NMR Studies of Differences in the Conformations and Dynamics of Ligand Complexes Formed with Mutant Dihydrofolate Reductases†

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ABSTRACT: Two mutants of *Lactobacillus casei* dihydrofolate reductase, Trp 21 → Leu and Asp 26 → Glu, have been prepared by using site-directed mutagenesis methods, and their ligand binding and structural properties have been compared with those of the wild-type enzyme. ^{1}H, ^{13}C, and ^{31}P NMR studies have been carried out to characterize the structural changes in the complexes of the mutant and wild-type enzymes. Replacement of the conserved Trp 21 by a Leu residue causes a decrease in activity of the enzyme and reduces the NADPH binding constant by a factor of 400. The binding of substrates and substrate analogues is only slightly affected. ^{1}H NMR studies of the Trp 21 → Leu enzyme complexes have confirmed the original resonance assignments for Trp 21. In complexes formed with methotrexate and the mutant enzyme, the results indicate some small changes in conformation occurring as much as 14 Å away from the site of substitution. For the enzyme–NADPH complexes, the chemical shifts of nuclei in the bound coenzyme indicate that the nicotinamide ring binds differently in complexes with the mutant and the wild-type enzyme. There are complexes where the wild-type enzyme has been shown to exist in solution as a mixture of conformations, and studies on the corresponding complexes with the Trp 21 → Leu mutant indicate that the delicately poised equilibria can be perturbed. For example, in the case of the ternary complex formed between enzyme, trimethoprim, and $NADP^{+}$, two almost equally populated conformations (forms I and II) are seen with the wild-type enzyme but only form II (the one in which the nicotinamide ring of the coenzyme is extended away from the enzyme structure and into the solvent) is observed for the mutant enzyme complex. It appears that the Trp 21 → Leu substitution has a major effect on the binding of the nicotinamide ring of the coenzyme. For the Asp 26 → Glu enzyme there is a change in the bound conformation of the substrate folate. Further indications that some conformational adjustments are required to allow the carboxylate of Glu 26 to bind effectively to the N1 proton of inhibitors such as methotrexate and trimethoprim come from the observation of a change in the dynamics of the bound trimethoprim molecule as seen from the increased rate of the flipping of the ^{13}C-labeled benzyl ring and the increased rate of the N1–H bond breaking.

Dihydrofolate reductase (dhfr) is the target enzyme for antifolate drugs such as the antineoplastic drug methotrexate (MTX) and the antibacterial drug trimethoprim [for reviews see Blakley (1985) and Feeney (1986)]. We have previously used high-resolution NMR spectroscopy to study complexes of the *Lactobacillus casei* enzyme with substrates, substrate analogues, and coenzymes with the aim of understanding the factors controlling the specificity of binding. Signals from individual protons in more than 24% of the amino acid residues in the protein have now been assigned, and a substantial number of specific resonance assignments have also been made for nuclei in the bound ligands. These latter assignments have allowed us to use ^{13}C, ^{15}N, and ^{31}P NMR to determine the ionization states of several ligands bound to dihydrofolate reductase (Roberts et al., 1981; Birdsall et al., 1977a; Bevan et al., 1985) and to elucidate their conformations (Cayley et al., 1979). Multiple coexisting conformations of enzyme–ligand complexes have also been characterized in solution (Gronenborn et al., 1981; Birdsall et al., 1982), and the dynamics of both the bound ligand and the protein (Searle et al., 1988) have been explored in some detail. NMR has proved to be a useful technique for monitoring changes in the mode of binding that accompany structural modifications of substrate analogues (Cayley et al., 1979; Antonjuk et al., 1984; Birdsall et al., 1984a,b; Hammond et al., 1987). These structure activity studies have now been extended to a series of site-directed mutants of dihydrofolate reductase. In this paper two mutant enzymes are compared with the wild-type enzyme in order to investigate how a single-point mutation can affect the mode of ligand binding, the conformation, and the dynamic properties of a bound ligand and the populations of conformational states in solution.

Trp 21 is a conserved residue in all the reported sequences of dihydrofolate reductase from bacterial and vertebrate sources. In the X-ray crystal structure of the *L. casei* dhfr–NADPH–methotrexate complex (Bolin et al., 1982), this

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residue is seen to be fairly close to both the coenzyme (3.9 Å from Trp 21 Cξ2 proton to the amide nitrogen of NADPH) and inhibitor binding sites (4.6 and 4.7 Å from the indole NH of Trp 21 to the N1 proton and N8 of MTX). By use of site-directed mutagenesis, residue Trp 21 has been changed to a leucine in order to allow us to investigate the role of Trp 21 in its complexes with substrates and substrate analogues; this mutant enzyme is designated dhfr W21L.

The second mutant, Asp 26 → Glu (dhfr D26E), was chosen for study for two main reasons. First, Asp 26 has been proposed as the possible proton donor in the catalytic reaction (Stone & Morrison, 1984). Second, the Asp 26 carboxyl group is important in the binding of inhibitors: it forms an ion pair interaction with the N1 proton and one of the 2-amino group protons of methotrexate, trimethoprim, and other 2,4-diaminopyrimidine substituted inhibitors of dihydrofolate reductase.

Materials and Methods

Enzyme Preparations and Purifications. The *L. casei* dhfr gene was cloned into *Escherichia coli* and fully sequenced as described by Andrews et al. (1985). The D26E and W21L mutants were made by site-directed mutagenesis (Andrews and Davies, unpublished results) using the modification of gapped duplex method originally developed by Kramer et al. 1984. Details concerning the fermentation conditions for the mutants W21L and D26E and the purification of the proteins are supplied as supplementary material.

Both proteins were shown to be homogeneous by the presence of a single band on an SDS–polyacrylamide gel. When the enzyme concentrations were determined by methotrexate titration (Dann et al., 1976), dhfr D26E was found to have the same extinction coefficient as the wild-type enzyme, whereas that for the dhfr W21L was significantly lower. The UV spectrum of dhfr W21L was therefore characterized and the molecular extinction coefficient determined. For dhfr W21L this was found to be $\epsilon_{280} = 23.5 \times 10^3$ M^{-1} cm^{-1} as compared to a wild-type value of $\epsilon_{280} = 30.5 \times 10^3$ M^{-1} cm^{-1}.

Sample Preparation. Enzyme was prepared for NMR study by lyophilizing twice from D_2O to replace exchangeable protons with deuterium. The final concentration of dhfr W21L in the NMR samples varied from 0.5 to 1.0 mM, and the concentration of dhfr D26E was 1.3 mM. All samples were in 0.35 mL of ^2H_2O containing 500 mM KCl, 1 mM EDTA, and 50 mM potassium phosphate at pH* 6.5, except for enzyme complexes containing folate, for which the KCl concentration was reduced by dialysis to 100 mM. The notation pH* denotes a pH reading uncorrected for the deuterium isotope effect on the glass electrode.

Measurement of Binding Constants. The binding of ligands to wild-type and mutant dhfr was measured fluorometrically at 25 °C by using the methods described previously (Birdsall et al., 1978, 1983a). The solutions contained 0.1–4.0 μM enzyme in 500 mM KCl and 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol], pH 6.0. The affinity of dhfr for folate is decreased by high ionic strength, and it was sometimes necessary to reduce the KCl concentration from 500 to 100 mM when folate binding was measured.

