Nuclear Magnetic Resonance Study of Interaction of Ligands with *Streptococcus faecium* Dihydrofolate Reductase Labeled with [γ-^{13}C]Tryptophan†

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ABSTRACT: Dihydrofolate reductase from *Streptococcus faecium* has been labeled with [γ-^{13}C]tryptophan. We have determined changes occurring in the chemical shifts and line widths of the four resonances of the ^{13}C NMR spectrum of the labeled enzyme, due to its interaction with various ligands. These include the coenzyme, NADPH and related nucleotides, folate and its polyglutamate derivatives, and many inhibitors including methotrexate and trimethoprim. In addition, paramagnetic relaxation effects produced by a bound spin-labeled analogue of 2′-phosphoadenosine-5′-diphosphoribose on the tryptophan C$^{\gamma}$ carbons have been measured. Distances calculated from the relaxation data have been compared with corresponding distances in the crystallographic model of the NADPH–methotrexate ternary complex of *Lactobacillus casei* reductase. The paramagnetic relaxation data indicate that the two downfield resonances (1 and 2) correspond to tryptophans (W_A and W_B) that are more remote from the catalytic site, and from the crystallographic model these are seen to be Trp-115 and Trp-160. The upfield resonances (3 and 4) that show broadening due to chemical exchange correspond to closer residues (W_C and W_D), and these are identified with Trp-6 and Trp-22. However, the relaxation data do not permit specific assignments within the nearer and farther pairs. Although resonance 3, which is split due to chemical exchange, was formerly assigned to Trp-6, data obtained for the enzyme in the presence of various ligands are better interpreted if resonance 3 is assigned to Trp-22, which is located on a loop that joins elements of secondary structure and forms one side of the ligand-binding cavity. Resonance 4 is then assigned to Trp-6, which is located in a hydrophobic region on the opposite side of the β sheet to the binding cavity. Evidence to support these assignments is as follows: (1) NADPH does not affect the line width of resonance 4 and therefore does not influence dynamic behavior of W_D, but Trp-22 interacts with NADPH in the crystal structure. (2) Pyrimethamine has a greater effect than other pyrimidine inhibitors on the chemical shift of W_C. This is consistent with the expected proximity of the chlorophenyl group to Trp-22. (3) The expected proximity of the charged guanidino group of Arg-116 to Trp-6 explains the upfield shift of resonance 4, and internal motion of the guanidino group can explain the exchange broadening of resonance 4. (4) Finally, if W_C is identified with Trp-22, the observed splitting of resonance 3 into two peaks can be explained in terms of cis–trans isomerism of the peptide bond between Leu-20 and Pro-21. Comparisons of the effects of different ligands suggest different modes of binding in certain cases. Examples are methotrexate and its chloro derivatives vs. the diaminopyrimidine inhibitors, pyrimethamine vs. other diaminopyrimidine inhibitors, folates vs. methotrexate, and $NADP^+$ vs. NADPH.

We have previously reported (London et al., 1979a; Groff et al., 1981) results of a nuclear magnetic resonance study of the dihydrofolate reductase (DHFR)[1] from *Streptococcus faecium* in which the enzyme was biosynthesized with 90% enrichment of ^{13}C in the C$^{\gamma}$ positions of tryptophan residues. Our earlier reports dealt with NMR spectra for the reductase in the absence of ligands. These spectra exhibited four resonances, which we assigned numbers 1–4, and we have designated the corresponding residues W_A, W_B, W_C, and W_D. Analysis of those spectra indicated that in the uncomplexed enzyme two of the four tryptophan residues undergo exchange between stable conformations. The exchange process for one of these residues (W_C) is slow on the NMR time scale, and the microenvironments of the C$^{\gamma}$ position in the two states of this residue are quite similar with respect to magnetic and electric fields. The other tryptophan (W_D) is exchanging much faster, and the two stable states are associated with such different chemical shifts that they must correspond to large changes in electric field effects.

W_D was tentatively identified as Trp-22, which is the only tryptophan in *S. faecium* DHFR not involved in secondary structure (β sheet or α helix). It was postulated that movement of this more flexible section of backbone to which Trp-22 is attached is primarily responsible for the exchange process and that the electric field is caused by the charged carboxyl group of Asp-9. Identification of W_C is more speculative, but it was tentatively suggested that it is Trp-6. This residue is in a hydrophobic region immediately below the active site cavity, and the corresponding tryptophan in the *Lactobacillus casei* structure has no charged residues closer than 8.7 Å.

Examination of the effects of ligand binding offers a means of further investigating the dynamic processes that occur in this interesting and important enzyme. They also contribute additional evidence concerning the assignment of the four resonances in the NMR spectra to the various tryptophan

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[1] Abbreviations: DHFR, dihydrofolate reductase; NMR, nuclear magnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ESR, electron spin resonance; MTX, methotrexate; THF, tetrahydrofolate.

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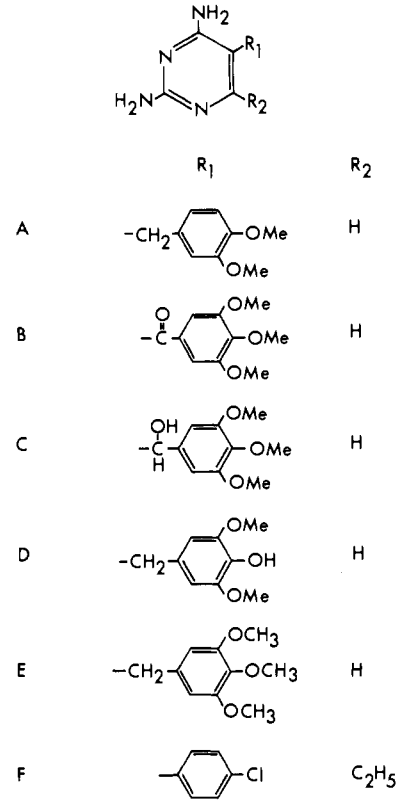

FIGURE 1: Structures of 2,4-diaminopyrimidine inhibitors used in this study: (A) diaveridine; (B) Ro 20-0570/002; (C) Ro 20-0657/000; (D) Ro 5-9552/001, (E) trimethoprim; (F) pyrimethamine.

residues in the sequence. Although our previous identification of W_C with Trp-6 and of W_D with Trp-22 is consistent with the present data, identification of W_C with Trp-22 and of W_D with Trp-6 appears to fit the data better. On the basis of this assignment, the slow dynamic processes monitored by W_C could correspond to cis–trans isomerization of the peptide bond between Leu-20 and Pro-21.

