& Fee (1978b), this cysteine does not exist as a persulfide as reported by Calabrese et al. (1975). Briggs & Fee (1978b) have indicated that the results reported by the latter workers are artifacts arising from oxidative conditions employed during isolation by procedures including removal of the hemoglobin by denaturation with solutions of ethanol–chloroform.

The sequence for human superoxide dismutase reported in the present study is similar to those portions of the partial structure given by Barra et al. (1978) except for residue 47, at which position these workers report a glycine. We have found a valine at this position, which is in agreement with what is noted for the analogous position in the bovine enzyme.

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Proton Nuclear Magnetic Resonance Studies of the Effects of Ligand Binding on Tryptophan Residues of Selectively Deuterated Dihydrofolate Reductase from *Lactobacillus casei*†

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ABSTRACT: We have prepared a selectively deuterated dihydrofolate reductase in which all the aromatic protons except the C(2) protons of tryptophan have been replaced by deuterium and have examined the ^{1}H NMR spectra of its complexes with folate, trimethoprim, methotrexate, $NADP^+$, and NADPH. One of the four Trp C(2)-proton resonance signals (signal P at 3.66 ppm from dioxane) has been assigned to Trp-21 by examining the NMR spectrum of a selectively deuterated *N*-bromosuccinimide-modified dihydrofolate reductase. This signal is not perturbed by NADPH, indicating that the coenzyme is not binding close to the 2 position of Trp-21. This contrasts markedly with the ^{19}F shift (2.7 ppm) observed for the ^{19}F signal of Trp-21 in the NADPH complex with the 6-fluorotryptophan-labeled enzyme. In fact the crystal structure of the enzyme·methotrexate·NADPH shows that the carboxamide group of the reduced nicotinamide ring is near to the 6 position of Trp-21 but remote from its 2 position. The nonadditivity of the ^{1}H chemical-shift contributions for signals tentatively assigned to Trp-5 and -133 indicates that these residues are influenced by ligand-induced conformational changes.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate by using NADPH as a coenzyme. The enzyme is of considerable pharmacological interest, being the target for "antifolate" drugs such as methotrexate and trimethoprim. As part of a wider program aimed at understanding the factors influencing the binding of ligands to dihydrofolate reductase, we have been examining the NMR spectra of complexes of the enzyme with its coenzyme, substrates, and substrate analogues. The success of such studies depends on the detection of signals from nuclei

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in individual residues in the complex. Signals from proton nuclei in the bound ligands can be detected by transfer of saturation experiments between free and bound species (Bendall et al., 1977; Cayley et al., 1979; Hyde et al., 1980) and by obtaining difference spectra of complexes formed with deuterated and nondeuterated ligands (Baldo et al., 1975; Cayley et al., 1979). Signals from individual amino acid residues of the protein are much more difficult to detect and assign because most of the signals are badly overlapped by resonances from other amino acid residues. London et al. (1979) have incorporated [γ-^{13}C]tryptophan into dihydrofolate reductase from *Streptococcus faecium* and detected the ^{13}C resonances from tryptophans in the labeled enzyme. Several workers (Markley et al., 1968; Putter et al., 1970; Crespi et al., 1968, 1973) have shown how protein ^{1}H NMR spectra can be simplified by selective deuteration, and we have previously used this method to study the five tyrosine residues in *Lactobacillus casei* dihydrofolate reductase (Feeney et al., 1977).

In this paper we describe how tryptophan residues can be monitored in a similar manner by examining a sample of dihydrofolate reductase in which all the aromatic protons except the C(2) protons of tryptophan have been replaced by deuterium; this enables the signals from the four tryptophan residues to be detected directly. One of the tryptophan residues (Trp-21) is of particular interest since selective chemical modification of this residue with *N*-bromosuccinimide has been shown to inactivate the enzyme and markedly decrease the binding of NADPH (Liu & Dunlap, 1974; Freisheim et al., 1977; Thomson et al., 1980).

