Nuclear Magnetic Resonance Studies on Bacterial Dihydrofolate Reductase Containing [*methyl*-^{13}C]Methionine†

Raymond L. Blakley,* Lennie Cocco, Robert E. London, Thomas E. Walker, and N. A. Matwiyoff

ABSTRACT: [*methyl*-^{13}C]Methionine has been incorporated with high efficiency by *Streptococcus faecium* var. *Durans* strain A into dihydrofolate reductase isoenzyme 2. In the ^{13}C NMR spectrum of the purified enzyme the resonances corresponding to the seven methionine residues are partially resolved into three composite peaks. Denaturation with urea collapses these into a single peak centered at 15.32 ppm, whereas the resonance of free methionine is at 15.04 ppm. Spectra of the free enzyme, its complex with methotrexate, and its complex with methotrexate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) have been simulated, permitting more accurate estimates of line widths and nuclear Overhauser enhancement (NOE) values. These, together with the T_1 values, cannot be explained solely by the effects of macromolecular tumbling and very rapid rotation of the methionine methyl group about its axis. A model assuming, in addition, the occurrence of free rotation about the methionine CH_2–S bond is also unsatisfactory, and it is concluded that internal rotation about the CH_2–S bond is highly restricted so that the methyl group oscillates through a relatively narrow angular range. Complex formation with NADPH produced rather small changes in the spectrum of the native enzyme, probably due to conformational transitions in the enzyme. However, $NADP^+$ produced several changes, including movement of one resonance downfield by at least 1.7 ppm. The latter is probably due to a combination of the effects of the nicotinamide ring current and of charge transfer between the nicotinamide ring and the methionine sulfur. This marked downfield shift is not seen in ternary complexes, presumably because the pteridine ring displaces the methionine residue from the proximity of the nicotinamide ring. Binding of methotrexate or other inhibitors produces upfield movement of several methionine resonances with consequent improved resolution of peaks in the binary and ternary complexes. The binding behavior of the carboxymethylated enzyme, the ^{13}C NMR of the carboxymethylated enzyme and its complexes, and the results of x-ray diffraction with *Escherichia coli* reductase together suggest that Met-28 and -50 correspond to two of the resonances shifted upfield by methotrexate binding. Since binding of folate causes fewer and smaller upfield shifts than inhibitors, substrate binding differs significantly from inhibitor binding. Dihydrofolate and 5-formyltetrahydrofolate cause still smaller changes in the spectrum presumably because of smaller pteridine ring currents.

The structure of dihydrofolate reductase is currently under intensive study in a number of laboratories. Interest in this enzyme derives in part from its clinical relevance. The reductase is the target of methotrexate, a drug used in the treatment of various types of cancer, and bacterial reductase is strongly and specifically inhibited by trimethoprim which is now used extensively in combination with sulfa drugs for treating a variety of infectious diseases.

Although studies of the amino acid sequence, the effects of chemical modification, and x-ray diffraction of the crystalline protein have made important contributions to understanding the structure of the reductase, NMR studies are able to make a unique contribution. The method does not perturb the structure of the enzyme and is potentially capable of giving information about the internal motions of amino acid residues and about the interactions of residues at the active site with a great variety of substrates and inhibitors in binary or ternary complexes under many conditions of pH, ionic strength, temperature, etc. It may also provide insights into the conformational changes induced in the protein by ligand binding.

Of the various approaches involving NMR to the study of enzyme structure, the incorporation into the protein of a single amino acid enriched with ^{13}C in a specific position has many advantages. It does not perturb the protein structure as in the case of studies on proteins modified by ^{13}C-labeled reagents. The protein ^{13}C NMR spectrum is comprised of only as many resonances as there are residues of the labeled amino acid in the molecule, and (with proton decoupling) there are no multiplets. If the number of residues is not too large, it is conceivable that the resonances may all be resolved from each other under favorable conditions. This simplicity of the spectra, together with the greater chemical-shift range, greatly facilitates the interpretation of spectral data.

