp47	Trp74	Trp133
apo	−45.6	−44.8	−49.2	−45.0	−46.9
MTX	−46.7	−44.6	−49.5	−44.8	−47.5
NADPH	−44.0	−45.0	−49.3	−43.1	−46.9
MTX–NADPH	−44.2	−45.0	−49.6	−42.9	−47.2
DHF	−46.4	−44.8	−49.6	−45.1	−47.1
DHF–$NADP^+$	−45.1	−46.5	−49.6	−43.0	−47.1
denatured	−46.1	−46.3	−46.3	−46.4	−46.5

[a] Data for the MTX and NADPH complexes and apo-DHFR are shown in Figure 1 and were collected under the conditions defined in the legend for Figure 1.

double in line width as the spectrometer frequency doubles. The line width of Trp22 increases by approximately a factor of 3, indicating that chemical shift anisotropy may be a significant relaxation pathway for Trp22 in the apoprotein, but not in the MTX complex where all of the resonances have the same line widths.

Changes in chemical shift may reflect changes in the environment of the fluorine nucleus due to either the presence of substrate, conformational changes, or both. Table 2 lists the chemical shifts of the wild-type [6-^{19}F]tryptophan-labeled protein in the absence or presence of various ligands. In the apoprotein and all ligand complexes, the resonances assigned to Trp47 and Trp133 have been shifted to higher field and those of the other tryptophans to lower field relative to the chemical shift of [6-^{19}F]fluorotryptophan in aqueous solution (−46.3 ppm). While all of the resonances undergo changes in chemical shift upon ligand binding, they do so to very different extents. Two of the peaks, those assigned to Trp22 and Trp74, undergo particularly dramatic chemical shift changes upon ligand binding. The chemical shift of the resonance assigned to Trp22 is the most affected, undergoing a 1.1 ppm shift to higher field in the presence of MTX alone and a 1.3 ppm shift to lower field in the presence of both MTX and NADPH or NADPH alone. The resonance assigned to Trp74 also undergoes a dramatic chemical shift change, 1.9 ppm to lower field, in the presence of NADPH alone or NADPH and MTX, but only 0.2 ppm to lower field in the presence of MTX alone.

Trp30, Trp47, and Trp133 undergo less dramatic chemical shift changes. The resonance assigned to Trp30 shifts 0.2 ppm to lower field in the presence of MTX alone, but 0.2 ppm to higher field in the presence of NADPH alone or NADPH and MTX. The resonance assigned to Trp133 shifts 0.6 ppm to higher field in the presence of MTX alone, but shifts only negligibly in the presence of NADPH alone and only 0.2 ppm to higher field in the presence of both MTX and NADPH. That assigned to Trp47 shifts 0.3 ppm to higher field in the presence of MTX alone, 0.1 ppm to higher field in the presence of NADPH alone, and 0.4 ppm to higher field in the presence of MTX and NADPH.

Urea Dependence. Although the intrinsic fluorescence properties of [6-^{19}F]tryptophan-labeled wild-type apo-DHFR differ markedly from the properties of unlabeled wild-type apo-DHFR (data not shown), the urea denaturation curves monitored by intrinsic fluorescence were nearly identical for wild-type unlabeled and [6-^{19}F]tryptophan-labeled apo-DHFR. The denaturation curve for both labeled and unlabeled proteins was highly cooperative and could be fit to the two-state model previously used (Ahrweiler & Frieden, 1991). The ΔG_{H_2O} values (extrapolated to zero denaturant) were similar, and the denaturation midpoint was approximately 3.9 M urea under the conditions used in the NMR experiments for both the [6-^{19}F]tryptophan and the unlabeled wild-type apoprotein (data not shown). The denaturation midpoint observed by monitoring the sum of unfolded peak intensity by NMR spectroscopy could also be fit to the two-state model previously used and gave results similar to those observed by fluorescence changes for the apoprotein. For the MTX and MTX–NADPH complexes, similar data were obtained but the midpoint of the denaturation curve was 5 M urea.