In an earlier experiment we examined the ^{19}F NMR spectra of complexes of dihydrofolate reductase containing 6-fluorotryptophan (Kimber et al., 1977, 1978). Although this provides a convenient and sensitive method for monitoring the effects of ligand binding on tryptophan residues, there is always the possibility that the introduction of fluorinated residues could perturb the enzyme structure and its interactions with ligands. The selective deuteration approach described here has the important advantage that it is unlikely to cause any problems of this type.

Materials and Methods

^2H_2O (99.85 atom % ^{2}H) was obtained from Norsk Hydroelectrisk; methotrexate was from Nutritional Biochemicals Corp.; NADP$^+$, NADPH, and folate were from Sigma Chemicals; trimethoprim was from Wellcome Laboratories.

Deuterated phenylalanine, histidine, and tyrosine in which all the aromatic protons had been replaced by deuterium and α,4,5,6,7-pentadeuteriotryptophan were prepared as described by Griffiths et al. (1976). *L. casei* MTX/R was grown as described by Dann et al. (1976). For the preparation of the selectively deuterated enzyme, the casein hydrolysate in the growth medium described by Dann et al. (1976) was replaced by a mixture of pure L-amino acids, including the above deuterated amino acids in place of the normal aromatic amino acids. It has been shown previously that this strain of *L. casei* is auxotrophic for all the aromatic amino acids and that these are incorporated without change into dihydrofolate reductase (R. W. King and P. Scudder, unpublished experiments).

The selectively deuterated dihydrofolate reductase, containing tryptophan C(2) protons as its only aromatic protons, was purified as described by Dann et al. (1976). The purified enzyme was lyophilized twice from a ^2H_2O solution to remove most of the exchangeable protons and then redissolved to give approximately 0.5 mM enzyme solutions in ^2H_2O, containing 3 mM dioxane reference, 50 mM potassium phosphate, and 500 mM potassium chloride at pH* 6.8 (uncorrected meter reading). The ligands were added as weighed amounts of the solids; 1:1 complexes were formed for these tightly binding ligands by adding slightly more than 1 molar equiv (up to two equivalents) to the enzyme solutions.

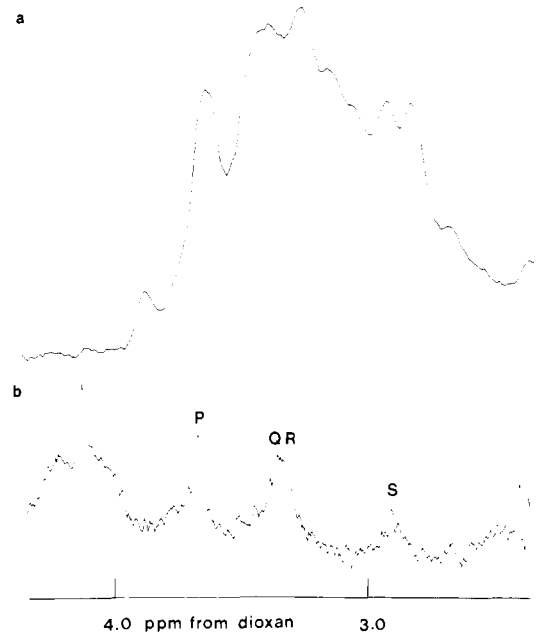


FIGURE 1: The aromatic region of the ^{1}H NMR spectrum at 270 MHz of *L. casei* dihydrofolate reductase at 11 °C. (a) Normal enzyme; (b) selectively deuterated enzyme with all aromatic protons replaced by deuterium except for C(2) protons of tryptophan.

The guanidine hydrochloride (used to facilitate the deuterium exchange of inaccessible NH protons in the protein) was lyophilized 6 times from ^2H_2O solution.

N-Bromosuccinimide (NBS) was used to chemically modify the enzyme. The reagent was crystallized twice from water, and a 20 mM stock solution was made up in ^2H_2O. By use of a long-needled Hamilton syringe, aliquots of this solution were added to an agitated solution of the enzyme in the NMR tube at 0 °C. A total of 3 molar equiv of *N*-bromosuccinimide was added. This leads to an 80% inactivation of the enzyme (Thomson et al., 1980), and Freisheim et al. (1977) have shown that only Trp-21 is chemically modified under these conditions.

