Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3849–3853, October 1974

Conformational Changes Induced in Dihydrofolate Reductase by Folates, Pyridine Nucleotide Coenzymes, and Methotrexate*

(220 MHz proton magnetic resonance/antifolates/ligand binding)

EDWARD J. PASTORE, ROY L. KISLIUK†, LAURENCE T. PLANTE, JOHN M. WRIGHT, AND NATHAN O. KAPLAN

Departments of Chemistry and Biology, The University of California, San Diego, La Jolla, Calif. 92037; and † Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111

Contributed by Nathan O. Kaplan, June 24, 1974

ABSTRACT **Dihydrofolate reductase (EC 1.5.1.3; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase) from antifolate-resistant *Lactobacillus casei* has been isolated in pure form and examined in solution by high resolution proton magnetic resonance spectroscopy. The 220 MHz proton magnetic resonance spectrum of this small enzyme (about 15,000 daltons) consists of several distinct resonance peaks that provide a sensitive nonperturbing probe of its conformational state. Comparison of catalytically active enzyme with preparations denatured in 6 M urea demonstrates dramatically the overall contribution of secondary and tertiary structure to its proton magnetic resonance spectra. More subtle differences existing among several catalytically active enzyme forms may also be readily differentiated by proton magnetic resonance spectroscopy, e.g., the spectra of the ligand-free enzyme and forms containing stoichiometric amounts of tightly bound folate and dihydrofolate, each obtained separately by affinity chromatography, are easily identified. Addition of ligands to these spectroscopically distinct forms may induce changes in their proton magnetic resonance spectra. For example, addition of equimolar dihydrofolate to the apoenzyme converts its relatively featureless aromatic proton magnetic resonance spectrum to one indistinguishable from that of the original enzyme–dihydrofolate binary complex obtained chromatographically. Interaction of the pyridine nucleotide coenzymes NADP$^+$ or NADPH or of the antifolate Methotrexate with apoenzyme induces additional distinct spectral changes. Enzyme–NADPH and enzyme–Methotrexate binary complexes, which have different aromatic region proton magnetic resonance spectra, are converted to ternary complexes having quite similar spectra by addition of Methotrexate and NADPH, respectively, thus suggesting that an ordered addition of ligands is not required.**

The principles of nuclear magnetic resonance (NMR) spectroscopy applied to enzymes evolved through investigation of readily available small hydrolytic enzymes of known structure, particularly in the laboratories of Jardetzky (2) and of Phillips (3). These principles are now being applied profitably to studies on a wide range of less well-characterized biologically important proteins (4). As an extension of our earlier proton magnetic resonance (PMR) studies of folate coenzymes (5, 6), as well as of pyridine nucleotides, this communication consists of an examination of dihydrofolate reductase (EC 1.5.1.3;5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase) and its interaction with stoichiometric amounts of pertinent ligands. Dihydrofolate reductase is well suited for NMR investigation because its low molecular weight, about 15,000 (7), permits direct examination of the protein itself during binding interactions. This enzyme is of special interest because it catalyzes a reaction between a reduced pyridine nucleotide coenzyme and a reduced folate (Formula 1) and because it is strongly inhibited by Methotrexate, a clinically important, antifolate, cancer chemotherapeutic drug (8).

$$\text{7,8-dihydrofolate} + \text{NADPH} \rightleftharpoons \text{NADP}^+ + \text{tetrahydrofolate} \quad [1]$$

These studies have been greatly facilitated by development of a rich enzyme source (9, 10) and an effective affinity column purification procedure (11). While low molecular weight enzymes have been examined successfully by NMR spectroscopy during the past several years (2, 3), the present report is the first detailed study of a small enzyme catalyzing a reaction involving the participation of ligands such as folate and pyridine nucleotide coenzymes; the ensuing results should, therefore, be of general interest to mechanistic studies.