Experimental Procedures

Materials. PADP-spin-label [adenosine 2′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy)] was synthesized as previously described (Cocco & Blakley, 1979). Methotrexate and 2,4-diaminopyrimidine were purchased from the Nutritional Biochemical Corp. Adenosine 2′,5′-diphosphate, 2′-phosphoadenosine-5′-diphosphoribose (PADPR), $NADP^+$, NADPH, and sodium ascorbate were obtained from Sigma. 3′-Chloro- and 3′,5′-dichloromethotrexates were gifts from Dr. E. C. DeRenzo of Lederle Laboratories. Trimethoprim, pyrimethamine, and diaveridine were gifts from Dr. J. J. Burchall of the Burroughs Wellcome Co. Ro 5-9552/001, Ro 20-0657/000 and Ro 20-0570/002 (trimethoprim congeners) were gifts from Dr. W. E. Scott of Hoffmann-La Roche Inc., Nutley, NJ. Structures of the pyrimidine inhibitors are shown in Figure 1. Folyldiglutamate and folylhexaglutamate were gifts from Dr. C. M. Baugh, University of South Alabama. Other chemicals and the dihydrofolate reductase labeled with [γ-^{13}C]tryptophan were obtained as previously described (Groff et al., 1981).

Methods. NMR spectra at 25.2 MHz were recorded on a Varian XL-100 spectrometer at 15 °C as previously described (Groff et al., 1981). Enzyme solutions for use in NMR measurements were concentrated by ultrafiltration and then dialyzed at 5 °C against a solution containing 0.05 M potassium phosphate buffer, pH* 7.3, 0.02% sodium azide, 0.5 M potassium chloride, 1 mM EDTA, and 10–20% D_2O. The enzyme concentration was in the range of 0.75–1.1 mM. Concentrated solutions of the ligands were prepared in 50 mM potassium phosphate buffer, pH 7.3, and the concentrations determined spectrophotometrically. A sufficient amount of the concentrated ligand solution was added to the solution of the ^{13}C-labeled enzyme to convert 90–95% of the enzyme to the complex. This amount was calculated from dissociation constants determined by fluorescence titration (Gleisner & Blakley, 1975) and from the K_i values obtained in initial velocity measurements. In the latter, dihydrofolate was the variable substrate in the case of pteridines and pyrimidines (M. Schrock, J. Thomas, and R. L. Blakley, unpublished results), and NADPH was the variable substrate in the case of nucleotides (Cocco & Blakley, 1979).

Details of enzyme and ligand concentrations are as follows: no ligand with 0.95 mM DHFR; 1.1 mM folate with 0.95 mM DHFR; 1.1 mM folyldiglutamate with 0.77 mM DHFR; 1.2 mM folylhexaglutamate with 0.77 mM DHFR; 2.6 mM 5-formyltetrahydrofolate with 0.77 mM DHFR; 1.1 mM methotrexate with 0.95 mM DHFR; 1.0 mM 3′-chloromethotrexate with 0.97 mM DHFR; 1.0 mM 3′,5′-dichloromethotrexate with 0.97 mM DHFR; 1.5 mM 2,4-diaminopyrimidine with 0.97 mM DHFR; 0.8 mM trimethoprim with 0.76 mM DHFR; 0.8 mM diaveridine with 0.77 mM DHFR; 0.8 mM Ro 20-0570/002 with 0.78 mM DHFR; 0.8 mM Ro 20-0657/000 with 0.76 mM DHFR; 0.8 mM Ro 5-9552/001 with 0.77 mM DHFR; 0.8 mM pyrimethamine with 0.76 mM DHFR; 1.1 mM NADPH with 0.95 mM DHFR; 1.3 mM PADPR with 0.77 mM DHFR; 1.2 mM 2′,5′-ADP with 0.95 mM DHFR; 1.8 mM $NADP^+$ with 0.97 mM DHFR; 1.1 mM PADP-spin-label with 0.95 mM DHFR; 1.1 mM methotrexate and 1.1 mM NADPH with 0.95 mM DHFR; 1.1 mM methotrexate and 1.2 mM 2′,5′-ADP with 0.81 mM DHFR; 1.0 mM dichloromethotrexate and 1.0 mM NADPH with 0.97 mM DHFR; 1.5 mM diaminopyrimidine and 1.8 mM $NADPH^+$ with 0.97 mM DHFR; 0.8 mM trimethoprim and 1.4 mM NADPH with 0.76 mM DHFR.

Chemical shifts are expressed in ppm with respect to external tetramethylsilane and were determined by computer analysis of the transformed spectra. They are accurate to ±0.08 ppm, or better in the case of the sharper resonances. The intensity of the resonances was estimated from the area under the peaks as determined either by computer integration or by "cut and weigh" procedure.

Calculation of Distances of Tryptophan Residues from Bound Spin-Label. The calculation of distances from a nitroxide spin-label is straightforward and has been carried out previously (Wien et al., 1972). The theoretical contribution to the line width is given by

$$\text{line width} = \frac{1}{\pi T_{2M}} = \frac{S(S+1)\gamma_C^2 g^2 \beta^2}{15\pi r^6}\left(4\tau_C + \frac{3\tau_C}{1+\omega_C^2\tau_C^2}\right) \quad (1)$$

where terms containing ω_S, the Larmor frequency of the electron, have been dropped as discussed by Wien et al., ω_C = 25.2 MHz, and

$$\frac{1}{\tau_C} = \frac{1}{\tau_R} + \frac{1}{\tau_S}$$

where τ_S is the electron relaxation time and τ_R the rotational correlation time of the enzyme. We have previously measured the value of τ_R at similar conditions of concentration and temperature for the [*guanido*-^{13}C]arginine-labeled dihydrofolate reductase to be 2×10^{-8} s (Cocco et al., 1978). A direct

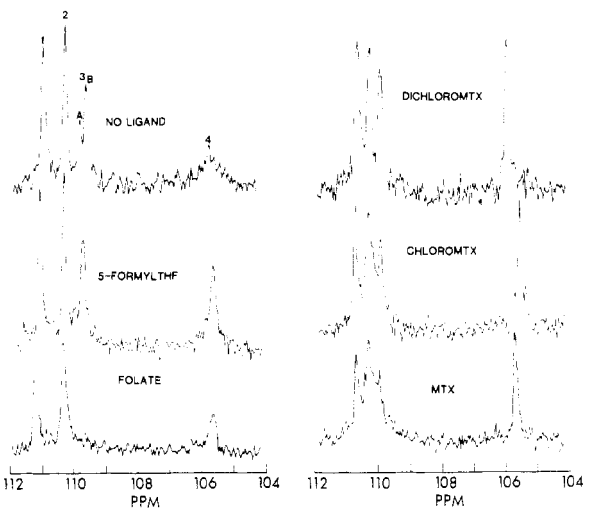

FIGURE 2: ^{13}C NMR spectra of binary complexes of pteridine ligands with *S. faecium* dihydrofolate reductase labeled with [γ-^{13}C]tryptophan. The number of transients for the respective spectra was as follows: without ligand, 63 309; 5-formyltetrahydrofolate, 148 280; folate, 62 105; 3′,5′-dichloromethotrexate, 59 365; 3′-chloromethotrexate, 139 132; methotrexate, 79 031. For other details, see Methods.