Despite these advantages, relatively few reports have appeared of ^{13}C NMR studies on enzymes labeled by incorporation of a specifically labeled amino acid. [2-^{13}C]Histidine has been incorporated biosynthetically into tryptophan synthetase (Browne et al., 1973a,b), α-lytic protease (Hunkapiller et al., 1973a,b, 1975), and hemoglobin (London et al., 1975) and by synthesis of labeled S peptide into ribonuclease S′ (Chaiken et al., 1974). In most of these cases rather broad resonances were obtained, and in the only case where more than one labeled residue was present (tryptophan synthetase) a broad envelope was observed rather than discrete resonances. Labeling of alkaline phosphatase with 90% enriched [4-^{13}C]histidine (Browne et al., 1976) gave a much better spectrum with resolution of eight His resonances, but no ligand

† From the Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52242, and the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545. *Received December 15, 1977.* This research was performed under the auspices of the United States Department of Energy and was supported in part by the United States Public Health Service Research Grant CA 13840 (R.L.B.) from the National Cancer Institute of the National Institutes of Health, and the National Institutes of Health Research Grant 1P07 RR-00962-01 (NAM) from the Division of Research Resources, Department of Health, Education, and Welfare. R.L.B. gratefully acknowledges support by a John Simon Guggenheim Memorial Foundation Fellowship during the period in which this work was carried out. T.E.W. gratefully acknowledges a postdoctoral Fellowship (5 F22 CA 00971-02) from the National Cancer Institute.

0006-2960/78/0417-2284$01.00/0

effects were reported. We have previously reported the observation of a partially resolved spectrum for dihydrofolate reductase containing 90% enriched [*guanidino*-^{13}C]arginine, together with effects on the spectrum of ligand binding (Cocco et al., 1977).

In the present paper we report ^{13}C NMR data for dihydrofolate reductase of *Streptococcus faecium* labeled by in vivo incorporation of [*methyl*-^{13}C]methionine. The data permit some conclusions about the internal motions of the methionine residues in the protein and about the interaction of methionine residues with bound ligands.

Experimental Procedure

Materials. [*methyl*-^{13}C]Methionine was synthesized as described by Melville et al. (1947) from L-*S*-benzylhomocysteine (Sigma) and [^{13}C]methyl iodide (90% enriched, Kor Isotopes). Yields of 90–93% based on methyl iodide were obtained and the product was >99% pure as judged by amino acid analysis. L amino acids for bacterial growth medium, adenosine 2′,5′-diphosphate, nicotinamide mononucleotide, $NADP^+$ and NADPH were obtained from Sigma. 2′-Phosphoadenosine 5′-diphosphoribose (PADPR[1]) was prepared from $NADP^+$ by the method of Ben-Hayyim et al. (1967). Fractions from the Dowex-1 column were pooled, the pH was adjusted to 7.0 with 1 N LiOH, and the solution was concentrated to an oil under reduced pressure. The oil was taken up in methanol and the solution evaporated to dryness under reduced pressure. This process was repeated three times. The residue was taken up in a minimum volume of methanol, 5 volumes of acetone was added, and the mixture was kept at 5 °C for several hours. The precipitate was recovered by centrifugation and suspended in about 40 mL of acetone–methanol (5:1), and the suspension was centrifuged. This procedure was repeated until the supernatant was free of chloride and the precipitate finally dried in vacuo. About 9 mg was obtained from 28 mg of $NADP^+$. Methotrexate was purchased from Nutritional Biochemicals, and aminopterin from the International Chemical and Nuclear Co. was purified by the method of Loo (1965). 3′,5′-Dichloromethotrexate was a generous gift from Dr. E. C. DeRenzo of Lederle Laboratories. [^{12}C]Urea depleted of carbon 13 was a generous gift from Dr. T. W. Whaley of the Los Alamos Scientific Laboratory.