MATERIALS AND METHODS

Chemicals were obtained as follows: NADP$^+$ and NADPH, Calbiochem, La Jolla, Calif.; folate, Sigma Chemical Co., St. Louis, Mo.; Methotrexate was a gift from Lederle, Pearl River, N.Y.; deuterium oxide (100%), Diaprep, Inc., Atlanta, Ga. Dihydrofolate was prepared by the procedure developed by Blakley (12). Dihydrofolate reductase was assayed spectrophotometrically (13), and protein was determined by the microbiuret procedure (14). Large-scale bacterial fermentations were carried out at the New England Enzyme Center, Boston, Mass.

Enzyme Purification. Dihydrofolate reductase was obtained from Methotrexate- and dichloromethotrexate-resistant strains of *Lactobacillus casei* developed by Crusberg, Leary, and Kisliuk (9). Their purification procedure (10) was followed through the hydroxylapatite chromatography step. Fractions containing dihydrofolate reductase activity were pooled and purified to homogeneity by affinity chromatography on pteroyllysine–Sepharose (11). By this procedure either apoenzyme or forms containing stoichiometric amounts of tightly bound folate (E–folate) or dihydrofolate (E–di-

* A preliminary report of these results has been presented (ref 1).

Abbreviations used: PMR, proton magnetic resonance; TSP, sodium trimethylsilyl [2,2,3,3-D$_4$]propionate; ppm, parts per million of the magnetic field; δ, chemical shift measured in ppm downfield relative to TSP; E, enzyme.

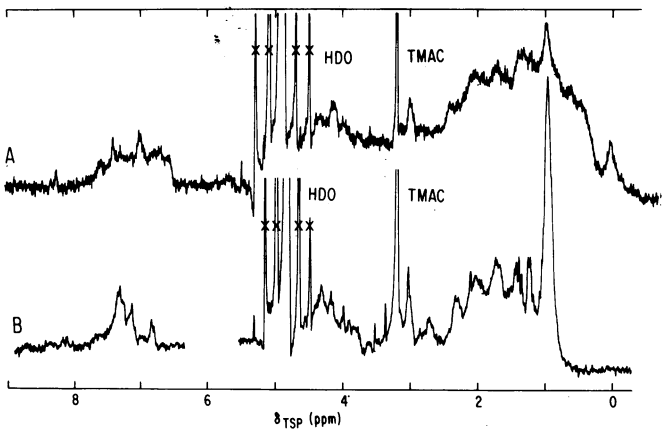

FIG. 1. Single-scan 220 MHz PMR spectra of 2 mM dihydrofolate reductase at 20° in D_2O containing 50 mM potassium phosphate (pH 6.8). (A) Enzyme obtained by elution with dihydrofolate; (B) the same enzyme form denatured in 6 M urea. TMAC, tetramethyl ammonium chloride.

hydrofolate) may be obtained by use of different elution buffers.

Proton Magnetic Resonance (PMR) Spectroscopy. PMR spectra were obtained with a Varian HR-220 NMR spectrometer operating in the field sweep mode. Signal-to-noise enhancement was achieved by time averaging with a Fabritek 1074 Time Averaging Computer. Enzyme (2 mM) in 50 mM potassium phosphate buffer (pH 6.8) was lyophilized, dissolved, and adjusted to its original volume with deuterium oxide and examined in 5-mm probe tubes at about 20°. Chemical shifts were calibrated with internal tetramethyl ammonium chloride and related to sodium trimethylsilyl [2,2,3,3,-D_4]propionate (TSP) by adding 700 Hz.