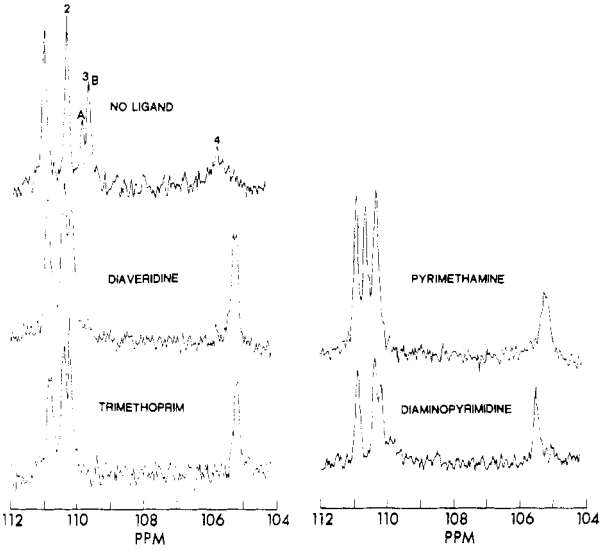

FIGURE 3: ^{13}C NMR spectra of binary complexes of 2,4-diaminopyrimidine inhibitors with *S. faecium* dihydrofolate reductase labeled with [γ-^{13}C]tryptophan. The number of transients for the respective spectra was as follows: without ligand, 63 309; diaveridine, 161 243; trimethoprim, 350 117; pyrimethamine, 176 729; 2,4-diaminopyrimidine, 59 300. For other details, see Methods.

ESR measurement of the enzyme–PADP-spin-label complex yielded a T_2 value of 18 ns, corresponding to $\tau_S = 5 \times 10^{-9}$ s (Cocco & Blakley, 1979). With these values τ_C can be calculated to be 4×10^{-9} s, and eq 1 can be reduced to the relation

$$r = 16.35(T_{2M})^{1/6} \quad (2)$$

where the value of r is given in angstroms. For measurement of the line-width differences in spectra as accurately as possible, spectra were simulated by Du Pont 310 curve resolver with a plotting accessory.

Results

Effect of Binding of Pteridine Substrates and Heterocyclic Inhibitors on $^{13}C^{\gamma}$ Chemical Shifts. As previously described (Groff et al., 1981; London et al., 1979a), the NMR spectrum of uncomplexed *S. faecium* DHFR that has been labeled with [γ-^{13}C]tryptophan consists of four well-resolved resonances, two of which (3 and 4) have characteristics that indicate that the corresponding residues are undergoing chemical exchange. The tryptophan corresponding to resonance 3 (W_C) is undergoing quite slow exchange, while W_D (corresponding to resonance 4) is undergoing more rapid exchange. This spectrum is shown in Figure 2 together with spectra of binary complexes of the enzyme with the substrate, folate, and various substrate analogues. The spectrum of the complex of the enzyme with dihydrofolate, the major substrate, could not be determined because of decomposition of the substrate.

It may be seen from Figures 2 and 3 that the binding of these ligands causes significant changes in the chemical shifts of resonances 1, 3, and 4. These changes in chemical shift due to binding of substrates and inhibitors are summarized in Table I. Resonance 1 is shifted downfield slightly (0.16–0.20 ppm) by folate and its polyglutamate forms and upfield by inhibitors (0.10–0.35 ppm). Resonance 2 is essentially unchanged by the binding of substrates or inhibitors, but some of the most significant changes are observed in the case of resonance 3. Complexes with folate or its polyglutamyl derivatives exhibit spectra in which the double structure of resonance 3 has disappeared and the single resonance is shifted under resonance 2. Spectra of complexes of 2,4-diaminopyrimidine and its derivatives (trimethoprim, diaveridine, Ro 20-0570, Ro 20-0657, Ro 5-9552, and pyrimethamine) also show a simple resonance 3 shifted downfield by 0.39–0.58 ppm for resonance 3B. A significantly larger downfield shift of 0.93 ppm is obtained for the enzyme–pyrimethamine complex. In the case of methotrexate and its derivatives spectra of the binary complexes show resonance 3B moved downfield 0.21–0.28 ppm (Figure 2). The double structure seems to be still present in the spectra of methotrexate and of 3′-chloromethotrexate complexes despite overlap with resonance 2. This result is confirmed by the spectra of the methotrexate binary complex obtained in Tris buffer in the absence of KCl, in which resonances 2 and 3 do not overlap (J. P. Groff, R. E. London, and R. L. Blakely, unpublished results). However, in the case of dichloromethotrexate there is little, if any, sign of splitting of resonance 3 (Figure 2). Moreover, the downfield shift decreases with substitution, i.e., in the order methotrexate > 3′-chloromethotrexate > 3′,5′-dichloromethotrexate (Table I, Figure 2).

Table I: Chemical Shifts in ^{13}C NMR Spectra of Binary Complexes of Substrates or Inhibitors with DHFR Labeled with [γ-^{13}C]Tryptophan

ligand	resonance 1	2	3	4
none	111.11	110.40	110.00 109.80	105.63
folate	111.31	110.40[a]		105.67
folyldiglutamate	111.31	111.37[a]		105.56
folylhexaglutamate	111.28	110.37[a]		105.60
5-formyl-THF	111.15	110.37	109.77	105.64
methotrexate	110.79	110.40	110.40 110.08	105.71
3′-chloromethotrexate	110.82	110.39	110.17 110.01	105.57
3′,5′-dichloromethotrexate	110.80	110.40	110.07	106.00
2,4-diaminopyrimidine	110.95	110.40	110.24	105.44
trimethoprim	110.85	110.44	110.38	105.21
diaveridine	110.90	110.38	110.19	105.17
Ro 20-0570/002	110.76	110.45	110.28	105.21
Ro 5-9552/001	110.92	110.37[a]		105.13
Ro 20-0657/000	110.85	110.43	110.31	105.12
pyrimethamine	111.01	110.41	110.73	105.21

[a] Area under the peak corresponds to two resonances.

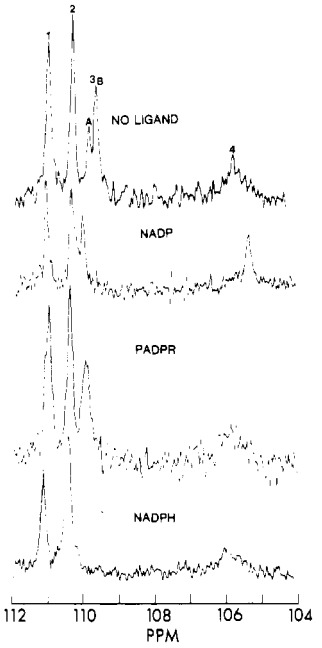

FIGURE 4: ^{13}C NMR spectra of binary complexes of nucleotides with *S. faecium* dihydrofolate reductase labeled with [γ-^{13}C]tryptophan. The number of transients for the respective spectra was as follows: without ligand, 63 309; $NADP^+$, 30 107; PADPR, 107 869; NADPH, 80 205. For other details, see Methods.

Table II: Chemical Shifts in ^{13}C NMR Spectra of Binary Complexes of Coenzyme or Coenzyme Analogues with DHFR Labeled with [γ-^{13}C]Tryptophan

ligand	resonance 1	2	3	4
none	111.11	110.40	110.00 109.80	105.63
NADPH	111.19	110.44[a]		105.87
PADPR	111.06	110.42	109.99	105.68
2′,5′-ADP	111.03	110.40	109.92	105.79
$NADP^+$	111.15	110.44	110.08	105.36

[a] Area under the peak corresponds to two resonances.