Figure 2 shows the ^{19}F NMR spectra of native apo-DHFR in the presence of increasing concentrations of urea. As the urea concentration increases from 0 to 3.5 M, the resonance assigned to Trp22 continuously shifts 0.4 ppm to higher field (toward its position in the denatured protein) and the line width narrows. Such changes in chemical shift and line shape may be indicative of fast exchange between native-like and unfolded-like environments (Rao, 1989) for this region of the protein, or they might indicate that the region of the protein around Trp22 steadily assumes a more unfolded-like environment at urea concentrations (1.5 and 2.5 M) where little unfolded protein is observed by fluorescence. The other tryptophans remain near the apo chemical shifts, indicating little structural change in those regions. Both native and unfolded forms of DHFR are observed to be present at urea concentrations near the denaturation midpoint (3.0–4.5 M urea). The presence of distinct native and unfolded chemical shifts indicates that exchange between most regions in the native and unfolded forms appears to be at least 10-fold slower than the difference between the chemical shift in the native and denatured form (slower than 50 s^{-1}). This is simply an upper bound; the actual rate of exchange may be much slower.

The resonances in the spectrum of DHFR denatured by 7.8 M urea (top spectrum) represent five tryptophan residues and were assigned by observing the spectrum of denatured Trp to Phe mutant DHFRs. Three of the five resonances have chemical shifts distinct from that of free 6-fluorotryptophan (at −46.3 ppm). A similar result was observed in 6 M guanidium hydrochloride (data not shown), indicating that the small remaining chemical shift dispersion observed in the spectrum of the urea-denatured protein is not a function of the denaturant used or the result of residual structure, but is probably due to the effect of the local amino acid sequence. A similar result was observed for the two tryptophan residues of [6-^{19}F]tryptophan-labeled intestinal fatty acid binding

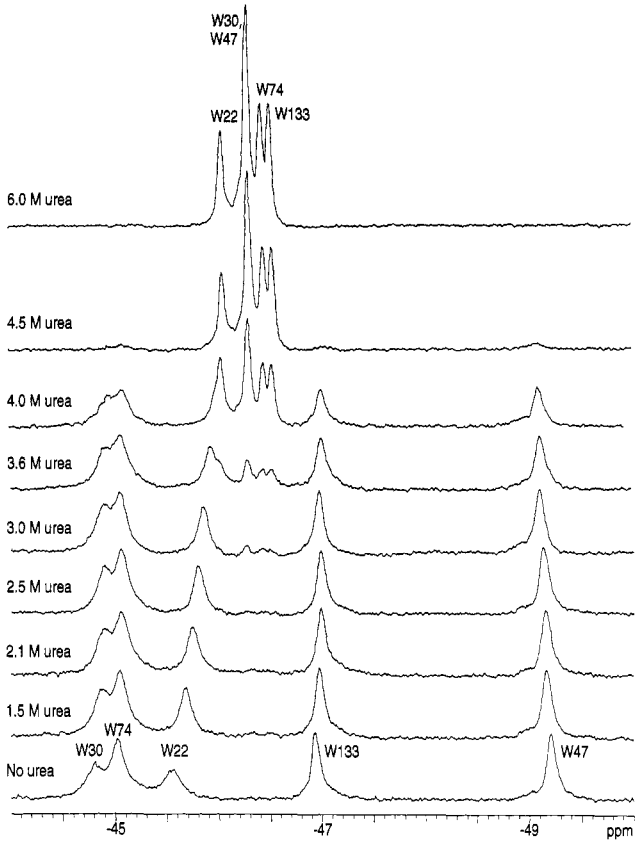

FIGURE 2: ^{19}F NMR spectra of apo-DHFR in the presence of the indicated concentration of urea. Spectra were collected at 22 °C on a Varian VXR500 spectrometer equipped with a Nalorac indirect detection probe. All spectra were collected without proton decoupling, with a recycle time sufficient to allow the recovery of equilibrium intensity between transients, and referenced to an internal standard of 1 mM [4-^{19}F]phenylalanine as described in the text. Each spectrum represents 500 transients of 0.8 mM DHFR in 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2. Each sample was placed in urea and allowed to denature for 1 h prior to collecting the spectrum. Each spectrum was collected with a spectral width of 6547 Hz and 5248 complex data points and processed with 5-Hz line broadening and zero-filling to 8192 points prior to Fourier transformation. The resonance assigned to Trp22 can be seen to sharpen and move toward its chemical shift in the denatured state as a function of urea concentration. Distinct native and unfolded resonances can be seen for the other resonances at intermediate concentrations of urea.