Growth of Bacteria. Streptococcus faecium var. *Durans* strain A was grown on a chemically defined medium of the following composition (concentrations in g/100 L): Gly, 8.2; Ala, 18.4; Ser, 26.3; Thr, 25.0; Pro, 22.0; Val, 28.5; Ile, 31.7; Leu, 34.9; Phe, 20.8; Tyr, 33.0, Cys, 3.0; Asp, 40.7; Glu, 46.4; Lys, 33.4; Asn, 37.5; His, 11.5; Trp, 30.0; Arg, 16.9; [*methyl*-^{13}C]Met, 1.7; sodium citrate, 3500; Tween 80, 6.3; glutathione, 0.3; adenine, 0.6; guanine, 0.6; uracil, 0.6; xanthine, 1.2; K_2HPO_4, 400; $MgSO_4$, 25; $FeSO_4 \cdot 7H_2O$, 1.3; $MnSO_4 \cdot H_2O$, 1.3; glucose, 2500; thiamin hydrochloride, 0.025; calcium pantothenate, 0.05; nicotinic acid, 0.05; pyridoxine hydrochloride, 0.25; *p*-aminobenzoic acid, 0.063; biotin, 0.002; riboflavin, 0.063; folic acid, 0.0001.

Enzyme Preparation. The 100-L culture was chilled below 15 °C and harvested when growth over a 30-min interval became negligible. The unwashed cells were suspended in 1500 mL of 0.01 M Tris-HCl buffer, pH 7.3, containing 0.02% azide, and broken by three passages through a Manton-Gaulin mill with cooling to 10 °C between successive passages. After centrifugation (Sorvall 3S head, 8500 rpm, 60 min), the sedimented material was resuspended in 400 mL of the same buffer and treated with 100 mg of lysozyme (Sigma) and 20 mg of deoxyribonuclease I (Sigma). The suspension was stirred overnight at 5 °C before centrifuging as before. The supernatant was combined with that from the previous centrifugation and isoenzyme 2 purified from this extract by the method of Nixon and Blakley (1968) with the following modifications. After treatment with protamine sulfate (Krishell Laboratories), the protein solution was concentrated by ultrafiltration (Amicon UM-10 membrane) rather than by ammonium sulfate precipitation, which occasionally gave poor recovery. Chromatography on hydroxylapatite was omitted. After purification on DEAE-Sephadex, the pooled active fractions were dialyzed against 0.05 M potassium phosphate buffer, pH 7.3, concentrated by ultrafiltration to a volume of about 60 mL, and dialyzed against the same buffer overnight. The solution was passed through a pteroyl-L-lysine–Sepharose column prepared as described previously (Peterson et al., 1975a) and equilibrated with the same buffer. Approximately 20 mL of solution containing 370 mg of protein was passed through a column 2.3 × 36 cm. A small peak of contaminating protein preceded the enzyme. Fractions of maximum specific activity were combined and concentrated by ultrafiltration, and the concentration of pure enzyme was estimated by absorbance at 280 (ϵ 30 000). Before use for ^{13}C NMR, the enzyme preparation was dialyzed against 0.05 M potassium phosphate buffer, pH 7.5, containing 0.5 M KCl. In this solution the concentrated reductase was much more stable at temperatures of 10–25 °C and concentrations up to 1.2 mM could be used at 15 °C without the precipitation which otherwise occurred.

^{13}C NMR spectra were obtained with a Varian XL-100-15 spectrometer (25.2 MHz) operating in the Fourier transform mode and interfaced to a Nova 1210 computer. In general, spectra were recorded with a 500-Hz spectral width and 512 spectral points, a 90° pulse (45 μs), no pulse delay, and an acquisition time of 1 s. Except where indicated otherwise, a lock was provided by incorporation of 5–10% D_2O in the sample solution. This concentration of D_2O is unlikely to cause significant changes in protein conformation or relaxation times, and inclusion of D_2O in the sample has the advantage that the homogeneity is optimized over the entire volume. This concentration of D_2O required a pulse lock on the XL-100. No exponential multiplication of the free-induction decay was performed so that resolution was maximized. Chemical shifts are given in ppm with respect to external tetramethylsilane and are accurate to 0.05 ppm. Relaxation times were measured using a $180°-\tau-90°-T$ pulse sequence (Vold et al., 1968). NOE values were measured using a gated decoupling procedure (Freeman et al., 1972; Harris and Newman, 1976). Interpulse intervals of 4.0 or 6.0 s during which the decoupler was off were utilized to obtain the necessary accuracy in the gated NOE experiments (Opella et al., 1976; Harris and Newman, 1976). Peak areas were determined by computer integration or by Xeroxing the spectra, cutting, and weighing. The agreement between the two methods was generally excellent, and the reported values represent averages of the two determinations. Spectra were simulated on the Supernova computer using a program developed by W. E. Wageman at Los Alamos in order to obtain good estimates of the line widths.