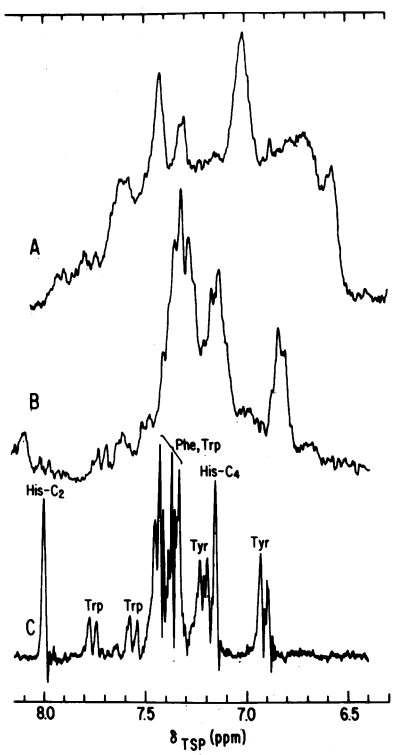

FIG. 2. Aromatic region 220 MHz PMR spectra of: (A) dihydrofolate reductase obtained by elution with dihydrofolate and (B) the enzyme denatured in 6 M urea, each time averaged over 16 scans; (C) mixture of free aromatic amino acids equivalent to 1 mM dihydrofolate reductase and in the same molar ratio as they occur in the enzyme [His 6, Phe 7, Tyr 4, and Trp 3 (15)], 9 scans. Conditions as in legend of Fig. 1.

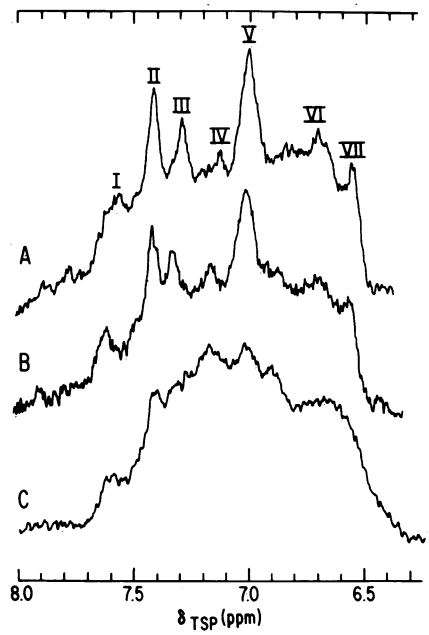

FIG. 3. Aromatic region 220 MHz PMR spectra of dihydrofolate reductase isolated by elution with (A) dihydrofolate, (B) folate, and (C) 0.5 M KCl (pH 8.0). Conditions as in legend of Fig. 1.

RESULTS AND DISCUSSION

The 220 MHz PMR spectrum of native dihydrofolate reductase in neutral aqueous solution shown in Fig. 1*A* reveals considerable fine structure typical of low molecular weight proteins and provides a powerful nonperturbing probe of protein structure (2). The contribution of secondary and tertiary structure to the PMR spectrum is revealed by comparing catalytically active enzyme with enzyme denatured in 6 M urea, i.e., the random coil configuration (Fig. 1*B*). The spectrum of the random coil protein is simplified by changes in resonance line shape and line position. Lines become narrower because of the presumed increase in local motional freedom of amino acid side-chain residues in the unfolded state, while line positions change because of alterations in local magnetic environments. The latter effects are ascribed to local fields set up by ring currents of aromatic amino acids. Amino acid side-chain residues held in close proximity to these rings may experience either increased or decreased shielding, depending on their particular geometrical orientation in these perturbed local magnetic fields (3). These anisotropic effects provide a very sensitive indicator of enzyme conformation. Note, for example, how the broad overlapping resonance bands between 0 and 2 ppm in the native enzyme (Fig. 1*A*) converge into one large narrow band centered at 0.9 ppm in the denatured form. This band, assigned to the methyl group resonances of leucine, isoleucine, and valine residues (3), provides a striking example of differential shielding attributable to peptide chain folding. Although these conformational changes are observable in both the aliphatic (δ = 0–4) and aromatic (δ = 6.5–8) regions, the