^1H NMR Spectroscopy. ^1H NMR spectra were recorded at 500 MHz with a Bruker AM500 spectrometer. The two-dimensional COSY and NOESY NMR spectra were recorded and analyzed following the procedures described previously (Hammond et al., 1986, 1987). Spectra were normally run at a sample temperature of 308 K. Chemical shift values (accurate to ±0.01 ppm) are reported relative to internal dioxane (1 mM) (which is 3.75 ppm from 5,5-dimethyl-5-silapentane-2-sulfonate at 308 K).

The dissociation rate constant of trimethoprim was estimated by measuring the transfer of magnetization from the bound to the free H6 signal as described previously (Forsen & Hoffman, 1963; Birdsall et al., 1981; Cayley et al., 1979). The rate of exchange of the pyrimidine N1 proton of trimethoprim with the solvent was measured from the temperature dependence of the N1 proton signal line widths in spectra of complexes examined in H_2O solution (Bevan et al., 1985; Searle et al., 1988).

^{13}C NMR Spectroscopy. [*m-methoxy*-^{13}C]Trimethoprim [[3′-*methoxy*-^{13}C]-2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine] was prepared essentially as described for the corresponding labeled brodimoprim (Cheung et al., 1986). The synthesis of ^{13}C-labeled [2,4a,6-^{13}C]methotrexate is described elsewhere (Cheung et al., 1987). The ^{13}C NMR spectra of [*m-methoxy*-^{13}C]trimethoprim bound to dhfr were recorded at 100 MHz on a Bruker AM400 spectrometer over a temperature range of 273–308 K to observe the exchange processes. The ^{13}C NMR spectra of [2,4a,6-^{13}C]methotrexate alone and with dhfr W21L were recorded at 125 MHz on a Bruker AM500 spectrometer at a temperature of 298 K. The data for the wild-type dhfr complexes were recorded on a Bruker WH270 spectrometer at 67.9 MHz. All ^{13}C spectra were recorded as previously described (Cheung et al., 1986) under conditions of ^{1}H broad band decoupling with a composite pulse low-power decoupler (Freeman et al., 1983). Chemical shifts are expressed in parts per million relative to external dioxane (1:1 dioxane/^2H_2O) with downfield shifts reported as positive.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were recorded at 162 MHz on a Bruker AM400 spectrometer, under conditions of ^{1}H broad band decoupling with a composite pulse low-power decoupler (Freeman et al., 1983). Chemical shifts are expressed in parts per million ppm with respect to inorganic phosphate pH* 8.4 (2.94 ppm downfield from H_3PO_4) with downfield shifts positive. Spectra were obtained at 285 K with a spectral width of 10 kHz and 32K data points (final digital resolution 0.61 Hz/point): the pulse interval was 2.0 s and the flip angle 45°. A total of 30000–70000 transients were averaged, and an exponential multiplication corresponding to a 15-Hz line broadening was applied to the free induction decay before Fourier transformation.

Results

The wild-type dihydrofolate reductase enzyme is subsequently referred to as dhfr, while the mutant enzymes are designated dhfr W21L and dhfr D26E.

Properties of Dhfr W21L. The catalytic activity and binding properties of dhfr W21L will be described in detail in a subsequent paper. Changing Trp 21 to a leucine residue is seen to decrease the catalytic activity by a factor of 17; k_{cat} for dhfr is 25 s^{-1} but only 1.5 s^{-1} for the W21L mutant under the conditions described in Dann et al. (1976). The binding of folate, trimethoprim, and the oxidized coenzyme $NADP^+$ is influenced very little (by less than a factor of 3) by the replacement of Trp 21 with a leucine residue, but NADPH binding is decreased by a factor of 400.

NMR Studies of Dhfr W21L. The Binary and Ternary Methotrexate Complexes. Methotrexate binds tightly to dhfr ($K_a > 10^9$ M^{-1}): in the bound state it is protonated on N1, and this is reflected in its ^{13}C chemical shifts (Cocco et al., 1981). The inhibitor also binds tightly to dhfr W21L ($K_a > 10^9$ M^{-1}), and the ^{13}C chemical shifts of methotrexate bound to the mutant enzyme indicate that the inhibitor is protonated

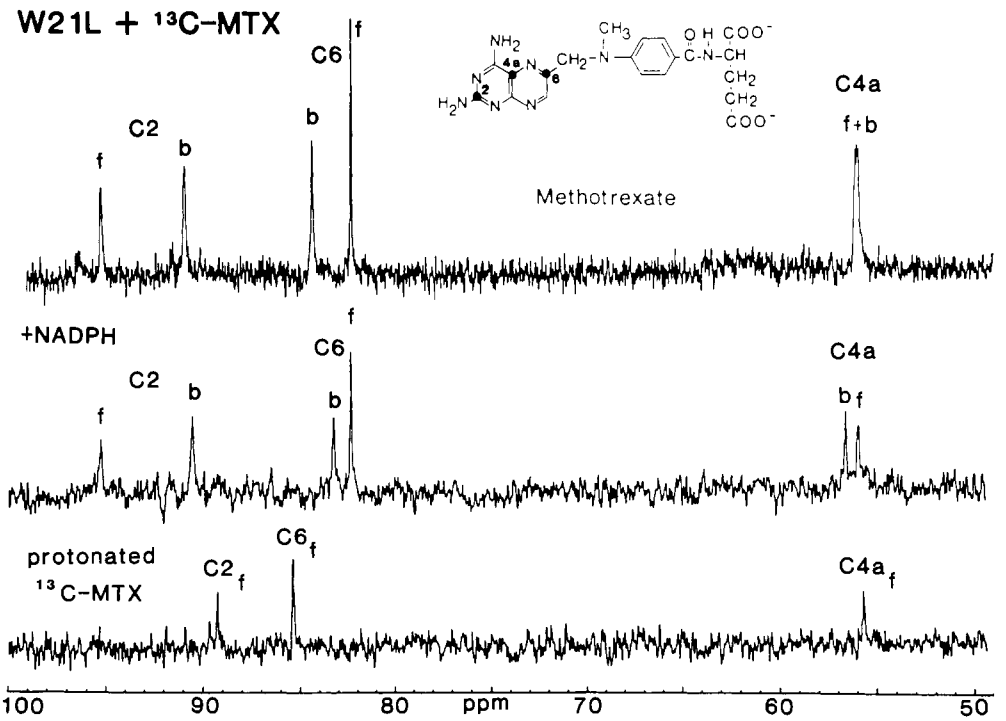

FIGURE 1: 125-MHz ^{13}C NMR spectra at 298 K of (top) a sample containing [2,4a,6-^{13}C]methotrexate and dhfr W21L in a ratio of 2:1 at pH 6.5* (bound and free methotrexate carbon resonances are indicated by b and f), (middle) same sample as in the top spectrum but with 2 equiv of NADPH added, and (bottom) [2,4a,6-^{13}C]methotrexate at pH* 2 in the absence of enzyme.

at N1 in the complex as was found with the wild-type enzyme (see Table I and Figure 1).