Results

Incorporation of Methionine into Dihydrofolate Reductase. In preliminary experiments *S. faecium* was found to have an

[1] Abbreviations used are: PADPR, 2′-phosphoadenosine 5′-diphosphoribose; MTX, methotrexate (4-amino-4-deoxy-10-methylfolic acid); $(NH_2)_2Pyr$, 2,4-diaminopyrimidine; Cl_2MTX, 2′,5′-dichloromethotrexate; NOE, nuclear Overhauser enhancement; EDTA, (ethylenedinitrilo)-tetraacetic acid; DEAE, diethylaminoethyl.

TABLE I: Chemical Shifts in ^{13}C NMR Spectra of [*methyl*-^{13}C]Methionine-Labeled Dihydrofolate Reductase (Ppm with Respect to External Tetramethylsilane).[a]

Enzyme			15.35		14.99	14.66			
Enzyme-NADPH			15.40	15.11		14.75	14.55 (s)		
Enzyme-$NADP^+$	17.08	15.68 (s)	15.39	15.24	14.95	14.73			
Enzyme-2′,5′-ADP		15.73 (s)	15.39		14.90	14.69			
Enzyme-2′,5′-ADP[b]		15.63 (s)	15.36	15.11 (s)	14.96	14.76			
Enzyme-2′,5′-ADP + NMN			15.39	15.08 (s)	14.92	14.71		14.31 (s)	
Enzyme-folate		15.47 (s)	15.32		14.89 (s)	14.71			
Enzyme-dihydrofolate			15.41		14.98	14.77			
Enzyme-5-formyltetrahydrofolate			15.36		14.91	14.60			
Enzyme-$(NH_2)_2$Pyr		15.87	15.37		14.94	14.65		14.23	
Enzyme-aminopterin			15.29	15.10	14.90		14.55	14.14	
Enzyme-aminopterin[b]			15.30	15.06	14.94		14.53	14.12	
Enzyme-MTX			15.37	15.24 (s)	14.95	14.74 (s)	14.57	14.18	
Enzyme-MTX[b]			15.34	15.16	14.97	14.80	14.57	14.16	
Enzyme-Cl_2MTX			15.39	15.06		14.75			
Enzyme-Cl_2MTX[b]			15.36	15.06		14.73			
Enzyme-dihydrofolate-$NADP^+$			15.40	15.17 (s)	15.01	14.74 (s)	14.62		
Enzyme-MTX-$NADP^+$ [b]			15.34	15.08			14.53	14.09	13.94
Enzyme-MTX-NADPH			15.40	15.21	15.09 (s)	14.57 (s)	14.47		13.87
Enzyme-MTX-NADPH[b]			15.36	15.24	15.03 (s)	14.68	14.44	14.31 (s)	13.96
Enzyme-MTX-NADPH[c]				15.26	15.05 (s)	14.94	14.40	14.07	
Enzyme-MTX-PADPR[b]			15.36	15.05	14.86 (s)		14.55	14.06	
Enzyme-$(NH_2)_2$Pyr-NADPH		15.50 (s)	15.34	15.09	14.88		14.59		13.73
Enzyme-$(NH_2)_2$Pyr-NADPH[b,d]			15.35	15.21		14.64			13.68
Enzyme-Cl_2MTX-NADPH[b]		15.60	15.38	15.15	14.89	14.70			