The ^{1}H NMR spectra were obtained at 270 MHz with a Bruker WH270 spectrometer operating in the Fourier transform mode. By use of a 4200-Hz spectral width, free induction decays (8K data points) were obtained and weighted with an exponential function to improve the signal-to-noise ratio in the spectrum obtained after Fourier transformation.

Results

As would be expected, the aromatic region of the NMR spectrum of the selectively deuterated enzyme has been much simplified compared with that for the natural enzyme; this is illustrated in Figure 1. In the deuterated enzyme, the only protons contributing to the low-field region of the spectrum should be the C(2) protons of the four tryptophan residues (Trp-5, -21, -133, and -158) and any "buried" NH protons which have escaped deuterium exchange in the ^2H_2O solution. The signals of these NH protons (from 4.0 to 5.5 ppm) could be removed by adding 0.5 M guanidine hydrochloride, which loosens the enzyme structure sufficiently to allow the NH protons to exchange rapidly with the ^2H_2O solvent. The only signals remaining are those at 4.13, 3.65, and 3.33 ppm and possibly the broad signal at 2.90 ppm (which falls under the

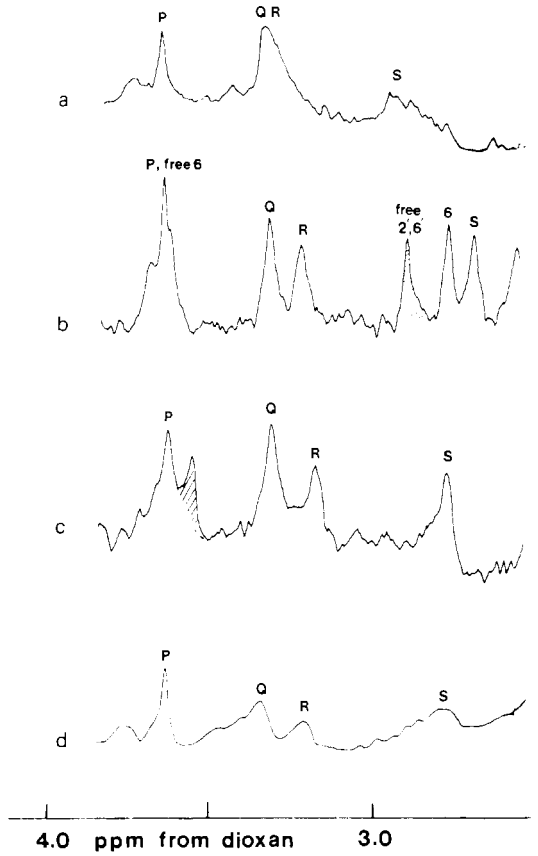


FIGURE 2: The aromatic region of the ^{1}H NMR spectrum at 270 MHz (25 °C) of selectively deuterated dihydrofolate reductase in various complexes. (a) Enzyme alone; (b) enzyme·trimethoprim; (c) enzyme·methotrexate; (d) enzyme·folate.

residual signal from the deuterated guanidine hydrochloride). The sharp signal at 4.13 ppm has been detected previously in samples containing fully deuterated tryptophan so that it cannot be a tryptophan C(2) proton resonance. The remaining signals, labeled P, Q, R, and S in Figures 1 and 2a, at 3.65, 3.33 (two protons), and 2.90 ppm are assigned to the four tryptophan C(2) protons. Three of these resonances are close to that of the tryptophan C(2) protons in the denatured protein (3.57 ppm; see below), but one has been shifted almost 0.7 ppm to high field. This high-field signal, S, is very broad, but it will be seen to sharpen considerably in complexes with the coenzyme and inhibitors.

Binary Complexes with Inhibitors and Substrates. In the spectrum of the trimethoprim complex (Figure 2b) two of the tryptophan signals (P and Q) have the same chemical shifts as in the free enzyme (Table I). It will be seen that these signals have the same chemical shifts in all the binary and ternary complexes so far examined. On addition of trimethoprim, signal R moves upfield by 0.11 ppm, and signal S sharpens considerably and shifts 0.22 ppm upfield. The sharp signals at 3.62 and 2.90 ppm arise from the H(6) and H(2′), H(6′) protons of free trimethoprim, while the signal at 2.75 ppm is from the H(6) proton in bound trimethoprim. This latter assignment has been made unequivocally by transfer of saturation experiments (Cayley et al., 1979); under these conditions the resonance of the 2′,6′ protons of bound trimethoprim is too broad to be observable (Cayley et al., 1979).