As can be seen from Figures 2 and 3 and Table I, effects on the chemical shift of resonance 4 are confined to the inhibitors: folate, its polyglutamate forms, and 5-formyltetrahydrofolate have no significant effects on the chemical shift of this resonance, although they cause significant decrease in line width (vide infra). Although methotrexate and 3′-chloromethotrexate also cause no significant change in the chemical shift of resonance 4, it is interesting to note that 3′,5′-dichloromethotrexate causes a noticeable downfield shift (0.37 ppm). By contrast, binding of the diaminopyrimidine inhibitors leads to upfield shifts of 0.19–0.51 ppm.

Effect of Binding of Coenzymes and Coenzyme Analogues on Chemical Shifts. The chemical shifts of the four resonances in spectra obtained with binary complexes of labeled DHFR with coenzyme or coenzyme analogues are illustrated in Figure 4 and listed in Table II. It may be seen that the chemical shifts of resonances 1 and 2 are not significantly changed in these binary complexes, but resonance 3 is affected in regard to both the line shape and chemical shift. In the binary complex with NADPH, resonance 3 has disappeared, this resonance having been shifted to a position coincident with resonance 2 (110.44 ppm), and there is no sign of a double structure. The binary complexes of PADPR and 2′,5′-ADP give spectra in which the double structure of resonance 3 is clearly absent (Figure 4), but resonance 3 is not displaced as far downfield as in the case of NADPH binary complex. In the case of the $NADP^+$ binary complex it is difficult to determine whether resonance 3 is complex or what the value of the chemical shift is, because of incomplete resolution from resonance 2. On the assumption that the downfield portion of a split resonance is largely obscured by resonance 2, the mean position of this resonance is shifted downfield by 0.3 ppm.

In the case of resonance 4 in spectra of binary complexes of NADPH, PADPR, and 2′,5′-ADP, there is a downfield shift of 0.03–0.24 ppm, whereas in the spectrum of the binary complex of $NADP^+$ resonance 4 is shifted upfield 0.27 ppm.

Chemical Shifts in Ternary Inhibitor–Nucleotide Complexes. Changes in chemical shifts of the four resonances in spectra of the labeled enzyme due to formation of ternary complexes are summarized in Table III, and representative spectra are shown in Figure 5. In the ternary complexes, as in the inhibitor binary complexes, resonance 1 in the NMR spectrum is shifted upfield, the exception being the trimethoprim–NADPH complex where there is a slight downfield

Table III: Chemical Shifts in ^{13}C NMR Spectra of Ternary Complexes of DHFR Labeled with [γ-^{13}C]Tryptophan

ligand	resonance 1	2	3	4
none	111.11	110.40	110.00 109.80	105.63
methotrexate–NADPH	110.91	110.40[a]		105.56
methotrexate–2′,5′-ADP	110.83	110.40[a]		105.60
3′,5′-dichloromethotrexate–NADPH	110.84	110.48[a]		105.58
methotrexate–$NADP^+$	110.60	110.52	110.12	105.63
2,4-diaminopyrimidine–$NADP^+$	110.83	110.63	110.52	105.40
trimethoprim–NADPH	111.19	110.07	110.35	105.12

[a] Area under the peak corresponds to two resonances.

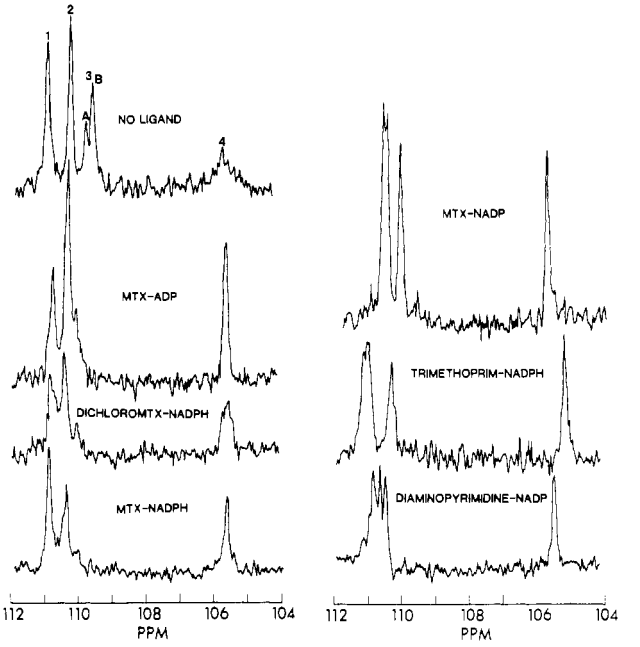

FIGURE 5: ^{13}C NMR spectra of ternary complexes of ligands with *S. faecium* dihydrofolate reductase labeled with [γ-^{13}C]tryptophan. The number of transients for the respective spectra was as follows: without ligand, 63 309; methotrexate–2′,5′-ADP, 71 980; 3′,5′-dichloromethotrexate–NADPH, 96 026; methotrexate–NADPH, 79 099; methotrexate–$NADP^+$, 59 270; trimethoprim–NADPH, 103 754; 2,4-diaminopyrimidine–$NADP^+$, 51 986. For other details, see Methods.

Table IV: Line Widths of Resonance 4 in ^{13}C NMR Spectra of Complexes of Ligands with DHFR Labeled with [γ-^{13}C]Tryptophan

ligand	$\nu_{1/2}$ (Hz)	ligand	$\nu_{1/2}$ (Hz)
Binary Complexes			
none	18.8	3′-chloromethotrexate	3.2
NADPH	18.0	3′,5′-dichloromethotrexate	3.7
$NADPH^+$	5.1		
PADPR	18.0	2,4-diaminopyrimidine	4.1
2′,5′-ADP	18.5	trimethoprim	4.3
folate	7.2	diaveridine	5.8
folyldiglutamate	4.6	pyrimethamine	5.8
folylhexaglutamate	5.8	Ro 20-0657/000	4.3
5-formyl-THF	5.1	Ro 5-9552/001	3.3
methotrexate	4.1	Ro 20-0570/002	6.7
Ternary Complexes			
methotrexate-NADPH	4.1	methotrexate-$NADP^+$	3.9
methotrexate-2′,5′-ADP	4.0	diaminopyrimidine-$NADP^+$	3.2
dichloromethotrexate-$NADP^+$	10.3	trimethoprim-NADPH	3.3

shift. In the spectra of the other ternary complexes the upfield shift was 0.20–0.51 ppm. In the spectrum of the $NADP^+$–methotrexate complex resonance 1 showed the largest upfield shift so far observed.