[a] Concentration of enzyme was 1.1 mM in most cases, 0.63 mM in others. Ligand concentrations (mM): 2′,5′-ADP, 1.25; NADPH, 2.0; $NADP^+$, 2.0; NMN, 15; folate, 2.0; dihydrofolate, 2.0; PADPR, 1.2; 5-formyltetrahydrofolate, 1.5; $(NH_2)_2$Pyr, 1.8; aminopterin, 0.86; MTX, 1.4; Cl_2MTX, 1.7 mM. Spectra were recorded at 15 °C unless otherwise indicated. Peaks labeled s appear as shoulders. [b] Spectrum recorded at 25 °C and corrected for 0.20-ppm lock shift. [c] Spectrum recorded at 38 °C and corrected for 0.46-ppm lock shift. [d] The mixture also contained 34 mM *p*-aminobenzoylglutamate.

absolute growth requirement for L-methionine, an observation indicating that the organism has negligible ability to synthesize this amino acid. Although percent incorporation of L-methionine from the medium into the bacteria was not measured, previous experience with the incorporation of [^{13}C]arginine indicated that when the culture was harvested at the point of growth cessation incorporation of the limiting amino acid was >90%. There appeared to be no significant dilution of ^{13}C in the methionine incorporated into the reductase. This conclusion was based on the following observations. In ^{13}C NMR spectra of the reductase over a 5000-Hz spectral width, no peaks due to natural abundance ^{13}C in other amino acids could be seen under conditions in which methionines gave strong signals. Furthermore, the signals from methionine residues were comparable in intensity to those from arginine residues in reductase that had incorporated 90% enriched [*guanidino*-^{13}C]arginine with negligible dilution (Cocco et al., in press).

Dissociation Constants of Ligand–Enzyme Complexes. Approximate dissociation constants were calculated from the inhibitory effects of the ligand under conditions in which linear competitive inhibition was obtained. With NADPH as the variable substrate, 2′,5′-adenosine diphosphate, PADPR, and nicotinamide mononucleotide were found to have K_i values of 35 ± 5 μM, 28 ± 9 μM, and 0.8 mM, respectively. With dihydrofolate as the variable substrate, 2,4-diaminopyrimidine and 5-formyltetrahydrofolate had K_i values of 30 ± 3 μM and 0.1 mM, respectively. These values were used to calculate the total concentration of ligand required to convert at least 95% of the enzyme to complex. Such a concentration was used in ^{13}C NMR experiments. *p*-Aminobenzoylglutamic acid did not give any measurable inhibition at a concentration of 1 mM.

^{13}C NMR Spectrum of Reductase. The spectrum of the enriched dihydrofolate reductase at 15 °C in the absence of ligands is shown in Figure 1. The resonances of the seven methionine residues are partially resolved into three composite peaks at 15.35, 14.99, and 14.66 ppm, respectively (Figure 1, Table I). The central peak shows signs of partial resolution into a doublet. Line widths for the resolved peaks are of the order of 2.5–5 Hz, but some of the underlying resonances are probably broader.

A sample of the enzyme in H_2O was treated with 6 M [^{12}C]urea and a ^{13}C NMR spectrum obtained using a D_2O capillary for a lock. The denatured enzyme showed complete collapse of the spectrum to a single peak centered at 15.32 ppm. This suggests that the sharp resonance at 15.35 ppm in the spectrum of the native enzyme is due to one or more methionine residues which have no more constraint on their freedom of motion than in the random-coil configuration formed in the presence of urea. On the other hand, [*methyl*-^{13}C]methionine showed a single sharp resonance at 15.04 ppm either in the presence of buffer and KCl or when added to an enzyme–dichloromethotrexate–NADPH sample. A sample of [*methyl*-^{13}C]methionine mixed with denatured enzyme in 6 M [^{12}C]urea gave both the high- and low-field peaks. When this sample was heated overnight at 60 °C, the free methionine reacted with cyanate present in the urea to form the carbamyl derivative (Stark et al., 1960) and the high-field (free methionine) resonance shifted to the low-field position. The significance of these data is discussed later.