The chemical shift of the imidazole 2-proton of His 28 in the complex with dhfr W21L is the same as that measured in the complex with dhfr (Birdsall et al., 1977a; Wyeth et al., 1980), and we conclude that the methotrexate γ carboxylate group is making the same interaction with this residue in both complexes.

The chemical shift of the pteridine H7 of methotrexate bound to dhfr is very sensitive to the relative orientation of the pteridine and the benzoyl rings (Hammond et al., 1987). The bound H7 resonance of [2,4a,6-^{13}C]methotrexate is easy to assign because of the presence of the 9-Hz coupling to $^{13}C6$. Table I shows that the chemical shift of methotrexate H7 is identical whether it is bound to wild type or to dhfr W21L, and therefore the relative orientation of the two rings of methotrexate is most likely to be the same in complexes with the two enzymes. Forming the ternary complex by addition of NADPH slightly alters the shift of H7. In dhfr W21L, we observe an NOE from the H7 of bound methotrexate to signals in the aliphatic region, two of which have chemical shifts and COSY connectivities characteristic of the methyls of Leu 27, as previously observed with the wild-type enzyme (Hammond et al., 1986), indicating that the orientation of the pteridine ring in its binding site is similar in the two cases.

An overall comparison of the complexes of methotrexate with the mutant and wild-type enzymes can be obtained from their 2D ^1H NOESY and COSY spectra. NMR signals have been assigned to amino acid residues throughout the protein structure and these can be used as molecular markers to observe structural differences in the mutant complexes. Figure 2 shows the corresponding parts of the aromatic regions of the COSY spectra of the complex of methotrexate with dhfr and the dhfr W21L. The COSY cross-peak connecting H4 and H5 of Trp 21 (Hammond et al., 1987) is clearly missing in the spectrum of the mutant complex. Several other peaks in both the aromatic and aliphatic regions of the spectrum have changed their positions, as seen in Figures 2 and 3. The

Table I: ^{13}C, ^1H, and ^{31}P Chemical Shifts of Methotrexate and Coenzyme Bound to Wild Type and Dhfr W21L

Binary Complex with [2,4a,6-^{13}C]MTX	C2	C6	C4a	H7
dhfr wild type	90.2	84.2	56.5	4.15
dhfr W21L	90.9	84.2	55.9	4.15
free MTX pH* 2	89.2	85.3	55.7	
free MTX pH* 6.5	95.1	82.4	55.9	4.95

Ternary Complex with [2,4a,6-^{13}C]MTX and NADPH	C2	C6	C4a	H7	2′P	P	P	A8
dhfr wild type	90.2	83.3	56.7	4.20	2.7	14.3	16.2[a]	4.24
dhfr W21L	90.5	83.1	56.5	4.08	2.62	14.0	16.6	4.23

Binary Complex with NADPH	N2	N6	A2	A8	A1′
dhfr wild type	3.57[b]	2.98[b]	3.57[b]	4.24	2.94[b]
dhfr W21L	3.74	2.89	3.62	4.24	3.00
free mononucleotide[b]	3.42	2.51	4.50	4.81	2.47

[a] Birdsall et al. (1981). [b] Hyde et al. (1980).

assigned signals that have shifted can be grouped into two categories. The first group arises from those protons that are close to Trp 21 in the enzyme and experience ring current shifts from this aromatic residue. These signals will thus be expected to have altered shifts in dhfr W21L even in the absence of any change in atomic positions; they include resonances from residues Leu 118, Leu 19, Leu 23, and Phe 122. The second group comprises signals from protons in residues that are remote from Trp 21 but whose chemical shifts are different by more than 0.05 ppm in the two enzyme complexes: these include residues Leu 27, Leu 54, Leu 4, Val 110, Val 115, His 153, and Tyr 29.

We conclude that methotrexate binds to dhfr W21L, forming the same ionic interactions as in wild-type enzyme and adopting essentially the same conformation. However, the amino acids in the binding site are not arranged around methotrexate in an identical manner, and this leads to small perturbations in ^{13}C shifts of bound methotrexate and also to small propagated conformational changes in the protein

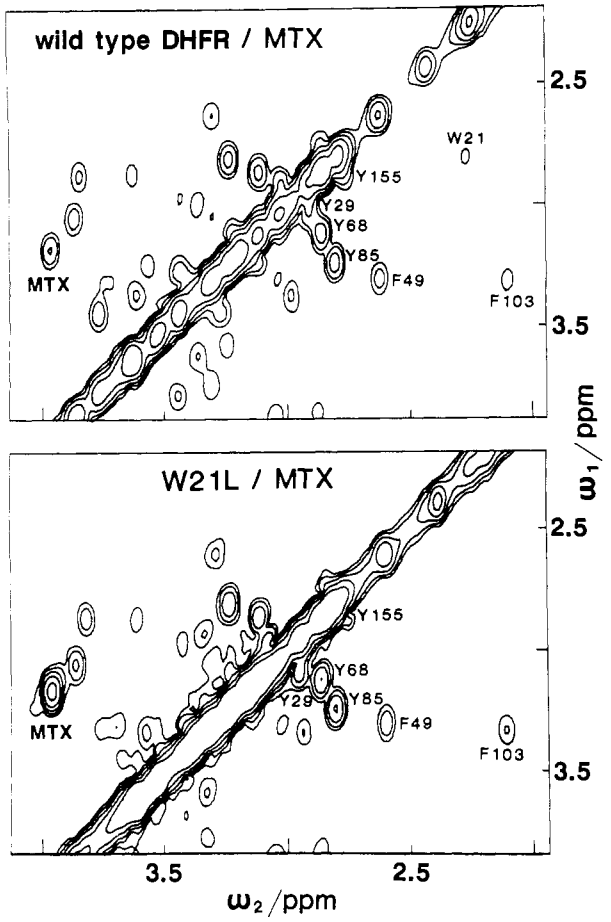

FIGURE 2: Aromatic regions of the 2D COSY ^1H contour plots for the methotrexate complex with (top) dhfr and (bottom) dhfr W21L at 308 K. The assignments of some cross-peaks to specific amino acid residues and free methotrexate are indicated.

structure that can be detected from the changes in chemical shift of residues remote from Trp 21. These small shift differences could be explained by differences in atomic position of only about 0.2 Å.

Dhfr W21L Coenzyme Binding. The ^1H NMR spectrum of the binary complex of NADPH with dhfr W21L contains much broader signals than those in the corresponding dhfr complex, probably due to exchange effects. Table I compares the chemical shifts of those coenzyme resonances observed in both complexes. The bound signals from the adenosine moiety are essentially identical, showing that this end of the molecule is probably binding in the same way to both dhfr and the W21L mutant. The nicotinamide ring protons have slightly different bound shifts in the two cases; the bound N2 resonance is 0.17 ppm downfield in the mutant complex, and the bound N6 signal is 0.09 ppm upfield. The ring current shift on the N2 proton due to Trp 21 has been calculated to be 0.04 ppm upfield, so substitution of that residue will contribute to the observed downfield shift of N2. The remainder of the shift difference may indicate a difference in the orientation of the nicotinamide ring in the binding site (but the shift differences are too small to be interpreted in detail).