In contrast to spectra of binary complexes where resonance 2 has the same shift as in the uncomplexed enzyme, spectra of four out of six of the ternary complexes show resonance 2 shifted downfield slightly (0.08–0.30 ppm). However, as in spectra of all the binary complexes, spectra of ternary complexes show resonance 3 moved downfield to varying degrees, and in some cases the double structure has clearly disappeared (Figure 5, Table III). The chemical shift of resonance 4 is unchanged by formation of ternary complexes involving pteridine inhibitors, but trimethoprim and 2,4-diaminopyrimidine in ternary complexes (as in binary complexes) cause significant downfield shifts of resonance 4.

Effects of Ligands on Line Widths of Resonance 4. Resonance 4 is characterized not only by its unusually large upfield shift but also by its large and temperature-dependent line width (Groff et al., 1981). In our first report (London et al., 1979a) we noted that formation of the binary complex of the enzyme with 3′,5′-dichloromethotrexate causes a dramatic narrowing of resonance 4, and a similar effect is seen in Figures 2–5. A summary of line widths at half peak height for resonance 4 is given in Table IV. It may be seen that in all binary complexes of the enzyme with inhibitors or folate derivatives spectra are generated in which resonance 4 is substantially sharpened. On the other hand the binding of nucleotides in binary complexes does not affect resonance 4 line width, with the single exception of $NADP^+$. In the case of ternary complexes the heterocyclic inhibitor still has its line-sharpening effect, but it is greatly decreased in the case of the dichloromethotrexate–NADPH complex.

Effect of PADP-Spin-Label on ^{13}C NMR Spectrum of Labeled DHFR. Figure 6 (top) shows the NMR spectrum of the binary complex of the [γ-^{13}C]tryptophan-labeled enzyme with PADP-spin-label, an analogue of PADPR in which the terminal ribose moiety is replaced by Tempo (tetramethylpiperidinyl-1-oxy). Resonances 3 and 4 are significantly broadened by the paramagnetic ligand while resonances 1 and 2 are much less affected. This indicates that C^γ of W_A and W_B must be further from the unpaired electron of the bound ligand than is C^γ of W_C and W_D. In order to make quantitative estimates of the distances of the tryptophan γ carbons from the nitroxide radical, it was necessary to determine line

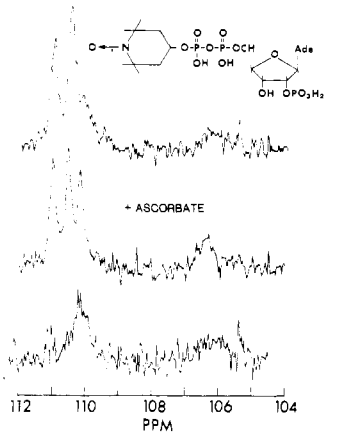

FIGURE 6: ^{13}C NMR spectra of binary complex of PADP-spin-label with *S. faecium* dihydrofolate reductase labeled with [γ-^{13}C]tryptophan. The ligand and enzyme were present at the same concentration. The middle spectrum was obtained after reduction of the nitroxide group by addition of ascorbate. Solid ascorbate (2 molar equivalents relative to spin-label) was dissolved in the sample in the NMR tube after recording the upper and lower spectra, and the mixture was left at room temperature for 10 min before collection of transients was recommenced. Previous EPR measurements indicated that this treatment completed abolished the paramagnetic signal. The bottom spectrum was obtained before ascorbate reduction but with a delay, τ, of 0.693 s in the pulse sequence $180°-\tau-90°-T$. The number of transients for the respective spectra was as follows: top, 142 456; middle, 109 589; bottom, 94 847. (Inset) Structure of PADP-spin-label, where Ade is adenine.

Table V: Effect of Bound PADP-Spin-Label on Spin–Spin Relaxation of C^γ in *S. faecium* DHFR Labeled with [γ-^{13}C]Tryptophan

	line width (Hz)			
resonance	PADP-spin-label	ascorbate reduced PADP-spin-label	T_{2M} (s)	r (Å)[a]
1	7.0	5.5	0.212	12.7
2	5.5	5.0	0.636	15.2
3	15.0	5.5	0.0335	9.3
4	26.5	14.0	0.0255	8.9

[a] On the assumption of an error of ±1 Hz in the line widths determined in the presence of PADP-spin-label, limits on the distances are as follows: for resonance 1, 11.6–15.2 Å; for resonance 2, 12.7–∞ Å; for resonance 3, 9.1–9.4 Å; for resonance 4, 8.8–9.0 Å.

widths in a spectrum of a binary complex that is very similar but diamagnetic. This was achieved by treating the PADP-spin-label–enzyme complex with sodium ascorbate, which reduces the nitroxide group. The spectrum of this diamagnetic complex is shown in Figure 6 (middle). Measured line widths and distances calculated from these line widths, as described under Methods, are shown in Table V.

In principle, calculation of the distances between the unpaired electron on the bound spin-label and C^γ of the four tryptophan residues can also be obtained from values of T_{1M}, the paramagnetic component of the spin–lattice relaxation time. We have previously reported on the spin–lattice relaxation behavior of the uncomplexed enzyme (Groff et al., 1981), but the reduced sensitivity associated with the paramagnetic broadening of the resonances in the PADP-spin-label complexed enzyme made such measurements unfeasible with the available instrumentation. Nevertheless, in order to provide a qualitative check on the distance estimates made from line

widths, we obtained a partially relaxed spectrum using a 180°–τ–90°–T sequence with the value of τ selected to null the resonances with T_1 values close to 1 s. Although only very approximate values can be derived from the resulting spectrum (Figure 6), it is apparent that in the presence of spin-label resonances 3 and 4 have T_1 values significantly shorter than 1 s, whereas resonances 1 and 2 have T_1 values of about 1 s, so that by comparison with values in the absence of spin-label (Groff et al., 1981) they are little affected by the bound spin-label.

When the complex of PADP-spin-label with enzyme was treated with NADPH (10% molar excess over reductase), the NMR spectrum changed to a spectrum very similar to that of the NADPH–reductase binary complex shown in Figure 4 except that resonance 3 was resolved from resonance 2. Within experimental error the widths of all resonances in this spectrum were similar to those of resonances in the spectrum of the NADPH complex in Figure 4.

Discussion

Distance of Tryptophans from Bound Spin-Label. The calculations of the distances of the tryptophan C^γ nuclei from the nitroxide radical in bound PADP-spin-label (shown in Table V) clearly indicate that W_C and W_D are significantly closer to the radical than W_A and W_B. A comparison of these results with crystallographic data can provide a basis for resonance assignment. Since the *S. faecium* reductase has not yet been crystallized, the analysis has been based on the structure of the *L. casei* enzyme (Matthews et al., 1978) by using the sequence alignment discussed previously (Blakley, 1981). According to this analysis, Trp-6, -22, -115, and -160 of the *S. faecium* 2 sequence correspond to Trp-5, Trp-21, Leu-114, and Tyr-155 of the *L. casei* enzyme. Furthermore, Trp-6 and Trp-22 are clearly closer in the crystallographic model to bound ligands than Trp-115 and Trp-160, so that resonances 3 and 4 correspond to Trp-6 and Trp-22, though not necessarily in that order.