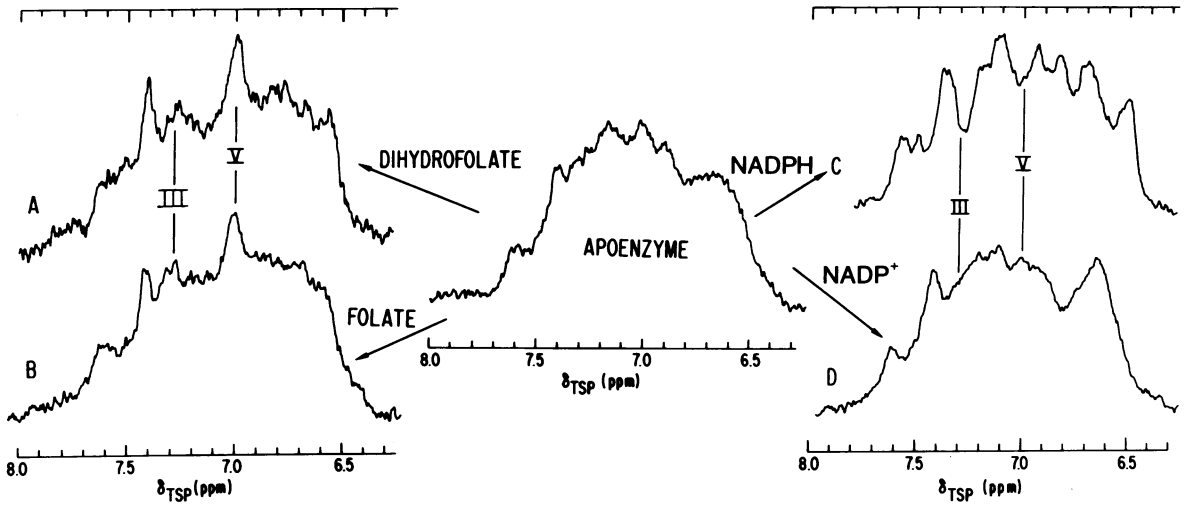

FIG. 4. Aromatic region 220 MHz PMR spectra (16 scans) of 2 mM dihydrofolate reductase apoenzyme in absence (center) and presence of stoichiometric amounts of (A) dihydrofolate, (B) folate, (C) NADPH, and (D) NADP+. Conditions as in legend of Fig. 1.

present discussion will be confined to the simpler aromatic region consisting of resonance lines from only the four aromatic amino acids.

Comparison of the expanded aromatic regions of native and denatured dihydrofolate reductases shown in Fig. 2*A* and *B* illustrates the degree to which peptide chain folding has affected the resonance lines of individual aromatic amino acid side-chain residues. The latter spectrum is similar to that of a solution of free aromatic amino acids present in the same molar ratio as they occur in the enzyme (Fig. 2*C*) (15). These results, which show that the characteristic dihydrofolate reductase spectrum is due in large measure to specific folding of its polypeptide chain, are thus consistent with earlier work on other low molecular weight enzymes (2, 3).

It is of greater interest to be able to distinguish different catalytically active protein conformations. In Fig. 3 are shown the aromatic regions of three different enzyme forms of dihydrofolate reductase of comparable specific activity isolated by affinity chromatography by different elution procedures (11). These three enzyme forms, which are readily differentiated by their ultraviolet absorbance spectra, are characterized by the presence or absence of stoichiometric amounts of nondialyzable folate or dihydrofolate†. The PMR spectrum of the enzyme–dihydrofolate binary complex (E–dihydrofolate) aromatic region is shown in Fig. 3*A*. The resonance envelope has been arbitrarily divided into seven regions labeled with Roman numerals in order to facilitate comparison with spectra of other enzyme forms. The corresponding chemical shifts (δ) are listed in Table 1. The aromatic regions of the enzyme–folate binary complex (E–folate) and apoenzyme are shown in Fig. 3*B* and *C*, respectively. The E–folate aromatic resonance is similar to that of the E–dihydrofolate complex, but can be distinguished from the latter by small δ changes to lower field (to the left) of peaks I, III, and V. The apoenzyme has no large peaks at II, III, and V, while region VII is featureless in contrast to the peak and sharp drop characteristic of the E–folate and E–dihydrofolate forms. Thus, different catalytically active enzyme forms obtained by affinity chromatography can be readily distinguished by the characteristic PMR profiles of their aromatic regions.