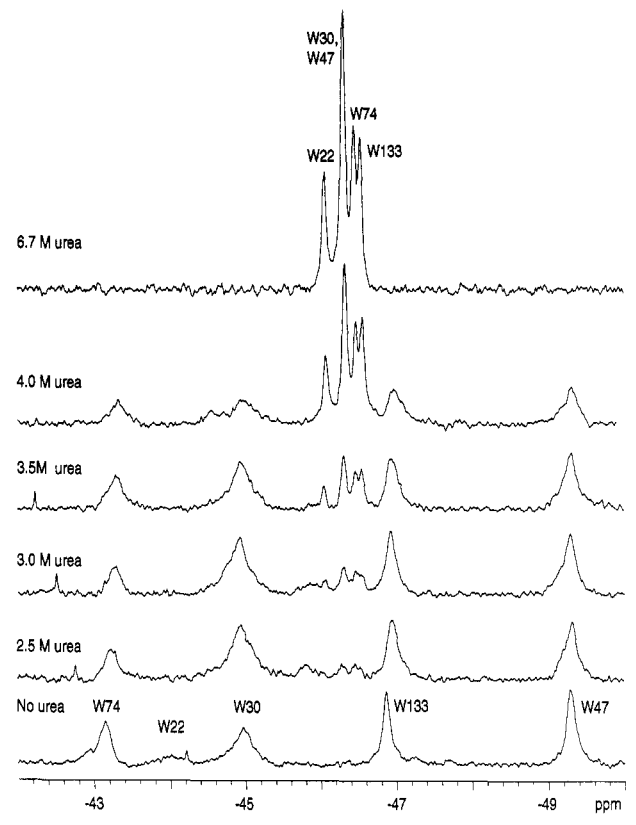

FIGURE 3: ^{19}F NMR spectra of the DHFR–NADPH complex as a function of urea concentration. All spectra were collected at 22 °C on a Varian VXR500 spectrometer equipped with a Nalorac indirect detection probe. Spectra were collected without proton decoupling and with a recycle time sufficient to allow the recovery of equilibrium intensity between transients and referenced to an internal standard of 1 mM [4-^{19}F]phenylalanine as described in the text. Each spectrum represents 2752 transients of 0.3 mM DHFR in 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2. Each sample was placed in urea and allowed to denature for 1 h prior to collecting the spectrum. Each spectrum was collected with a spectral width of 5704 Hz and 4544 complex data points and processed with 10-Hz line broadening and zero-filling to 8192 points prior to Fourier transformation. The broadened resonance assigned to Trp22 can be seen to diminish in intensity, while additional intensity appears and shifts it toward the position of Trp22 in the denatured state as a function of urea concentration. Distinct native and unfolded resonances can be seen for the other tryptophans at intermediate concentrations of urea.

protein (IFABP) in both urea and guanidine hydrochloride (Ropson & Frieden, 1992). Additionally, in that case, distinct chemical shifts were observed for two five-residue peptides containing the amino acid sequence surrounding the two tryptophans, indicating the extraordinary sensitivity of fluorine chemical shifts to changes in the environment of the fluorine nucleus.

Small changes in chemical shift (<0.1 ppm) occur for all of the tryptophan resonances in the presence of urea. The internal standard [4-^{19}F]phenylalanine undergoes a chemical shift change of similar magnitude (relative to an external reference; data not shown) as the urea concentration changes from 0 to 6.7 M, indicating that fluorine chemical shifts are somewhat sensitive to the chemical composition of the solvent.

Figure 3 shows the urea dependence of the protein–NADPH complex. The very broadened resonance of Trp22 disappears from its native chemical shift at low concentrations of urea. This observation may reflect behavior of Trp22 similar to that observed in the apoprotein. Here, however, the extreme line broadening of the resonance for Trp22 and the chemical shift overlap of the shifted Trp22 resonance with Trp30 make the interpretation of these results difficult.

Figure 4 shows the ^{19}F NMR spectra of native DHFR complexed with MTX and NADPH in the presence of increasing concentrations of urea. Similar results were obtained for the MTX binary complex (data not shown). As in the apoprotein, distinct resonances are observed for the native and unfolded proteins at intermediate concentrations of urea. In both the apoprotein and the ligand complexes, the total integrated intensity remains approximately the same (within 10%) at all urea concentrations. However, in marked contrast to the results for the apoprotein, all regions of the protein, including the region around Trp22, appear to unfold at the same urea concentration and to exchange between native and unfolded environments at the same slow rate.