Dhfr W21L–Trimethoprim–NADP$^+$ Complex. NMR experiments have shown that this complex with wild-type enzyme exists in solution as a mixture of approximately equal amounts of two conformational states that differ primarily in the mode of binding the coenzyme (Gronenborn et al., 1981; Birdsall et al., 1984). In the state designated form I, the nicotinamide ring is bound specifically to the enzyme. In the other state (form II), the conformation of the pyrophosphate backbone has been altered such that the nicotinamide ring has swung out of the coenzyme binding pocket and extends into solution. As Trp 21 is positioned near both the coenzyme and the inhibitor binding sites, it is of interest to ask whether the conformations of the ternary complex formed between NADP$^+$, trimethoprim, and dhfr W21L are different from those of the corresponding dhfr complex.

The most reliable way of measuring the relative populations of the two forms is from the relative areas of the NADP$^+$ pyrophosphate ^{31}P signals, which have distinct chemical shifts in each form. Figure 4 shows the ^{31}P spectra of the NADP$^+$–trimethoprim ternary complexes formed with dhfr and dhfr W21L. The dhfr complex (Figure 4b) shows a resonance from the 2′-phosphate group at 2.71 ppm and two pairs of signals from the pyrophosphate, one pair from each conformation. The subspectrum for each state (Gronenborn et al., 1981; Birdsall et al., 1984) is illustrated in the figure by the stick diagram below the experimental spectra. The sample of dhfr W21L with NADP$^+$ and trimethoprim contained 1.7 molar equiv of NADP$^+$, and thus the spectrum (Figure 4a) shows signals from free NADP$^+$ at −0.08 ppm (2′PO_4 group) and at 14.0 ppm (pyrophosphate group) that overlap part of the region of interest. However, it is clear that in this spectrum there is a pyrophosphate signal from form

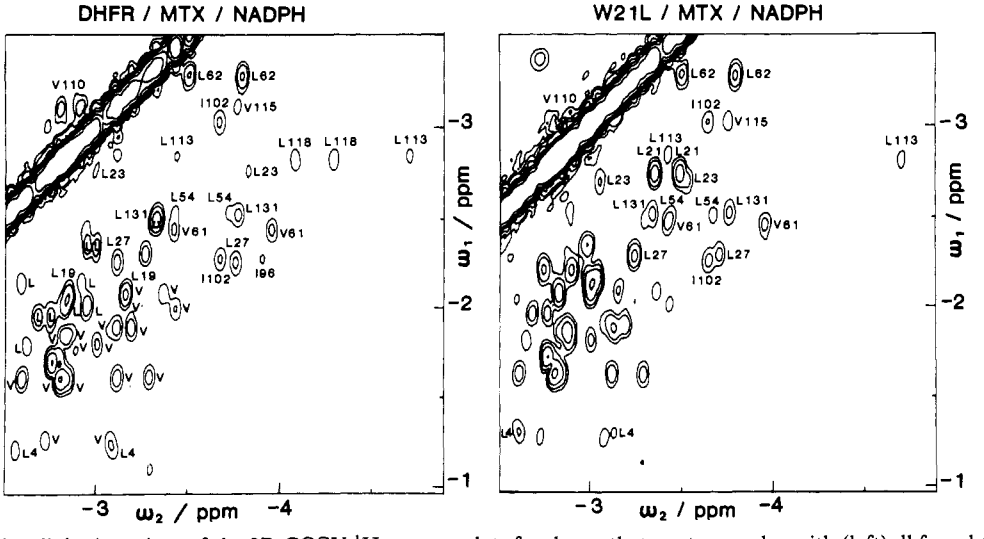

FIGURE 3: Part of the aliphatic regions of the 2D COSY ^1H contour plots for the methotrexate complex with (left) dhfr and (right) dhfr W21L at 308 K.

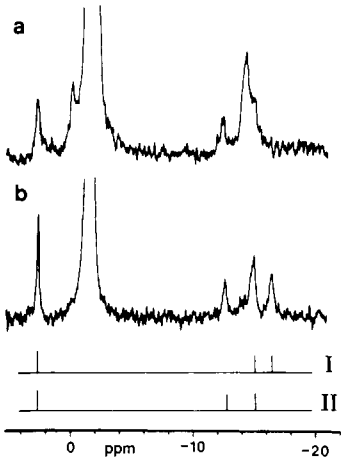

FIGURE 4: 160-MHz ^{31}P NMR spectra at 285 K of a sample containing (a) dhfr W21L, trimethoprim, and $NADP^+$ in a ratio 1:2.7:1.8 and (b) wild-type dhfr, trimethoprim, and $NADP^+$ in a ratio 1:2.5:0.9. The subspectra from forms I and II are indicated by the stick diagrams.

Table II: Chemical Shifts (ppm) of Trimethoprim and $NADP^+$ Bound to Dhfr and Dhfr W21L

	wild-type dhfr		
	form I	form II	dhfr W21L
trimethoprim			
H6[a]	−1.3	ND[e]	−1.08
H2′6′[a]	−1.5	ND[e]	−0.63
^{13}C 2[b]	1.5	1.21	1.30
^{13}C 5[b]	−0.25	1.31	1.35
	−2.2	−0.7	−0.61
$NADP^+$[c]			
N2	0.89	−0.16	−0.07
N6	0.84	−0.08	−0.02
N4	1.06	0.10	0.11
N5	0.85	0.02	0.06
N1′	−0.43	ND[e]	0.04
A8	0.57	−0.70	−0.67
A2	−0.91	−0.93	−0.89
A1′	0.49	ND[e]	0.53
P–P[d]	−0.7	1.7	1.90
	−2.2	−0.7	−0.61

[a] Chemical shifts expressed in ppm relative to protonated 2,4-diaminopyrimidine for H6 and 1-alkyl-3,4,5-trimethoxybenzene (Cayley et al. (1979). [b] Chemical shifts expressed in ppm relative to corresponding shift in free protonated TMP (Cheung et al. (1986). [c] Chemical shift expressed in ppm relative to corresponding shift of free nicotinamide mononucleotide (NMN) and AMP (Hyde et al., 1980a). [d] Chemical shifts expressed in ppm relative to free $NADP^+$. [e] ND, not determined.

II at −12.3 ppm, while there is no signal from form I at −16.4 ppm. This shows that the mutant complex has a preference for form II over form I of greater than 10:1 (an amount of form I of less than 10% would not be detected under the prevailing signal/noise conditions).

This conclusion is confirmed by the ^{13}C and ^1H NMR studies of this complex. For example, only one set of signals for the nicotinamide ring protons of the bound $NADP^+$ are observed for the complex with dhfr W21L. All the measured chemical shifts for nuclei of the bound ligands are summarized in Table II; it can be seen that there is very close agreement between the chemical shifts in form II of the dhfr complex and those in the complex with dhfr W21L.