The enzyme·methotrexate complex gives a spectrum (Figure 2c) quite similar to that of the trimethoprim binary complex. Again, only signals R and S are affected, being shifted only slightly more and slightly less, respectively, than in the trimethoprim complex (see Table I). Signal S again shows considerable line narrowing on formation of the complex. The signal at 3.57 ppm arises from Trp residues in a small amount of denatured enzyme which was present in this sample. No signals from the protons of the benzoyl ring of bound methotrexate were detected in the spectrum; however, the possibility that some of them resonate at the same frequency as one or more of the tryptophan residues cannot be excluded because accurate intensity measurements are difficult to make in these spectra.

Table I: ^{1}H NMR Chemical Shifts[a] of Tryptophan C(2)-Proton Resonances in Selectively Deuterated Dihydrofolate Reductase and Its Complexes with Ligands at 25 °C

ligand	chemical shift (ppm)			
	P	Q	R	S
none	−0.14	0.18	0.18	0.61[b]
trimethoprim	−0.14	0.18	0.29	0.83
methotrexate	−0.15	0.18	0.32	0.73
folate (11 °C)	−0.15	0.15	0.27	0.71
$NADP^+$	−0.14	0.21	0.60	0.60
NADPH	−0.15	0.19	0.32	0.68
NADPH·methotrexate	−0.15	0.17	(0.50)[c] (0.44)[c]	0.63

[a] Chemical shifts measured in parts per million (±0.02) upfield from the C(2)-proton resonance of *N*-acetyltryptophanamide. [b] Very broad signal ($\nu_{1/2}$ = 25 Hz); error in shift ±0.07 ppm. [c] Assignment is uncertain because of overlap with signals from bound NADPH.

Addition of 1 molar equiv of the substrate folate gave a spectrum (Figure 2d) in which the chemical shifts of all the Trp C(2) proton resonances are very similar to those in the enzyme·methotrexate complex. However, signals Q, R, and S are much broader for the folate complex, probably due to exchange effects.

Binary Complexes with Coenzyme. The spectrum of the $NADP^+$ complex (Figure 3b) indicates that only signal R has been affected. This signal is again shifted upfield but by a much larger amount (0.42 ppm) than in other complexes. No signals from the A(2) and A(1′) protons of bound $NADP^+$ were detected; although these protons are known from saturation-transfer experiments to resonate in this spectral region (at 3.64 and 3.03 ppm), they give rise to very broad resonances at 25 °C (Hyde et al., 1980). There is a small signal at 3.18 ppm which has not been assigned.

The complex of the enzyme with the reduced coenzyme gives a spectrum (Figure 3c) in which the tryptophan signals have very similar shifts to those in the methotrexate·enzyme complex: the signals from R and S are again shifted upfield with S sharpening considerably. Additional signals arise from protons of bound NADPH at 3.59 [A(2)] and 2.99 ppm [A(1′), N(6)] and under signal P at 3.66 ppm [N(2)]. The assignments for these protons in the bound coenzyme have been determined previously by using transfer of saturation experiments (Hyde et al., 1980).

Enzyme·NADPH·Methotrexate Complex. In the spectrum of this complex (Figure 3d) only signal R has changed its position from that in the free enzyme. Although signal S is again sharp, its chemical shift is the same as that in the free enzyme, that is, downfield from its corresponding position in the binary complexes [0.10 ppm in the enzyme·methotrexate and 0.05 ppm in the enzyme·NADPH complexes (Table I)].