We also examined binary complexes of E–folate and E–dihydrofolate obtained by adding the ligands to solutions of the apoenzyme. As shown in Fig. 4*A*, addition of an equimolar amount of dihydrofolate to apoenzyme restores the aromatic PMR spectrum at all seven regions to that of E–dihydrofolate obtained chromatographically (Fig. 3*A*). The large peaks at points II, III, and V reappear with the same chemical shifts and relative amplitudes, and the characteristic shape of the trailing edge, area VII, is also restored. Addition of an equimolar amount of folate to apoenzyme, however, does not restore the characteristic aromatic PMR spectrum of the E–folate complex obtained chromatographically. As shown in Fig. 4*B*, the ligand does alter the apoenzyme aromatic PMR spectrum, but peaks II, III, and V and the region labeled VII are not completely restored even when the ligand:enzyme ratio is increased 4-fold. Thus, despite their similar affinities for dihydrofolate reductase determined kinetically (8), folate and dihydrofolate differ in their ability to induce conformational changes in the apoenzyme.

Pyridine nucleotide coenzymes also bind and induce distinct changes in the apoenzyme PMR spectrum. As shown in Fig. 4*C*, addition of equimolar NADPH to apoenzyme generates a number of peaks with chemical shifts and relative amplitudes different from those induced by folates described above. Note particularly that the peaks seen in the E–dihydrofolate spectrum (Fig. 4*A*) at δ = 7.01 (labeled V) and at δ = 7.29 (labeled III) are absent in the E–NADPH spectrum (Fig. 4*C*), while several new peaks have appeared at δ = 7.38, 7.10, 6.94, 6.82, and 6.50. NADP+ also induces changes in the apoenzyme aromatic PMR spectrum, but these changes are less extensive than with NADPH. The most

TABLE 1. *Aromatic resonances of dihydrofolate reductase*[a]

δ^*_{TSP} (ppm)						
I	II	III	IV	V	VI	VII
7.57	7.42	7.29	7.13	7.01	6.70	6.57

[a] See Fig. 3.

† Microbiological assays for folate and dihydrofolate were performed by Wilhelmina Korevaar.

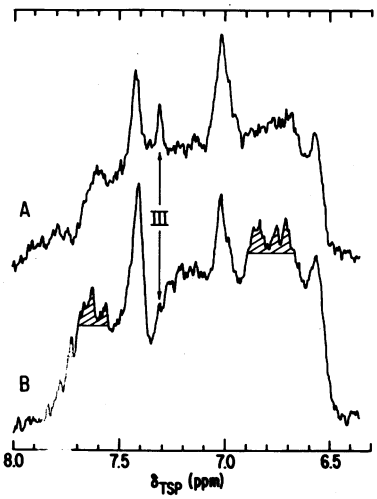

FIG. 5. Aromatic region 220 MHz PMR spectra of 2 mM dihydrofolate reductase: (A) enzyme–dihydrofolate (8 scans) and (B) the same enzyme in the presence of 4 mM Methotrexate (11 scans). Hatched areas are attributable to free dihydrofolate and Methotrexate.

noticeable effects (Fig. 4*D*) are (*a*) the appearance of a broad depression centered at δ = 6.8 ppm in place of peak VI (δ = 6.7) seen in the E–dihydrofolate binary complex and (*b*) a broad peak (δ = 6.65) near region VII (δ = 6.57). As was observed with the E–folate complex this E-$NADP^+$ spectrum is also stable and is not further changed by increasing the $NADP^+$:enzyme ratio to four. Thus, each ligand interacts differently with the apoenzyme, forming a binary complex that has a distinct conformation easily recognized by its characteristic aromatic PMR envelope.

The ability to distinguish a number of active enzyme forms provides a powerful basis for directly examining the nature of enzyme–ligand interaction in unique detail. One may examine binary complexes formed between apoenzyme and analogs of substrate and cofactors. One may also examine the interaction of various binary complexes with other small molecules in displacement reactions or in the formation of ternary complexes. Such data could provide evidence of multiple binding sites and might also distinguish between random and ordered ligand addition sequences. We report below the results of several such experiments.