The pattern of NOE effects provides evidence for the conformation about the nicotinamide glycosidic bond of bound $NADP^+$. In form I of the dhfr–$NADP^+$–trimethoprim complex, NOE effects are seen between protons N1′ and N2, indicating that the glycosidic bond to the nicotinamide ring is in the anti conformation. However, in form II, NOE effects

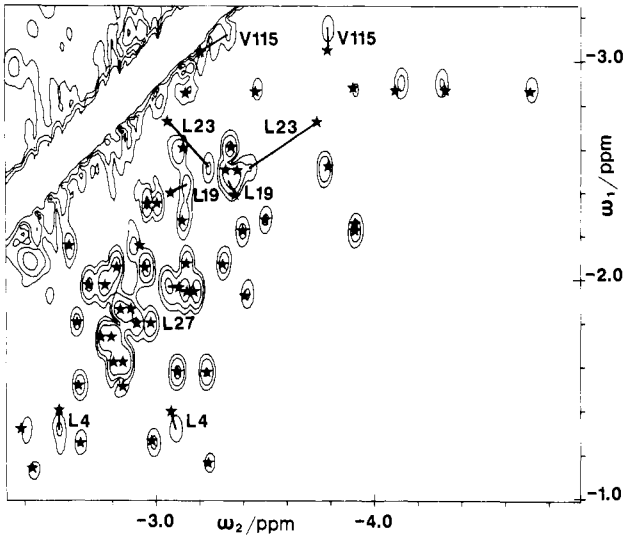

FIGURE 5: High-field region of the 2D COSY ^1H contour plot for the dhfr D26E trimethoprim complex with the position of the cross-peaks in the corresponding wild-type enzyme complex indicated by stars.

are observed between proton N1′ and *both* N2 and N6 (Birdsall et al., 1984), indicating a mixture of syn and anti conformations. The complex with dhfr W21L shows the latter pattern, indicating a mixture of syn and anti forms with flexibility about the glycosidic bond; this, together with the very small bound shifts, suggests that the nicotinamide ring is not in close contact with the enzyme. Substitution of Trp 21 with a leucine has made the interaction of the nicotinamide ring with the protein much less favorable and destabilized form I of the complex.

Properties of Dhfr D26E. This mutant has 90% of the specific activity of wild-type enzyme under the normal assay conditions compared with the 5% activity measured for dhfr W21L; its kinetic characteristics will be described in detail in a subsequent paper. The affinity for the inhibitor trimethoprim has decreased by only a factor of 2 in dhfr D26E, suggesting that the carboxyl group of Glu 26 is able to interact with the N1–H and the 2-amino group of trimethoprim in a similar way to the carboxyl of Asp 26 in dhfr.

NMR Studies of the Dhfr D26E–Trimethoprim Complex. The trimethoprim–dihydrofolate reductase complex has been extensively studied by NMR. We have previously determined the conformation of the ligand bound to the enzyme (Cayley et al., 1979) and characterized its conformational dynamics (Searle et al., 1988). The 2D ^1H NMR COSY spectra of the trimethoprim complex with dhfr and dhfr D26E are remarkably similar. Figure 5 shows the high-field methyl regions of the COSY spectrum of the dhfr D26E complex with the position of the cross-peaks in the dhfr complex indicated schematically by stars. A large proportion of the signals have identical chemical shifts, and of the assigned resonances, only those from residues Leu 4, Leu 19, Leu 23, Tyr 29, Val 115, and Trp 21 show significant changes in chemical shift (>0.04 ppm, Table III). The differences between the chemical shift of many of the protons in the trimethoprim complexes of dhfr and dhfr D26E are of similar size to that caused by only a 0.2-Å movement, as judged by the gradient factors [defined in Hammond et al. (1987)]. The overall conformation of the complex has not changed in this mutant. Changes in atomic position are probably very small and are consistent with the idea that helix B and the diaminopyrimidine ring move slightly away from one another to accommodate the extra methylene group in D26E.

Table III: Assigned Resonances with Different Chemical Shifts (>0.04 ppm) in the Trimethoprim Complex with Wild Type and Dhfr D26E

		shift differences[a] (ppm)
Leu 4	HG	−0.09
	HMD1	
	HMD2	
Leu 19	HG	0.04
	HMD1	0.07
	HMD2	
Leu 23	HG	−0.21
	HMD1	−0.31
	HMD2	0.18
Val 115	HB	0.10
	HMD1	
	HMD2	0.12
Trp 21	$H\epsilon_3$	0.08
	$H\xi_3$	0.03

[a] Negative shift means that the chemical in the mutant complex is downfield of the corresponding one in the wild-type complex.

Table IV: ^1H Chemical Shift and Dynamic Properties of Trimethoprim Bound to Wild Type and Dhfr D26E

	bound chemical shifts	
	wild type	dhfr D26E
H6[a]	−1.10	−1.03
H2′6′[b]	−0.60	−0.55
k_{off}[c] (s^{-1})	1.7	7
K_a[c] (M^{-1})	2.0×10^7	0.9×10^7
$k_{flipping}$[d] (s^{-1})	80	320

[a] 308 K ppm shift relative to model compound (Cayley et al., 1979). [b] 318 K ppm shift relative to model compound (Cayley et al., 1979). [c] 298 K. [d] Rate of flipping of the trimethoxybenzyl ring about C7–C1′ in trimethoprim bound to the enzyme at 274 K.

The chemical shifts of the H6 and H2′6′ protons of bound trimethoprim have been used previously to define its conformation (Cayley et al., 1979; Birdsall et al., 1983b). Table IV compares the chemical shifts of these protons determined by transfer of saturation; it is clear that these shifts are very similar in the complexes with dhfr and dhfr D26E. The difference in shifts could be accounted for by only a 3° change in the two torsion angles defined by the atoms C4–C5–C7–C1′ for τ_1 and by C5–C7–C1′–C2′ for τ_2; defining such small changes quantitatively is beyond the accuracy limits of the method. Evidence that the N1 position of trimethoprim is protonated when bound to dhfr D26E comes from comparing the observed chemical shift of the C2 resonance of [^{13}C]-2-trimethoprim with that from the dhfr complex (see Table II) (Roberts et al., 1981; Cheung et al., 1986).

Although the trimethoprim conformation is essentially the same in the two complexes, the small structural changes that accompany the Asp 26 → Glu substitution do lead to differences in the dynamics of the ligand–protein interactions. The dissociation rate constant of trimethoprim, k_{off}, measured from the transfer of saturation experiments on H6 at 298K, is increased by a factor of 4 (Table IV). Since the K_a of trimethoprim is decreased by a factor of 2, the association rate constant must also be affected. We have also measured the rate of flipping of the benzyl ring of trimethoprim by analysis of the line shape of the ^{13}C resonance of selectively enriched [*m*-O^{13}CH$_3$]trimethoprim (Searle et al., 1988). In complexes with dhfr examined below 280 K, one observes two signals for the *m*-methoxy-labeled carbon corresponding to their two different environments on either side of the ring. When the temperature is increased, these two signals coalesce to one, behavior typical of exchange between two equally populated sites: the simplest process leading to this exchange would be a 180° flip of the benzyl ring. Figure 6 shows the ^{13}C spectra

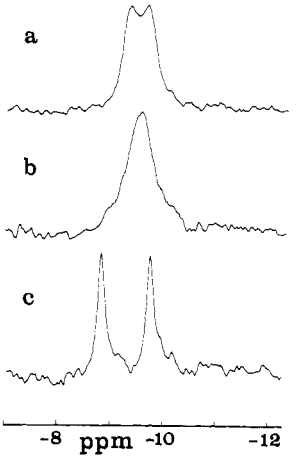

FIGURE 6: 100-MHz ^{13}C NMR spectra at 274 K of a sample containing 0.9 equiv of [*m*-O^{13}CH$_3$]trimethoprim and (a) wild-type dhfr, (b) dhfr D26E, and (c) dhfr D26E with 2 equiv of NADPH.