Complete resonance assignments cannot be made for several reasons: (1) The spatial coordinates of the C^γ nuclei of Trp-6, -22, -115, and -160 of the *S. faecium* dihydrofolate reductase are probably not exactly the same as those for the C^γ nuclei of Trp-5, Trp-21, Leu-114, and Tyr-155 in the *L. casei* reductase. (2) The conformation of the active site area is altered in the presence of different ligands and is consequently different in the methotrexate–NADPH ternary complex, for which the atomic coordinates are available, from that in the PADP-spin-label binary complex. PADP-spin-label is a linear competitive inhibitor of dihydrofolate reductase with respect to NADPH with a low K_i (7.7 μM; Cocco & Blakely, 1979). Furthermore, binding to only one site on the reductase is detectable, and this must therefore be the same site at which NADPH binds. The NMR data showing that NADPH displaces PADP-spin-label confirm this conclusion. Nevertheless, crystallographic data for the binary NADPH complex are not yet available. Even if they were, small but significant differences in the conformation of the NADPH and PADP-spin-label binary complexes would be anticipated due to the hydrophobic character of the Tempo group. (3) Several approximations are inherent in the spin-label approach, including the neglect of effects associated with possible internal motion of the spin-label, the anisotropic diffusion of the enzyme, and the assumption that the relaxation effects are exclusively dipolar in nature. Contribution to resonance broadening due to the "Curie spin" term discussed by Gueron (1975) has been neglected, but this is probably justified at the relatively low magnetic field, 2.35 T, at which the experiments were carried out. Error due to exchange broadening of the tryptophan resonances is probably small. In the first place very similar exchange broadening should occur for the reduced hydroxylamine form used for comparison. Secondly, such ligands are in slow exchange: the K_i for PADP–spin-label is low (7 μM), sharp resonances are seen for nuclei in side chains interacting with ligands (Blakley et al., 1978; Cocco et al., 1978), and when spectra were obtained for enzyme half-saturated with ligand, separate resonances were seen corresponding to free enzyme and to complex (L. Cocco and R. E. London, unpublished results). Although internal motion of the label could, in principle, reduce the effective rotational correlation time, τ_R, characterizing the interaction, in view of the relatively long distance between the bound nitroxide group and the labeled tryptophan γ carbons, error due to this is also likely to be small.

Table VI: Interatomic Distances (Angstroms) to C^γ Calculated from Atomic Coordinates for the Methotrexate–NADPH Ternary Complex of *L. casei* Dihydrofolate Reductase[a]

	Trp-5	Trp-21	Leu-114	Tyr-155
nicotinamide N-1	7.93	9.08	15.33	16.97
nicotinamide ribose C-1	9.21	9.06	16.84	18.22
NMR distances[b]	8.9 (W_D)	9.3 (W_C)	12.7 (W_A)	15.2 (W_B)

[a] Atomic coordinates from the X-ray crystallographic analysis (Matthews et al., 1978, 1979) were kindly provided by Dr. D. A. Matthews. [b] Distances were calculated from relaxation data for *S. faecium* reductase. The distance of the unpaired election of bound PADP-spin-label from C^γ of the tryptophan residues is shown in parentheses. Assignments are tentative, particularly for W_A and W_B, as indicated in the text.

Evidence that the method does give useful results is found in Table VI, which presents the calculated distances between C^γ of Trp-5, Trp-21, Leu-114, and Tyr-155 in the *L. casei* reductase ternary complex and two of the atoms in the nicotinamide ribonucleoside moiety of the bound NADPH. For the two closer residues, distances between the nicotinamide ribose C-1′ and the γ carbons of the residues noted above are found to approximate the distances between the nitroxide radical and the γ carbons of the corresponding tryptophan residues in the *S. faecium* reductase closely. The partially relaxed ^{13}C spectrum (Figure 6, lower trace) further indicates that the spin–lattice relaxation rates for two of the residues, W_C and W_D, are significantly shorter than the rates for the other two, W_A and W_B. Assignment of W_C and W_D to Trp-6 and Trp-22, and consequently W_A and W_B to Trp-115 and Trp-160, can therefore be made with confidence, but assignments within each pair are uncertain.

Chemical Shift Data. As discussed previously (Groff et al., 1981), the small shift differences observed for resonances 1, 2, and 3 in the spectrum of the unligated enzyme can be explained by a variety of factors. However, the relatively large upfield shift of resonance 4 requires the existence of a strong electric field contribution, presumably reflecting proximity to a charged side chain. If, as proposed previously (Groff et al., 1981), W_D is identified with Trp-22, which forms part of one side of the hydrophobic pocket where ligands bind, the electric field could reflect contributions of the carboxylate groups of Asp-27 and Asp-9. However, the carboxylate ions of both of these residues are rather distant, and Asp-27 is also present in the *L. casei* enzyme where it does not seem to cause a large upfield shift (Pastore et al., 1979, 1981). If resonance 4 is assigned to Trp-6, on the other hand, the upfield shift could be explained by the proximity of the indole ring of this residue to the guanidino side chain of Arg-116. By analogy with Trp-5 in the crystallographic model of the *L. casei* enzyme, Trp-6

in the *S. faecium* structure is in a hydrophobic region at the bottom of the binding cavity and on the opposite side of the β sheet to the latter. The probable proximity of the Arg-116 side chain to Trp-6 was overlooked in our previous analysis (Groff et al., 1981). This residue corresponds to Val-115 in *L. casei* reductase, the β carbon of which makes hydrophobic contact with Trp-5. The exact position of the guanidino group of Arg-116 in *S. faecium* reductase cannot be predicted with certainty, but the group is probably hydrogen bonded with Gln-8 or Gln-161 and likely to be 5–7 Å from C^{γ} of Trp-6. This distance also seems rather large to produce the upfield chemical shifts of resonance 4. However, it is not impossible that the charged guanidino group, if suitably oriented with respect to Trp-6, could produce the observed upfield shift of 4 ppm at a distance of 5 Å.

It must be concluded that the need for invoking shift contributions of proximate charged residues to explain the upfield position of resonance 4 does not provide a good basis for determining whether W_D corresponds to Trp-6 or Trp-22 but slightly favors the former assignment.

Dynamic Data. As discussed previously, resonance 3 is split into two components as a consequence of slow exchange between two enzyme conformations (Groff et al., 1981), and resonance 4 is strongly exchange broadened. Analysis of the data for resonance 4 on the basis of a two-state model gave rates of 90 and 150 s^{-1} corresponding to 5 and 25 °C, respectively. We have previously suggested (Groff et al., 1981) that the dynamic behavior of W_D would most likely correspond to the motion of the loop that joins βA to αB and contains Trp-22. This segment of the enzyme is not involved in secondary structure and consequently would have a relatively high degree of conformational mobility. Furthermore, it is one of two loops represented by relatively weak density in the 2.5-Å map for the binary complex of methotrexate with *Escherichia coli* reductase, which indicates significant motion within the crystal structure (Matthews et al., 1979). However, there is no evidence as to whether the motion of this segment of backbone would have a rate constant in the range required by the NMR data.