DISCUSSION

DHFR contains five tryptophan residues that are distributed throughout the whole molecule (Figure 5). The side chains of Trp47 and Trp74 are closest to each other in the native protein and approach to within 4 Å (Bolin et al., 1982).

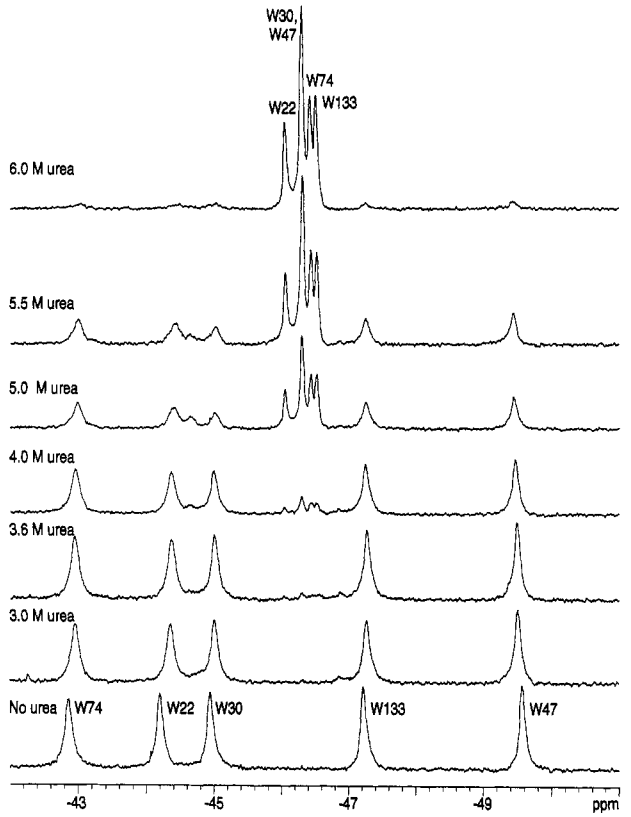

FIGURE 4: ^{19}F NMR spectra of the DHFR–NADPH–MTX complex as a function of urea concentration. All spectra were collected at 22 °C on a Varian VXR500 spectrometer equipped with a Nalorac indirect detection probe. Spectra were collected without proton decoupling using a recycle time sufficient to allow the recovery of equilibrium intensity between transients and referenced to an internal standard of 1 mM [4-^{19}F]phenylalanine as described in the text. Each spectrum represents 500 transients of 0.8 mM DHFR in 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 15% D_2O at pH 7.2. Each sample was placed in urea and allowed to denature for 1 h prior to collecting the spectrum. Each spectrum was collected with a spectral width of 6547 Hz and 5248 complex data points and processed with 5-Hz line broadening and zero-filling to 8192 points prior to Fourier transformation. All native resonances decrease in intensity equally with increasing urea concentration.

However, the 6-^{19}F positions on these two residues are >6 Å apart, a sufficient distance that coupling between these residues would not be expected to occur. Thus, changes in the chemical shifts of any of the ^{19}F resonances should reflect changes in the environment of different regions of the protein.

Early work with ^{19}F-labeled proteins attempted to interpret fluorine chemical shifts in terms of "buried" fluorine nuclei being shifted to lower field relative to the resonance of an unincorporated amino acid (Sykes & Hull, 1978). Later studies have shown that shifts to both higher and lower field occur in some systems (Luck & Falke, 1991). Although several groups have attempted to develop a theory to predict the direction and magnitude of protein-induced chemical shift changes (de Dios et al., 1993; Gregory & Gerig, 1991), each of these theories has only been tested with a single case and appeared to reach somewhat different conclusions about which factors are most important in predicting fluorine chemical shifts. Hence, we have chosen to employ the empirical approach of using mutant proteins created by site-directed mutagenesis to unambiguously assign the specific residues to resonances that undergo dramatic changes in line shape and chemical shift upon ligand binding.