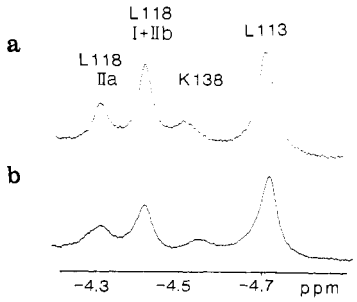

FIGURE 7: High-field region of the 500-MHz ^1H NMR spectra of the ternary complex formed with 2 equiv of folate, 2 equiv of NADP$^+$, and (a) wild-type dhfr at 313 K and (b) dhfr D26E at 308 K.

of the complexes of [*m*-O^{13}CH$_3$]trimethoprim with dhfr and dhfr D26E at 274 K; at this temperature spectrum a from the dhfr complex shows two signals, while spectrum b from dhfr D26E shows only a single coalesced signal. Unfortunately, it is not possible to lower the temperature sufficiently to observe separate signals from each conformation of the dhfr D26E trimethoprim binary complex, although two separate signals are clearly seen in the spectrum of the ternary complex with NADPH (Figure 6c). If we assume that the chemical shifts of the ^{13}C signals are the same in the binary as in the ternary complex, then it is possible to estimate a rate of 320 s^{-1} at 274 K for the flipping of the benzyl ring of trimethoprim in the binary complex. This suggests that the rate of this dynamic process is faster by a factor of 4 in dhfr D26E than in the normal enzyme.

It was also possible to estimate the rate of breaking and making of the hydrogen bond between the pyrimidine N1 proton of trimethoprim and the carboxylate group of residue 26 from measurements of the rate of N1 proton exchange, determined from the line width of the N1 proton signal as a function of temperature: this rate was increased by a factor of 50 in the dhfr D26E complex compared with its value in the dhfr complex [5 s^{-1} at 278 (Searle et al., 1988)].

NMR Studies on Dhfr D26E–Folate–NADP$^+$ Complex. The dhfr–folate–NADP$^+$ complex has been shown by NMR to exist in solution as a mixture of three conformational states called forms I, IIa, and IIb (Birdsall et al., 1982, 1987). The relative populations of these states are pH dependent, form I being predominant at low pH, while forms IIa and IIb (in constant relative proportions) predominate above pH 6. In previous papers we have suggested that Asp 26 might be the ionizable residue in the binding site responsible for the pH dependence of this conformational equilibrium (Birdsall et al.,

Table V: Difference between the ^1H Chemical Shifts (ppm) of Bound $NADP^+$ and Folate When Complexed with Dhfr and Dhfr D26E[a]

	conformation			
	IIa	IIa + IIb	IIb	I
folate				
7	−0.05		0.41	ND[b]
3′5′		−0.14		ND[b]
2′6′		−0.21		ND[b]
$NADP^+$				
N2	−0.02		0.04	0.02
N6	−0.06		0.06	0.03
N4	−0.05		−0.14	−0.04
N5	0.02		0.02	0.01
N1′	ND[b]		0.00	ND[b]
A8		−0.04		0.02
A2		−0.01		0.01
A1′		−0.03		ND[b]

[a] A negative shift means that the signal in the dhfr D26E complex is downfield of the corresponding signal in the wild-type enzyme complex. [b] ND, not detected.

1982). From intensity measurements on the Leu 118 methyl ^1H signals from different forms of the complex, it appears that in fact the conformational equilibrium remains essentially unchanged in the complex with the D26E enzyme (Figure 7). The ratio of populations of forms I to IIa can also be monitored directly from the area of the bound nicotinamide-4 proton signals at 6.85 and 6.75 ppm; this ratio also remains unchanged in the complexes with dhfr D26E.

Although the relative populations of the three conformational states in solution have not altered, there is evidence from the chemical shifts of the protons of bound folate and $NADP^+$ that at least one of the conformations of the dhfr D26E complex is not identical with its counterpart in the dhfr complex. The ligand resonances in forms IIa and IIb of the dhfr D26E–folate–$NADP^+$ complex were located by a 2D exchange experiment (Figure 8). The dotted lines indicate the connections between the H7 resonance of free folate and the two corresponding H7 signals of folate bound. The assignment to forms IIa and IIb (Table V) was based on the area of the resolved peak from form IIa in the 1D ^1H NMR spectrum. Similar connections can be made between the ^1H signals of bound and free $NADP^+$ in the 2D exchange spectrum. The chemical shifts of protons in the coenzyme and folate bound to dhfr D26E, and the corresponding shifts for the dhfr complex are compared in Table V. Only a single bound signal has been detected for each of the protons from the *p*-aminobenzoyl ring of folate (2′,6′ and 3′,5′) and the adenosine A2 and A8 protons of $NADP^+$. Although one must be careful in interpreting the absence of cross-peaks, in the free ligands, the T_1 values of the protons for which only single cross-peaks are observed are similar to those of protons for which two distinct cross-peaks are seen, and the simplest explanation appears to be that the chemical shifts of $NADP^+$, A2, A8, A1′ and folate 3′5′ and 2′6′ are the same in conformations IIa and IIb. The comparisons between dhfr and dhfr D26E for these protons are listed in the center column of Table V. Signals from form I do not appear in the 2D exchange spectrum at pH* 6.5 because of the slower exchange rate between this state and free ligand and the small population of this species at this pH value. However, at pH* 5 several bound signals from form I can be observed directly in the 1D spectrum and assigned by either a transfer of saturation experiment or a NOESY experiment. For most ligand protons, the difference between the chemical shifts in the dhfr and mutant complexes is not significant (<0.05 ppm). However, in form IIb the bound folate H7 proton signal is shifted upfield by 0.41 ppm in the dhfr D26E complex compared with its value in the dhfr complex. As the chemical shift of the pteridine H7 proton is greatly affected by changes in its orientation with respect to the benzoyl ring, this considerable shift perhaps suggests that folate is bound in a somewhat different conformation. The chemical shifts of the bound 2′6′ and 3′5′ protons are also different in the D26E complex. In addition, the resonances of the nicotinamide-4 proton in form IIb has been shifted downfield by 0.14 ppm. The proton at the 4-position of this ring would be expected to be very near the folate pteridine ring, and its change in chemical shift might reflect a change in the ring current shielding contribution at that position due to a different orientation of the pteridine or *p*-aminobenzoylglutamate ring (or the neighboring Phe 49).