The three signals in the region 3.01–3.07 ppm correspond to signal R and the N(2) and A(1′) signals from bound NADPH. The A(2) proton of bound NADPH resonates as a sharp signal at 3.59 ppm [this signal has been detected previously in our studies on complexes with other selectively deuterated enzymes (Feeney et al., 1977)]. The doublet at

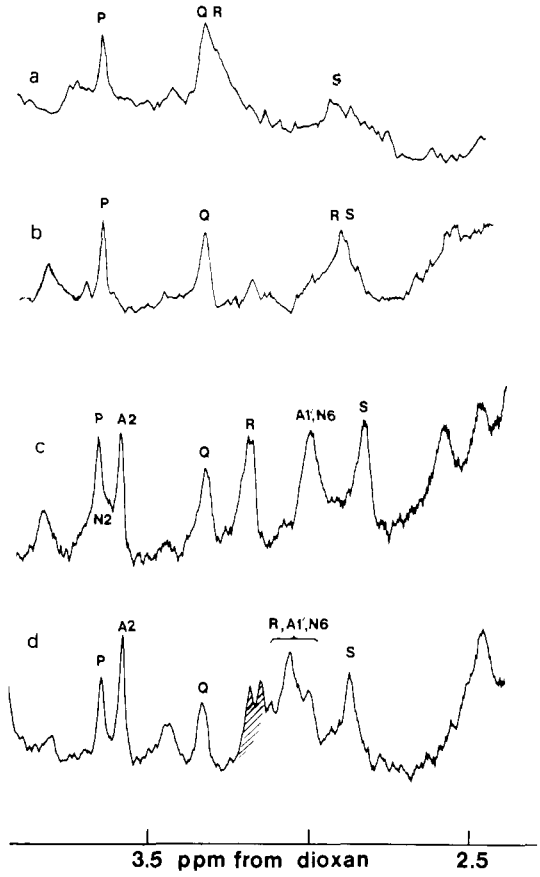
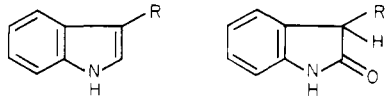


FIGURE 3: The aromatic region of the ^1H NMR spectrum at 270 MHz (25 °C) of selectively deuterated dihydrofolate reductase in various complexes. (a) Enzyme alone; (b) enzyme·$NADP^+$; (c) enzyme·NADPH; (d) enzyme·NADPH·methotrexate.

3.18 ppm arises from the ortho benzoyl protons of a small amount of free methotrexate present in this sample. It was not possible to identify with certainty any signals from the benzoyl protons of bound methotrexate although they could give rise to the signal at 3.45 ppm.

Effects of N-Bromosuccinimide Modification: Assignment of Trp-21. Liu & Dunlap (1974) have shown that modification of *L. casei* dihydrofolate reductase with *N*-bromosuccinimide (NBS) results in complete inactivation of the enzyme concomitant with oxidation of one tryptophan. Freisheim et al. (1977) have shown by sequence studies on the modified enzyme that the chemical modification occurs at Trp-21. Folate and its analogues bind to the modified enzyme almost as well as to the native enzyme, but the binding of NADPH is markedly weaker (Thomson et al., 1980).

Since the reaction of NBS with a tryptophan residue (I) removes the C(2) proton on formation of the oxindole (II),



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this provides a convenient method of assigning the C(2) proton of Trp-21 in the deuterated enzyme sample examined here.

In concentrated solutions (0.5 mM) the enzyme modified at a given NBS/enzyme ratio gives the same loss of activity and has the same number of modified tryptophans as that found at lower concentrations (0.01 mM) by Thomson et al. (1980). It is thus reasonable to assume that selective modification of Trp-21 is also occurring in the 0.5 mM enzyme solution. A concentrated solution (0.5 mM) of the enzyme modified with NBS to the extent of 80% inactivation was found to be unstable above 0 °C, and it was not possible to obtain its NMR spectrum. However, the modified enzyme is much more stable in the presence of methotrexate, and a ^1H NMR spectrum for the complex between the NBS-modified enzyme and methotrexate was obtained in which signal P had almost disappeared. This indicates that signal P arises from the principal tryptophan residue modified by NBS, identified as Trp-21 by Freisheim et al. (1977). Further evidence for this assignment is obtained by examining the product of NBS modification of the enzyme·methotrexate complex. This gives an improved spectrum (Figure 4b) which clearly shows that signal P has been greatly reduced in intensity and a small shift of signal Q has occurred. Some intensity remains at P because of the low level of NBS modification (80% inactivation). Liu & Dunlap (1974) have shown that the inactivation of the enzyme by NBS is unaffected by the presence of methotrexate. Our results, indicating that the same residue is modified in each case (signal P = Trp-21), are fully consistent with these findings.