Two of the resonances (Trp22 and Trp30) in the spectrum of the apoprotein are broadened relative to the other resonances. In the binary complex with NADPH, the resonances of Trp30 and Trp74 are broadened relative to Trp47 and Trp133, and the resonance of Trp22 almost disappears. Chemical exchange (or in this case, exchange between conformers) with different chemical shifts at a rate within an order of magnitude of the difference in frequency between the resonances of the different conformers can cause changes in the line shapes of the NMR spectra similar to those we observed. If the observed line broadening is a consequence of conformational exchange, lowering the temperature might be sufficient to further broaden or resolve the two resonances, while raising the temperature might be sufficient to increase the rate of exchange and narrow the line shape. We did not observe such changes, suggesting that if the observed line broadening is a consequence of exchange between different conformers, the rate of that exchange is not very temperature-sensitive.

Another possible explanation for the line broadening observed would be a significant contribution of chemical shift anisotropy to the relaxation pathway. Although line broadening due to conformational exchange would be expected to vary with magnetic field strength, chemical shift anisotropy in theory depends upon the square of the field strength (in practice, the observed line width in a macromolecule would be expected to vary less). Chemical shift anisotropy arises from asymmetry in the molecular motion surrounding a nucleus. Such motion can produce a fluctuating magnetic field, which can serve as a relaxation pathway. Although some field dependence for the line width is observed for all of the resonances, that of Trp22 is particularly striking, suggesting that chemical shift anisotropy may be a significant relaxation pathway for this nucleus. If so, this observation is still likely to reflect mobility in that region of the protein.

Trp22 is adjacent to a loop (residues 16–20) that is disordered in the crystal structure of the apoprotein (Bystroff & Kraut, 1991) and in the NADPH binary complex (Bystroff et al., 1990) and is itself in a region (residues 21–24) that is partially disordered in the apoenzyme (Bystroff & Kraut, 1991). Falzone et al. (1994) have suggested that a loop comprising residues 9–24 undergoes rapid exchange (35 s^{-1}) in the apoprotein. Thus, the line broadening we observe for Trp22 is consistent with these observations and probably reflects the motion of this region of the protein.

In the ternary complex, all of the resonances have the same line widths and intensities. This observation is consistent with crystallographic data, which show that the protein becomes more compact in the ternary complex (Bystroff & Kraut, 1991) and that all regions are clearly defined. The fact that Trp47 and Trp133 are not broadened in the apoprotein or any in ligand complex is consistent with the observations from crystallographic data, which show that these regions of the protein are ordered in all forms of the protein. We conclude that whether the line broadening observed for Trp22 and Trp30 in the apoprotein and for Trp30, Trp74, and especially Trp22 in the NADPH complex is caused by conformational exchange or is due to chemical shift anisotropy, its root cause is likely to be local motion in the region of the structure surrounding that tryptophan rather than the overall motion of the molecule.

The changes in chemical shift observed upon substrate binding may reflect changes in the environment of the fluorine nucleus due to either the presence of substrate, conformational change in the enzyme, or both. The enzyme is known to undergo conformational changes upon ligand binding, both from the crystal structures of various ligand complexes (Bolin et al., 1982; Bystroff & Kraut, 1991; Bystroff et al., 1990)

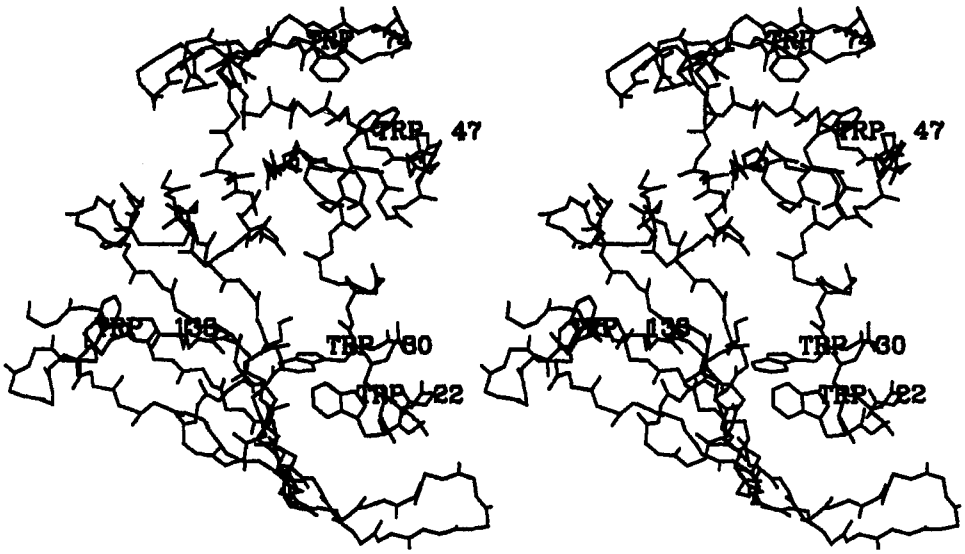

FIGURE 5: α-Carbon stereo diagram from the 2.3-Å crystal structure of apo-DHFR (Bystroff et al., 1990). The graphic was created using the program Insight. *E. coli* DHFR contains five tryptophan residues at positions 22, 30, 47, 74, and 133. The tryptophan side chains are shown. Residues 16–20 are disordered in the crystal structure and appear as a gap adjacent to Trp22.