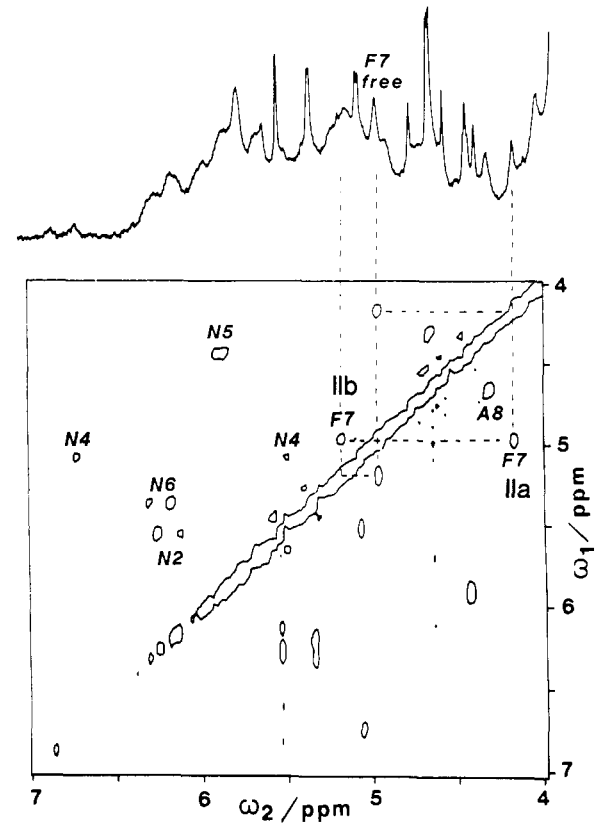

FIGURE 8: (Top) Low-field region of the 500-MHz ^1H NMR spectrum at 303 K of a sample containing dhfr D26E, $NADP^+$, and folate in a ratio of 1:2:2. The 7-proton resonance of free folate is indicated. (Bottom) Corresponding region of the 2D exchange spectrum, showing the cross-peaks linking the 7-proton resonances of free and bound folate connected by dashed lines. Cross-peaks linking free and bound $NADP^+$ are labeled N for nicotinamide and A for adenine ring protons.

Discussion

Crystallographic studies on complexes formed by ligands bound to normal and mutant *E. coli* dhfr (Howell et al., 1986) have revealed only local changes in conformation around the site of the amino acid substitution with smaller changes (up to 0.2 Å) at positions remote from the site of substitution. A broadly similar picture emerges from this present NMR study on complexes of ligands with *L. casei* dhfr and mutant dhfr enzymes. The NMR data on the dhfr W21L and D26E complexes indicate that while the substitution of a single amino acid is accompanied by large local perturbations in protein structure, there are only minimal effects on the overall structure. Although it is not yet possible to define these small changes precisely, one can identify those protons with large chemical shift gradient factors whose shifts are particularly sensitive to small movements. For example, the HB and

HMD2 protons of Val 115 have large gradient factors, and the observed shift differences in the trimethoprim complexes of dhfr and dhfr D26E could be explained by relative movements of only 0.2 Å between the Val 115 protons and the aromatic ring of the nearby Trp 5 residue. At the present stage of our understanding of structure–function relationships in proteins, it is difficult to assess whether or not these small movements have any functional significance. However, they might well be related to the perturbations in internal dynamic processes observed in these complexes.

Complexes with Dhfr W21L. The substitution of Trp 21 by Leu has dramatic effects not only on the catalytic process itself but also on coenzyme binding and on cooperativity in binding between coenzyme and substrate analogues (B. Birdsall, unpublished results). However, the substitution has relatively little effect on the binding of folate and substrate analogues in binary complexes with the enzymes. The NMR data reported here, when considered in conjunction with crystallographic data on the wild-type enzyme obtained by Matthews and co-workers (Matthews et al., 1979; Bolin et al., 1982) suggest structural explanations for the perturbations of ligand binding in dhfr W21L. The observation that the ^1H and ^{13}C chemical shifts of bound substrates and substrate analogues are very similar in the binary complexes with the wild-type and dhfr W21L enzymes confirms that the ligands are binding similarly to the normal and mutant enzymes. In order to explain the differences in catalysis and coenzyme binding, one must now look for structural differences involving the coenzyme binding sites. The ^1H chemical shifts of NADPH bound to dhfr and dhfr W21L indicate that most parts of the coenzyme structure bind similarly in the two cases, with the notable exception of the reduced nicotinamide ring itself. The chemical shifts of the protons of this ring (Table I) are clearly different in the two complexes, although substantial shift changes of the same sign are observed on binding in each case. This suggests that the reduced nicotinamide ring is probably still binding to the mutant protein within the same binding pocket but in a modified manner. The crystal structure data of Matthews and co-workers (1979) shows that the Cξ2 proton of Trp 21 has a hydrophobic interaction with the carboxamide nitrogen of bound NADPH. The simplest explanation of the observed binding and chemical shift data is that substitution of Leu for Trp at position 21, with the consequent loss of this interaction, leads to a change in the orientation of the reduced nicotinamide ring in the binding site. Since the reduced nicotinamide ring makes a major contribution to the binding energy of the coenzyme, this could result in a change in the binding affinity for NADPH. It could also account for the substantial decrease observed in the rate of hydride transfer, the rate-limiting step in the catalytic reaction for dhfr W21L (J. Andrews, unpublished results). The observations regarding coenzyme binding are reminiscent of the behavior seen earlier with reduced thio-NADPH, where the interaction between the coenzyme carboxamide group and the enzyme was altered by modification of the ligand rather than the enzyme. In the case of thio-NADPH there is a 500-fold reduction in coenzyme binding (Birdsall et al., 1980) and a substantial decrease in k_{cat} (Hyde, 1981). In this context it should also be mentioned that *L. casei* dhfr selectively modified at Trp 21 by *N*-bromosuccinimide shows a 200-fold reduction in NADPH binding but only small reductions in the binding of $NADP^+$ (3-fold) and substrate analogues (Thomson et al., 1980).

Complexes with Dhfr D26E. Because of the potentially important role of Asp 26 (Asp 27 in *E. coli*) in both catalysis and inhibitor binding, much attention has already been focused on site-directed mutants involving substitutions of this residue (Villafranca et al., 1983; Howell et al., 1986).

In the complex of D26E with folate and $NADP^+$ we have found that at least one (form IIb) of the three forms present has a different conformation from that in the complex with wild-type enzyme. This conformational difference is characterized by chemical shift differences in both the bound folate and $NADP^+$. However, the major conformational difference between form IIb and forms I and IIa in the complexes with dhfr, namely, the gross orientation of the pteridine ring, is retained in the complex with the mutant enzyme. Form IIb has its pteridine ring turned over by approximately 180° compared to its orientation in forms I and IIa: form IIb thus retains the catalytically functional orientation, while form I and IIa have the pteridine ring oriented as in the methotrexate–enzyme complex in a "nonproductive" conformational state (Charlton et al., 1979, 1985; Birdsall, Feeney, Tendler, Hammond, and Roberts, unpublished results).