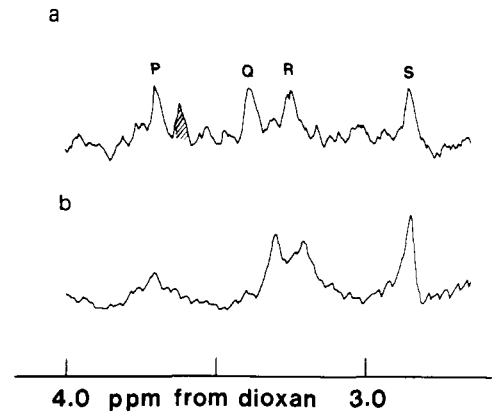

FIGURE 4: The aromatic region of the ^1H NMR spectrum at 270 MHz of selectively deuterated dihydrofolate reductase in various complexes. (a) Enzyme·methotrexate (25 °C); (b) enzyme·methotrexate after NBS modification of the complex (25 °C).

Discussion

Assignment of ^1H and ^{19}F NMR Signals from the Tryptophan Residues. In our previous studies of the 6-fluorotryptophan labeled enzyme (Kimber et al., 1977, 1978) three of the ^{19}F signals (labeled K, L, and M) were perturbed by ligand binding while another (N) was unaffected. Examination of the recently published crystal structure of the enzyme methotrexate·NADPH complex (Matthews et al., 1978) has provided tentative assignments of these signals (Matthews, 1979). Signals K and L have been assigned to Trp-5 and Trp-133; signal N has been assigned to Trp-158 and signal M to Trp-21. Signal M experiences a large downfield shift (2.7 ppm) in the presence of NADPH, which is known to bind with its reduced nicotinamide ring close to Trp-21 (Matthews et al., 1978).

Of the four ^1H resonances, one (signal P) has been unequivocally assigned to Trp-21 by the NBS-modification experiment. The remaining signals, Q, R, and S, can be tentatively correlated, on the basis of their behavior in the presence of ligands, with the ^{19}F signals K, L, and N. Thus signal Q, like signal N, is completely unaffected by ligand binding, while R and S, like K and L, are quite appreciably affected. It must be emphasized that such a correlation of shift behavior is a rather unsatisfactory method of assignment, particularly since the ^{19}F and ^1H NMR signals arise from nuclei at opposite ends

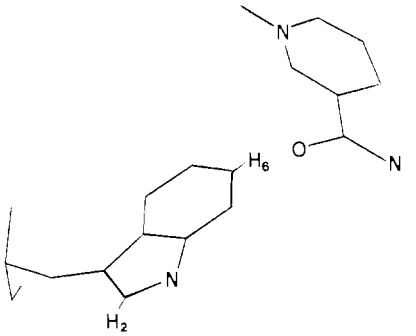

FIGURE 5: Diagram showing the orientation of Trp-21 and the reduced nicotinamide ring of NADPH as found in the crystal structure of the *L. casei* enzyme·methotrexate·NADPH complex (Matthews et al., 1978).

of the indole ring (see below). However, the assignment of Q to Trp-158 is consistent with all the data available at present.