Methotrexate, which binds to dihydrofolate reductase a 1000-fold more tightly than either folate or dihydrofolate, was added to the E-dihydrofolate binary complex in order to observe its effects on the enzyme PMR spectrum. The results show (Fig. 5) that this inhibitor does alter the aromatic PMR envelope. The benzene ring protons of dihydrofolate and Methotrexate add a small amount of resonance at I (δ = 7.57) and VI (δ = 6.70), indicated by cross-hatching in Fig. 5*B*, but the main resonance change is that of the enzyme itself. The relative amplitudes of the two major peaks, II (δ = 7.42) and V (δ = 7.01), have been reversed while peak III (δ = 7.29) has been completely displaced, leaving a deep notch in this region (δ = 7.35). Alterations of this magnitude in the enzyme PMR spectrum show that Methotrexate binding causes changes in protein conformation not seen with dihydrofolate despite the structural similarity of these two ligands. This direct physical evidence provides a basis for eventually explaining such interactions in molecular terms, since it should be possible to assign the resonance bands in this region to specific amino acid residues. Such assignments can be made by incorporating specifically labeled amino acids into the enzyme.

Knowledge of the ternary complex E–NADPH–Methotrexate is of particular importance in understanding the reaction and inhibition mechanisms of dihydrofolate reductase. Results of several preliminary experiments described below indicate that magnetic resonance may provide new information about the nature of this complex. One preparation of the ternary complex was formed from the E–folate binary complex by adding NADPH followed by Methotrexate. A second preparation was obtained by reversing the addition of NADPH and Methotrexate to the E–folate binary complex. Fig. 6*A* shows the results of addition of 2-fold excess of NADPH to the E-folate binary complex. (Note that NADPH has only one proton resonance in this region, while $NADP^+$ has none.) The major changes are a large loss of resonance at δ = 7.1, equalization of amplitude of the two major peaks, II and V, and a general narrowing of all peaks. This spectrum does not resemble the binary product formed from apoenzyme and

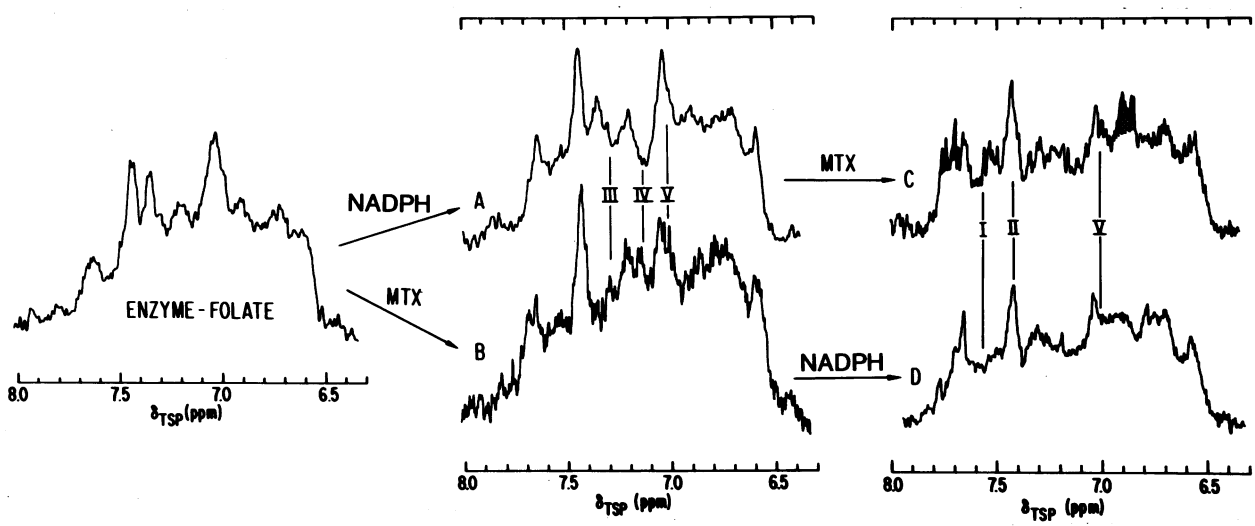

FIG. 6. Aromatic region 220 MHz PMR spectra of 2 mM dihydrofolate reductase. Enzyme–folate binary complex (left side), and the same enzyme in the presence of a stoichiometric amount of either (A) NADPH or (B) Methotrexate (Mtx) followed by the addition of (C) Methotrexate and (D) NADPH to (A) and (B), respectively.