and from a detailed study of its enzyme mechanism (Fierke et al., 1987; Penner & Frieden, 1987). The resonances assigned to Trp22 and Trp74 undergo the most pronounced changes in chemical shift upon ligand binding. Although it does not make direct contact with the ligand, Trp74 may be close enough to the phosphate on the 2′-position of the ribose of $NADP^+$ for the binding of NADPH to induce its pronounced shift to lower field if, as has been suggested (de Dios, 1993), electrostatic field effects make an important contribution to fluorine chemical shift dispersion. Although the 6-position itself is not in direct contact with the ligand, Trp22 makes a hydrophobic contact with the pyrimidine ring of methotrexate (Bystroff & Kraut, 1991). It is also located in a region that undergoes a large conformational change upon ligand binding. The pronounced (and opposite) changes in chemical shift and line shape observed for Trp22 upon ligand binding are probably caused by both changes in environment due to the presence of ligand and alteration in environment due to conformational changes. The other resonances show more subtle changes in chemical shift, which may be caused by alteration in environment due to conformational changes being propagated through the structure as a consequence of ligand binding.

The above results demonstrate that changes in the chemical shifts of assigned fluorine nuclei may serve as useful probes for the regions of a protein involved in ligand binding, even in the absence of structural information.

The changes in chemical shift and line shape as a function of urea concentration give information about the folding properties of the protein at equilibrium. For the apoprotein, the resonance assigned to Trp22 continually narrows, and its chemical shift moves toward its value in the denatured protein at urea concentrations well below the midpoint of the denaturation curve. Such a simultaneous change in line shape and chemical shift may indicate exchange between the native and unfolded states and can be modeled to provide information on the relative populations of the states of the system and the sum of the rates of exchange. Modeling studies using the program DNMR5 (LeMaster et al., 1989) and a two-state model indicate that this region exchanges at a rate of greater than 1000 s^{-1}, with a ratio of native/unfolded protein that shifts from 6 to 0.1 as the urea concentration increases from 1.5 to 3.5 M, the latter value being below the midpoint of the denaturation curve (3.9 M). For the rest of the protein, distinct native and unfolded resonances are observed at urea concentrations around the midpoint of the denaturation curve. These results indicate that while most of the protein is in slow exchange between the native and unfolded states, the region around Trp22 appears either to unfold at lower concentrations of urea (and exchange between the native and unfolded states at a much faster rate than the rest of the protein) or to assume a more unfolded-like environment at low concentrations of urea where the other tryptophan residues remain in a more native-like environment. The behavior observed for the NADPH binary complex, where the very broad Trp22 peak disappears at very low concentrations of urea, may have a similar explanation. In contrast, in the MTX and MTX–NADPH ligand complexes, all regions of the protein appear to exchange between the folded and denatured forms at the same slow rate. These differences in the behavior of the region of the protein around Trp22 and its altered behavior in the MTX ligand complexes demonstrate the usefulness of this technique, which allows us to monitor specific regions of the protein.

The use of 6-fluorotryptophan-labeled DHFR has allowed us to examine the behavior of amino acid side chains as a function of urea denaturation at equilibrium. Since the resolution and sensitivity of these spectra appeared to be sufficient to estimate the population of folded and unfolded residues from the sum of a small number of single transients, we hope to be able to utilize this technique to observe the behavior of individual amino acid side chains in real time during the folding process. We have designed, built, and obtained preliminary results from a stopped-flow device incorporated into a standard NMR spectrometer (Frieden et al., 1993; Hoeltzli et al., 1994). This technique may provide additional structural information about the folding mechanism of DHFR and may prove helpful in the study of other proteins, including proteins too large to be readily studied by hydrogen–deuterium exchange and proton NMR.

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