Effects of Ligand Binding on Trp-21. In view of the marked effects of oxidation of Trp-21 on NADPH binding (Thomson et al., 1980), the absence of any perturbation of the shift of its C(2) proton resonance (signal P) on binding the coenzyme is, at first sight, somewhat surprising. Clearly this part of the tryptophan side chain cannot be close to the aromatic rings of NADPH. The observed shift behavior contrasts markedly with that of the ^{19}F resonance tentatively assigned to the corresponding 6-fluorotryptophan (signal M), which shows very large shift changes on coenzyme binding. The changes in chemical shift of protein resonances on ligand binding are made up of contributions from direct effects of the ligand and from changes in magnetic environment resulting from ligand-induced conformational changes. Since a fluorine at the 6 position of an indole ring is some 6 Å from the proton at the 2 position, it would be quite possible for coenzyme binding to have a direct effect on the fluorine chemical shift of 6-F-Trp-21 but not on the 2-proton chemical shift of the same residue. This would imply that the indole ring of Trp-21 is oriented so that the 6 position (but not the 2 position) is close to the coenzyme. In the crystal structure of the enzyme·methotrexate·NADPH complex (Matthews et al., 1978), the only part of the enzyme molecule close to Trp-21 is the nicotinamide ring, and this is indeed close to the 6 but not to the 2 position, as illustrated in Figure 5. The nicotinamide ring is more than 8 Å from the 2 proton and would give rise to a ring-current shift of this proton resonance of less than 0.05 ppm. A fluorine in the 6 position, by contrast, would experience not only a downfield ring-current shift but also a substantial downfield second-order electric field shift [van der Waals' shift; see Kimber et al. (1977)].

The absence of a change in shielding of the 2 proton of Trp-21 on coenzyme binding implies not only the absence of a direct effect but also the absence of any effect arising from a conformational change. This proton must therefore be in a region of the structure where there are no large gradients of magnetic anisotropy and must not change its position appreciably with respect to neighboring atoms when NADPH binds. These conclusions are strongly supported by consideration of the crystal structures of the enzyme complexes (Matthews et al., 1977, 1978). In the *L. casei* enzyme·methotrexate·NADPH complex (Matthews et al., 1978) there are no aromatic rings within 6 Å of the C(2) carbon of Trp-21, and the only source of magnetic anisotropy which could contribute significantly to the C(2) proton shielding is the carbonyl group of the Trp-21–His-22 amide bond. There is also good evidence that NADPH binding does not appreciably change the relative positions of the C(2) proton and the amide bond of Trp-21; Matthews et al. (1978) have noted that the side-chain conformation for the Trp-21 residue in the *L. casei* enzyme·methotrexate·NADPH complex shows no discernible difference from that of the corresponding tryptophan residue (Trp-22) in the *Escherichia coli* enzyme·methotrexate complex (Matthews et al., 1977). Since the distance from the carbonyl group to the 2 proton is determined by the dihedral angles about the α–β and β–γ bonds, this local distance must remain constant on NADPH binding although there is movement of the main-chain "loop" which includes Trp-21 (Matthews, 1979). Residue 21 is clearly in direct contact with the coenzyme, and this is reflected in the chemical shift of nuclei in the "contact" area. However, there is no change in the *local* environment of the 2 proton and hence no change in chemical shift.

Effects of Ligand Binding on Trp-5 and -133. The ^1H NMR signals R and S (tentatively assigned to Trp-5 and -133) are perturbed by the binding of substrate analogues and coenzyme. In the spectrum of the free enzyme, signal S is a very broad signal displaced 0.7 ppm to high field of the position of a tryptophan C(2) proton from a solvent-accessible tryptophan. This large upfield shift may result from a ring-current effect from a neighboring aromatic residue. In the crystal structure of the enzyme complex with methotrexate and NADPH (Matthews et al., 1978), Trp-5 is the only tryptophan residue which is sufficiently close to an aromatic ring (Phe-103) to give the observed upfield shift of its C(2) proton resonance. Although signal S is broad in the spectrum of the free enzyme, it sharpens in all the complexes with inhibitors and coenzyme. One possible explanation is that in the free enzyme, the tryptophan is exchanging relatively slowly between two or more conformational states, whereas in the complexes, the tryptophan is in a single preferred conformation.