On this view the split resonance (resonance 3) would correspond to Trp-6. No specific hypothesis was advanced for the molecular basis of the splitting of resonance 3, but exchange of the tryptophan ring, or of a nearby perturbing side chain, between alternative preferred conformations would be presumed.

An alternative possibility is that the slow exchange kinetics observed for resonance 3 correspond to cis–trans isomerism of peptide bonds formed with proline. Such effects are commonly observed in proline-containing peptides and can produce splitting of resonances corresponding both to proline nuclei and to nuclei of other residues in the vicinity. For example, in the tripeptide Gly–Pro–Phe several of the phenylalanine resonances are split as a result of the cis–trans isomerism of the Gly–Pro bond, and the magnitude of the splitting is similar to that for resonance 3 (London et al., 1979b; Anteunis et al., 1981). Similarly, tyrosyl resonances of ACTH are split due to the cis–trans isomerism of the bond between Tyr-23 and Pro-24 (Toma et al., 1981). Although crystallographic evidence for the existence of cis peptide bonds in proteins has been reported, we are unaware of no precedent for evidence of the existence of an equilibrium involving *both* conformations in a globular protein structure. Clearly, such a situation would require that the residues involved be situated in a segment of the protein chain not involved in secondary structure. The loop connecting βA to αB fulfills this condition, and hence, the assignment of W_C to Trp-22 is consistent with this interpretation.

The NMR spectrum for the unligated reductase of *L. casei* labeled with [γ-^{13}C]tryptophan presented by Pastore et al. (1979, 1981) showed no evidence of a split resonance similar to that of resonance 3 in the *S. faecium* reductase spectrum. It is possible that overlap of resonances in the spectra presented might explain this result. If not, since the Pro-20–Trp-21 sequence in *L. casei* reductase corresponds to Pro-21–Trp-22 in *S. faecium* reductase, the difference in the splitting of the resonances from Trp-21 of *L. casei* and Trp-22 of *S. faecium* reductases requires an explanation. There are two possibilities: a different cis–trans equilibrium in the two enzymes; or absence of field change for the tryptophan C^{γ} of the *L. casei* reductase when isomerization occurs. The first possibility seems especially likely, since it would only require a destabilization of one of the isomers by about 1 kcal/mol to prevent observation of the corresponding resonance. The many sequence differences between the two reductases could easily account for such a difference in stability.

It is difficult to determine what conformational changes in the vicinity of the ligand-binding cavity would occur as the Leu-20–Pro-21 bond changes from the trans conformation (as in the *L. casei* reductase ternary complex) to the cis conformation. However, examination of graphic displays of the *L. casei* reductase structure seem to indicate that such isomerism would be sterically possible, especially in the absence of bound NADPH (D. A. Matthews, personal communication).

If this assignment is correct, the exchange broadening observed for resonance 4 would probably arise from internal motion of the Arg-116 side chain, motion of Trp-6 probably being relatively slow because this residue is involved in β sheet and the bulky indole side chain is in a tightly packed hydrophobic region. It should be noted that the increase in the line width of resonance 4 due to exchange is directly related to the chemical shift range of the various states among which C^{γ} of W_D is exchanging, which is about 100 Hz (Groff et al., 1981). This in turn is directly related to the large upfield shift of resonance 4.

Ligand Effects. As has been discussed previously (Blakley et al., 1978), the chemical shift changes of protein resonances resulting from complexation with ligands can be produced by either direct or indirect effects. In an analogous fashion, ligands can produce both direct and indirect effects on the dynamics of residues, the latter resulting from conformational changes induced by ligand complexation. Although the dual possibilities of direct and indirect effects prevent the use of ligand-induced parameter changes as absolute assignment aids, strong suggestive evidence can frequently be obtained.

We first consider the effects of NADPH and its analogues (Table II). Resonance 3 is only slightly shifted (<0.18 ppm) by the formation of binary complexes with $NADP^+$, 2′5′-ADP, or PADPR (Figure 4 and Table II), but NADPH produces a significant downfield shift. The extent of this shift cannot be determined accurately due to the overlap of resonances 2 and 3 in the spectrum of the NADPH complex. However, it is clear that the reduced nicotinamide ring of bound NADPH has a specific effect on W_C. Further, no evidence for the double structure of resonance 3 could be observed in any of the nucleotide complexes, so that binding of these ligands appears to inhibit the motion required for exchange. In contrast, $NADP^+$ has the largest effect on the chemical shift of resonance 4 (upfield shift of 0.27 ppm) and is also the only ligand in this series that produces some sharpening of resonance 4 (Table IV). The observation that NADPH (as well

as PADPR and 2′,5′-ADP) produces no significant sharpening of resonance 4 is significant in view of the known interactions of the nicotinamide ring of bound NADPH with Trp-21 and adjacent residues in the NADPH–MTX–DHFR complex formed by the *L. casei* enzyme (Matthews, 1979). According to the X-ray crystallographic results on this complex, Ile-13, Gly-14, Gly-17, His-18, Leu-19, and Trp-21 all interact with either the nicotinamide ring or the adjacent ribose, and these interactions would be expected to restrict the motion of this loop of backbone. In contrast, Leu-19 is the only residue in this loop of backbone in the *L. casei* reductase with which methotrexate interacts (Matthews et al., 1978), yet this inhibitor is efficient in retarding the chemical exchange behavior of resonance 4 as monitored by the line width (Table IV). If W_C is identified as Trp-22 and W_D as Trp-6, the data can be explained by assuming that NADPH binds to a particular conformation of the loop in which the Leu-20–Pro-21 bond has only one conformation (probably trans, since this is the conformation in the *L. casei* reductase crystallographic model). This would lead to the observed elimination of the splitting of resonance 3, while having only a small effect on the dynamic behavior responsible for the broadening of resonance 4.

In contrast to the results with NADPH, all inhibitors of the methotrexate and trimethoprim family produce dramatic sharpening of resonance 4 (Figures 2, 3, and 5; Table IV). 5-Formyltetrahydrofolate and folate also produce significant sharpening of this resonance. This marked effect of inhibitor binding is somewhat surprising if resonance 4 corresponds to Trp-6, since the latter is on the opposite side of the β sheet to the binding cavity. However, in studies on *L. casei* reductase into which fluorotryptophan had been incorporated, Kimber et al. (1977) reported that methotrexate binding produced a narrowing of all the fluorine resonances including the downfield resonance assigned to fluorotryptophan-5 (Matthews, 1979) and the resonance assigned to fluorotryptophan-133 (also on the opposite side of the β sheet to the bound ligand). Thus, binding of inhibitor does, in fact, cause sufficient change in conformation in the hydrophobic region on the distal side of the β sheet to decrease significantly side-chain motions responsible for chemical exchange of these residues.