In the present study we have also examined the effects on trimethoprim binding of the substitution of Asp 26 with a Glu residue and have found that this substitution does not result in a major perturbation of the trimethoprim binding, thus showing that the sequence difference at residue 26 (*L. casei* numbering), which is a conserved Asp residue in bacterial enzymes but a Glu residue in vertebrate enzymes (Matthews et al., 1985), does not in itself confer specificity of binding of trimethoprim to bacterial over vertebrate enzymes. The binding constant for trimethoprim, the conformation of the bound trimethoprim molecule, and the trimethoprim $^{13}C2$ and its N1 ^1H chemical shifts are very similar in the complexes with dhfr and dhfr D26E. Consideration of the ^1H chemical shifts of assigned residues on the protein provides no evidence that might indicate that the pyrimidine ring is occupying a different site in the two complexes. Some of the resonances that show significant changes in chemical shift (>0.04 ppm, see Table III) arise from protons that are close to residue 26; for example, several protons from the side chain of Leu 23 are only 2.6 Å from Asp 26. Other protons affected, such as those from Leu 19 or Phe 122, receive shielding contributions from Trp 21, and these would be perturbed if the helix B and its adjoining loop 21–25 move slightly to accommodate the extra methylene in the Glu 26 side chain. Although there are no major differences in the overall conformations of trimethoprim or its environment in the two complexes, the structural changes that do occur are sufficient to perturb the dynamic processes within the complexes. For example, the rate of breaking of the hydrogen bond between the pyrimidine N1 proton and the carboxylate of residue 26 is increased 50-fold in the complex with the mutant enzyme. The observed differences in chemical shifts of protein signals in the two complexes are consistent with small movements of helix B, and since this contains residue 26, these movements together with the increased flexibility of the side chain of Glu compared with Asp could be implicated in the increased rates of breaking and reforming of this interaction.

Another dynamic process that is perturbed in this complex is the flipping of the trimethoprim benzyl ring, which is 4 times faster in the D26E mutant complex. Since this process can only take place following a conformational change in the complex that allows the trimethoprim τ_1 torsion angle (C4–C5–C7–C1′) to change by at least 60° (Searle et al., 1988), the mutation at residue 26 obviously slightly perturbs the energy barriers to the required conformational changes in the protein. It is likely that the rates of these conformational

fluctuations are sensitive indicators of slight structural differences between normal and mutant enzymes.

Conformational Equilibria. We have previously reported the existence of two conformations (forms I and II) in the enzyme–trimethoprim–$NADP^+$ complex: as mentioned earlier, the crucial difference between these is that in one form (form II) the $NADP^+$ nicotinamide ring is no longer binding within its normal binding pocket but is extending into solution. If the Trp 21 interaction with the $NADP^+$ carboxamide nitrogen atom is important in maintaining the nicotinamide ring within its binding pocket, then one might expect a perturbation of this conformational equilibrium in complexes with dhfr W21L. In fact, our results indicate that in this complex with the dhfr W21L enzyme the only detectable conformation is form II. This behavior is exactly the same as that seen for the complex of the normal enzyme with trimethoprim and thio-$NADP^+$. Thus, a comparison of the complex of the normal enzyme and the thioamide coenzymes with complexes formed by the dhfr W21L and normal coenzymes shows similarities in the k_{cat} and also in the positions of conformational equilibria in trimethoprim–coenzyme complexes. These results provide a clear indication that the interactions between the nicotinamide carboxamide group and the protein are important in controlling these processes.

In earlier work we also reported the presence of three coexisting conformational states in the complex enzyme–folate–$NADP^+$ and showed that the populations of the conformations depended on pH. We suggested that the ionization of Asp 26 might be responsible for the pH dependence. We have now found that the conformational equilibrium is the same in the ternary complexes at pH 6.5 with the normal and the D26E mutant enzyme. Thus, if Asp 26 in dhfr is responsible for the pH dependence, then Glu 26 in D26E would need to act in a similar way. A definitive conclusion on the importance of Asp 26 in controlling the pH dependence of this conformational equilibrium must await the results of studies on the enzyme–folate–$NADP^+$ complex formed with the Asp 26 → Asn enzyme currently in progress.

Acknowledgments

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Supplementary Material Available

Fermentation conditions for the mutants W21L and D26E and details of the purification of the proteins (3 pages). Ordering information is given on any current masthead page.

Registry No. MTX, 59-05-2; dhfr, 9002-03-3; TMP, 738-70-5; NADPH, 53-57-6; NADP, 53-59-8; L-Trp, 73-22-3; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Leu, 61-90-5; folate, 59-30-3.

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Protein Dynamics from Chemical Shift and Dipolar Rotational Spin-Echo ^{15}N NMR†

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ABSTRACT: The partial collapse of dipolar and chemical shift tensors for peptide NH and for the amide NH at cross-link sites in cell wall peptidoglycan, of intact lyophilized cells of *Aerococcus viridans*, indicates NH vector root-mean-square fluctuations of 23°. This result is consistent with the local mobility calculated in typical picosecond regime computer simulations of protein dynamics in the solid state. The experimental root-mean-square angular fluctuations for both types of NH vectors increase to 37° for viable wet cells at 10 °C. The similarity in mobilities for both general protein and cell wall peptidoglycan suggests that one additional motion in wet cells involves cooperative fluctuations of segments of cell walls, attached proteins, and associated cytoplasmic proteins.

Computer simulations of the atomic mobilities of the component parts of crystalline and globular proteins have revealed extensive internal dynamics in the solid state at room temperature (Swaminathan et al., 1982; van Gunsteren & Karplus, 1982; Olejniczak et al., 1984; Karplus, 1987). Time-dependent determinations of the atomic Cartesian coordinates show that both main-chain and side-chain carbons and nitrogens have root-mean-square fluctuations of the order of 0.5 Å. Fluctuations in various protein dihedral angles are between 8 and 15°. Such internal rotational motions may have a role in the functions of some proteins (McCammon et al., 1979).

Experimental evidence for internal motions of this sort is available from temperature-dependent Debye–Waller factors of X-ray analysis of crystallizable proteins (Willis & Pryor, 1975; Northrup et al., 1980a,b; van Gunsteren & Karplus, 1982). In general, there is qualitative agreement between the extent of motion revealed by X-ray analysis and computer simulation of molecular dynamics. Differences may be due to contributions to the Debye–Waller factors from crystal disorder or to the limitation of the simulations to time scales shorter than 100 ps.

In this paper, we report the characterization by ^{15}N nuclear magnetic resonance (NMR)[1] of the mobility of proteins in intact lyophilized (10% water by weight) and wet cells (90% water by weight) of the bacterium *Aerococcus viridans* tagged with both specific and general ^{15}N labels. Since the static peptide dipolar NH coupling is known accurately, the diminution of this coupling by molecular motion (of frequency greater than the scaled NH 12-kHz coupling) is an unambiguous direct measure of protein mobility. The partial collapse by motion of the dipolar tensor can be measured in a dipolar rotational spin-echo ^{15}N NMR experiment (Munowitz & Griffin, 1982; Schaefer et al., 1983, Cross & Opella, 1985). The peptide ^{15}N chemical shift tensor is also partially averaged

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[1] Abbreviations: NMR, nuclear magnetic resonance; CP, cross-polarization; MAS, magic-angle spinning; MREV-8, designation of a specific multiple-pulse homonuclear decoupling sequence; μ and ρ, chemical shift anisotropy parameters; θ, torsional angular excursion.

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