The chemical-shift perturbations on signal S (Trp-5) caused by inhibitor and coenzyme binding are not additive. Upfield shifts are observed in both the enzyme·methotrexate (0.12 ppm) and enzyme·NADPH (0.07) complexes, but in the ternary complex this signal is in the same position as in the free enzyme. This behavior is perhaps most easily explained by a ligand-induced conformational change rather than a direct interaction. In this case, where the proton is in a position to experience large anisotropic shielding effects, relatively minor changes in the relative orientation of the Phe-103 aromatic ring and the Trp-5 C(2) proton could cause the observed shifts. The indole side chain of Trp-133 is well removed from the active site of the enzyme (Matthews et al., 1978; Matthews, 1979), and it seems unlikely that the shift perturbations of signal S arise from direct interactions. The observed upfield shifts for signal R are very similar for the binary complexes with folate (0.09 ppm), methotrexate (0.14 ppm), and trimethoprim (0.11 ppm), suggesting that in each case a similar conformational change is influencing this tryptophan residue.

Conclusions

The C(2) proton signal from Trp-21 has been assigned in the spectrum of a selectively deuterated NBS-modified dihydrofolate reductase. The absence of any shift perturbation on this signal (signal P at 3.66 ppm) on addition of NADPH suggests that the coenzyme is not very close to the 2 position of Trp-21. In the NADPH complex with the 6-fluorotryptophan labeled enzyme the ^{19}F signal from Trp-21 is markedly affected by the coenzyme binding (2.7-ppm shift), indicating that the 6 position is oriented toward the coenzyme. These results agree with the crystal structure of the enzyme·methotrexate·NADPH complex where the carboxamide group of the reduced nicotinamide ring of NADPH is near

to the 6 position of Trp-21 but remote from its 2 position.

The nonadditivity of the ^1H and ^{19}F NMR chemical shift contributions in binary and ternary complexes for signals tentatively assigned to Trp-5 and -133 indicates that these residues are influenced by ligand-induced conformational changes. One of these residues experiences similar conformational changes in the presence of either substrates or inhibitors.

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Kinetic Mechanism of Glutamate Dehydrogenase†

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ABSTRACT: Initial velocity patterns and dead-end inhibition studies with oxalylglycine suggest that the addition of NADPH, keto acid, and ammonia occurs with obligatory order. For monocarboxylic keto acids, the keto acid–ammonia initial velocity pattern is equilibrium ordered because k_{off} is much greater than V_{max}. α-Ketoglutarate gives substrate inhibition that is uncompetitive vs. either NADPH or ammonia in the absence of $NADP^+$, but with high $NADP^+$ is noncompetitive vs. NADPH and uncompetitive vs. ammonia. The inhibition is partial on both slopes and intercepts, showing that ketoglutarate slows down but does not prevent $NADP^+$ release, and that it forms a weak but kinetically competent E–ketoglutarate complex. The apparent ordered combination of NADPH and keto acid thus results from strong synergism in binding. Deuterium isotope effects, together with the substrate inhibition patterns for ketoglutarate, show that addition of amino acid and $NADP^+$ is random, with $NADP^+$ being released from the ternary complex more rapidly than either glutamate or norvaline. With norvaline, hydride transfer is a major rate-limiting step, while with glutamate a step preceding hydride transfer is slower than hydride transfer. The equilibrium ^{18}O isotope effect is 1.031 ± 0.006 (^{18}O enriching in ketoglutarate relative to water), but no kinetic ^{18}O isotope effect was seen.

The kinetic mechanism of glutamate dehydrogenase (EC 1.4.1.3) has been examined by steady-state kinetics (Engel & Dalziel, 1969, 1970; Engel & Chen, 1975), transient kinetics (Colen et al., 1972, 1977; Jallon et al., 1975) and equilibrium isotope exchange studies (Silverstein & Sulebele, 1973). It has been proposed that both the forward reaction (glutamate oxidation) and the reverse reaction (α-ketoglutarate reduction) involve random binding of ligands. For the forward reaction it is thought that the rate-limiting step is release of NADPH from an abortive E–NADPH–glutamate complex when the glutamate concentration is high or release of α-ketoglutarate from E–NADPH–ketoglutarate under other conditions (di Franco, 1974; Silverstein & Sulebele, 1973; Colen et al., 1975). The rate-limiting steps of the reverse reaction have not been well characterized. In this report, various kinetic techniques and the alternate substrates norvaline and α-ketovalerate were employed to further investigate the kinetic mechanism of glutamate dehydrogenase. In the following paper (Rife &

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