The trimethoprim series is also interesting in terms of the effects on the chemical shift of resonance 3. This resonance is shifted significantly further downfield by pyrimethamine than by any of the other inhibitors in this series (Figure 1). In fact, the shift of resonance 3 is 0.33 ppm further downfield in the binary DHFR–pyrimethamine complex than in any of the other complexes observed. Assuming that the diaminopyrimidine moiety of each inhibitor binds in a very similar orientation relative to the enzyme, this difference most probably reflects differing orientations of the various aromatic substituents. Since examination of the crystallographic model of the *L. casei* ternary complex (Matthews et al., 1979) indicates that the aromatic substituents are closer to Trp-21 than to Trp-5, the data are consistent with the assignment of Trp-22 in the *S. faecium* enzyme to resonance 3. The larger downfield shift of this resonance in the pyrimethamine complex is then ascribed to a direct ring current contribution by the chlorophenyl group.

Resonances 1 and 2. The effects of the various ligands on resonances 1 and 2 are small, but the most significant trend is the lack of sensitivity of the chemical shift of resonance 2 in binary complexes of any of the ligands tested. Where the ligands contain aromatic rings and therefore have ring currents associated with them in a powerful magnetic field, and when they also contain charged groups, as in the case of the ligands considered here, the absence of any change in chemical shift when the ligands bind is a reliable indication that the ^{13}C-enriched side chain is relatively remote from the ligand-binding site. In addition, it must be concluded that conformational transitions induced in the binary complexes do not affect the region of the protein containing W_B. In contrast, small but significant downfield shifts for W_B are observed in four of the ternary complexes (Table III), most probably indicating that in these complexes a ligand-induced conformational transition in the enzyme has produced indirect effects. Such differences between the binary and ternary complexes are consistent with crystallographic differences in the conformations of the binary and ternary complexes (Matthews et al., 1979). W_A also shows small changes in chemical shift (+0.20 to −0.35 ppm) when pteridines form binary complexes (Table I), no changes when nucleotides bind (Table II), and a maximum change (−0.51 ppm) in a ternary complex.

On the assumption that the *S. faecium* and *L. casei* reductases have similar conformation of the backbone and that W_A and W_B, corresponding to Trp-115 and Trp-160, occupy positions corresponding to Leu-114 and Tyr-155 in the *L. casei* structure, W_A and W_B are positioned at the end of the ligand-binding cavity, beneath the α helix over which the benzoylglutamate moiety of pteridine ligands is draped. They probably have their rings stacked on either side of Phe-30, and they are approximately equidistant from the heterocyclic ring of bound inhibitors. Assignment of resonances 1 and 2 to residues in these positions is consistent with all the available data, but evidence for the specific assignment of each of these resonances is relatively weak and inconclusive at present. Although the assignment of W_A and W_B to Trp-115 and Trp-160, respectively, is suggested by the distance data obtained with the PADP-spin-label (Table VI), an error of ±1 Hz in the line widths measured for the binary complex with the spin-label corresponds to a distance range of 11.6–15.2 Å for resonance 1 and 12.7–∞ Å for resonance 2, precluding definitive assignments.

Different Modes of Ligand Binding Inferred from NMR Data. Comparison of the chemical shifts of the various tryptophan residues in the various ligand complexes is, in principle, able to provide evidence as to whether related ligands bind differently. One example of this is seen when the chemical shift of resonance 4 is compared in binary complexes of different inhibitors (Table I). Whereas δ for W_D in complexes of methotrexate, 3′-chloromethotrexate, and 3′,5′-dichloromethotrexate is almost the same as for the uncomplexed enzyme and for binary complexes of folate and its polyglutamates, there are significant upfield shifts for complexes of all the diaminopyrimidines. It seems likely that the diaminopyridine inhibitors bind somewhat differently from methotrexate and its derivatives in the cavity and thus cause different indirect effects on W_D. This conclusion is strengthened by the different chemical shifts of all four resonances in the spectrum of the methotrexate–NADPH ternary complex compared with those of the trimethoprim–NADPH ternary complex (Table III). Similarly, all four chemical shifts for the methotrexate–$NADP^+$ ternary complex differ from those for the 2,4-diaminopyrimidine–$NADP^+$ complex (Table III). It should also be noted that there are some significantly different effects of methotrexate binding and 2,4-diaminopyrimidine binding on the ^{13}C NMR spectrum of *S. faecium* DHFR labeled with [*methyl*-^{13}C]methionine (Blakley et al., 1978).

When the effect of ligands in ternary complexes on the line width of resonance 4 is examined (Table IV), one of the most

surprising anomalies occurs with the dichloromethotrexate–NADPH–DHFR complex. For this complex, $\nu_{1/2}$ is 10.3 Hz compared with 3.2–4.1 Hz for the other ternary complexes examined, including the methotrexate–NADPH complex. This result is the more unexpected since the binary 3′,5′-dichloromethotrexate complex gives a line width of 3.7 Hz for resonance 4. The binding of NADPH interferes with the line-narrowing effect of 3′,5′-dichloromethotrexate in a manner presumably related to the bulk of the chlorine atoms.

Other differences in ligand effects are probably due more directly to differences in the structure of the ligands than to differences in orientation of the ligands in the binding cavity. The different effects of folates as compared with inhibitors on the chemical shift of resonance 1 are most readily understood in terms of the positive charge on N-1 of the heterocyclic ring of bound inhibitors (Cocco et al., 1981a, 1981b). No such charge is present on bound folates. These two groups of ligands also have different effects on the chemical shifts of resonances 3 and 4, which may in part also arise from the charged ring of the inhibitors. However, another important contributing factor to these different effects is the fact that the folates apparently bind in the cavity with the pteridine ring in an orientation approximately 180° different from that of the ring of methotrexate in the ternary complex with NADPH and *L. casei* DHFR. This follows from the results of Charlton et al. (1979) taken together with the crystallographic data of Fonticilla-Camps et al. (1979) and also from the data of Armarego et al. (1980).

The different chemical shift of resonance 3 in the spectrum of the binary NADPH complex from that for the complex of 2′,5′-ADP or PADPR can also be explained simply on the basis that only the nicotinamide riboside moiety of NADPH is inserted into the active site cavity. Since 2′,5′-ADP and PADPR lack this moiety, it is not unexpected that they produce little conformational change in the cavity region as monitored by tryptophan-6, -22, and -160.

Complexes of NADPH and $NADP^+$ differ significantly as indicated by a number of aspects of their ^{13}C NMR spectra: in the binary complexes δ values for resonance 4 are significantly different; in the methotrexate complexes δ values for resonances 1 and 3 are different; and the line width of resonance 4 in the binary complexes is markedly different. Some marked differences were previously observed in the ^{13}C NMR spectra of the two binary complexes with the [*methyl*-^{13}C]-methionine-labeled *S. faecium* enzyme (Blakley et al., 1978). Many of these different effects of the binding of $NADP^+$ and NADPH are probably due to the electric field effect of the positively charged nicotinamide ring in $NADP^+$ and ring current in the nicotinamide ring of $NADP^+$, but as has previously been argued (Blakley, 1981), the reduced nicotinamide ring of NADPH may be oriented differently in the active site cavity than the charged oxidized ring of $NADP^+$. It is possible that electrostatic attraction may draw the charged nicotinamide ring further into the cavity toward the carboxylate group of Asp-27, with consequent restriction of the motion responsible for chemical exchange of Trp-6.

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