NADPH (Fig. 4*C*). Addition of an equivalent amount of Methotrexate to the E-folate binary complex is shown in Fig. 6*B*. Methotrexate induces a large change, resulting in a spectrum corresponding closely to the E–Methotrexate complex formed from E–dihydrofolate (Fig. 5*B*). The major changes include reversal of the amplitude of II and V and a loss of resonance signal in region III.

Methotrexate and NADPH were added to the samples of Fig. 6*A* and *B*, respectively, and spectra of the resulting ternary complexes are shown in Fig. 6*C* and *D*. In Fig. 6*C* the hatched-out areas are the resonance contributions of the benzene rings of the excess Methotrexate used in this experiment. It can be seen that the spectra in each case, though arising from complexes having distinctly different spectra (Fig. 6*A* and *B*), are quite similar, e.g., in each the major peak is II (δ = 7.42) and each has quite sharp peaks at δ = 7.65 and δ = 7.13; the region between II and V is essentially flat and area VII is unchanged. Because of the known tight binding of NADPH and Methotrexate and the quite different conformations they induce (shown above), it is somewhat surprising to find that these ternary complexes formed by different paths have aromatic region PMR profiles that are similar. Although these data are preliminary, the convergence of the two spectra shown in Fig. 6*C* and *D* suggest that for the *L. casei* dihydrofolate reductase, the order of ligand addition is random.

E.J.P. gratefully acknowledges the assistance and encouragement of Prof. Morris Friedkin throughout this work. We are indebted to Lisa Nelson and Yvette Gaumont for excellent technical assistance. This work was supported in part by personal funds of E.J.P. and by grants from the National Science Foundation (NSF GB 41533), the National Institutes of Health (CA 10914, CA 11449, CA 11683-05, and RR-00757), and from the American Cancer Society (BC-60-P).

1. Pastore, E. J., Kisliuk, R. L., Plante, L. T., Kaplan, N. O. & Friedkin, M. (1973) *Fed. Proc.* **322**, 119.
2. Roberts, G. C. K. & Jardetzky, O. (1970) *Advan. Protein Chem.* **24**, 447–545.
3. McDonald, C. C. & Phillips, W. D. (1970) "Fine structure of proteins and nucleic acids," in *Biological Macromolecules*, eds. Fasman, G. D. & Timasheff, S. N. (Dekker, New York), Vol. 4, pp. 1–48.
4. Lasker, S. E. & Milvy, P. eds. (1973) *Ann. N.Y. Acad. Sci.* **222**, 1–1124.
5. Pastore, E. J., Friedkin, M. & Jardetzky, O. (1963) *J. Amer. Chem. Soc.* 3058–3059.
6. Pastore, E. J. (1971) *Ann. N.Y. Acad. Sci.* **186**, 43–54.
7. Gundersen, L. E., Dunlap, R. B., Harding, N. G. L., Freisheim, J. H., Otting, F. & Huennekens, F. M. (1972) *Biochemistry* **11**, 1018–1023.
8. Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines* (American Elsevier, New York).
9. Crusberg, T. C., Leary, R. P. & Kisliuk, R. L. (1970) *J. Biol. Chem.* **245**, 5292–5296.
10. Leary, R. P. & Kisliuk, R. L. (1971) *Prep. Biochem.* **1**, 47–54.
11. Pastore, E. J., Plante, L. T. & Kisliuk, R. L. (1974) in *Methods in Enzymology* (Academic Press, New York), in press.
12. Blakley, R. L., (1960) *Nature* **188**, 231–232.
13. Pastore, E. J. & Friedkin, M. (1961) *J. Biol. Chem.* **237**, 3802–3810.
14. Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222.
15. Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971) *Ann. N.Y. Acad. Sci.* **186**, 153–165.