Effect of Ligands on ^{13}C Shifts

Effect of Nucleotide Binding on Chemical Shifts. The changes in the ^{13}C NMR spectrum of the labeled enzyme due to binding of the reducing substrate, NADPH, were relatively slight (Figure 1 and Table I). Nevertheless, it is clear that there has been a significant downfield shift of one or more resonances in the central group. 2′,5′-Adenosine diphosphate, which

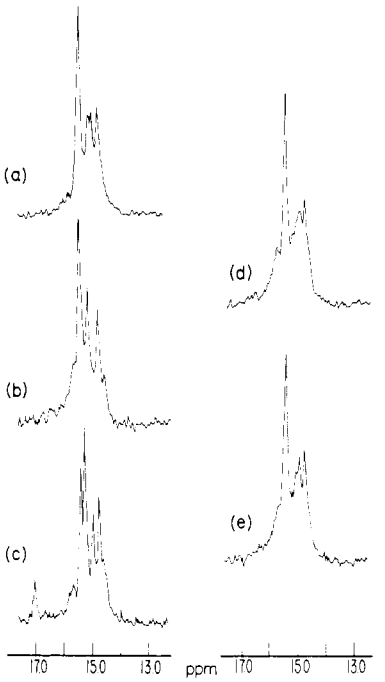

FIGURE 1: ^{13}C NMR spectra of dihydrofolate reductase labeled with [*methyl*-^{13}C]methionine and of complexes of the enzyme with various nucleotides. Spectra were recorded at 15 °C. For other details, see Table I. (a) Uncomplexed enzyme; (b) enzyme-NADPH; (c) enzyme-NADP$^+$; (d) enzyme-2′,5′-ADP; (e) enzyme-2′,5′-ADP-NMN.

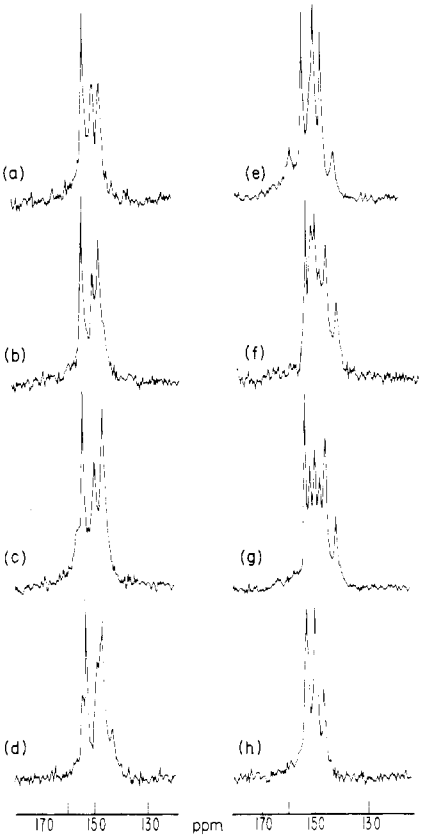

FIGURE 2: ^{13}C NMR spectra of binary complexes of pteridine substrates and inhibitors with dihydrofolate reductase labeled with [*methyl*-^{13}C]-methionine. Spectra were recorded at 15 or 25 °C as indicated. In the latter case a 0.20-ppm correction for lock shift has been applied. For other details, see Table I. (a) Uncomplexed enzyme, 25 °C; (b) enzyme-dihydrofolate, 15 °C; (c) enzyme-formyltetrahydrofolate, 15 °C; (d) enzyme-folate, 15 °C; (e) enzyme-diaminopyrimidine, 15 °C; (f) enzyme-aminopterin, 25 °C; (g) enzyme-methotrexate, 25 °C; (h) enzyme-dichloromethotrexate, 25 °C.

corresponds to a portion of the NADPH molecule, causes an even smaller effect on the spectrum. The most marked nucleotide effect is produced by NADP$^+$ which causes the appearance of a resonance shifted downfield to 17.08 ppm. Although the original position of this peak is unknown, this must correspond to a change in chemical shift of at least 1.7 ppm. This is the largest ligand-induced shift that we have observed to date for ^{13}C in the enzyme and it is interesting to consider the underlying mechanism (see Discussion). It results in a range of 2.27 ppm for the chemical shifts in this binary complex.

Effect of Pteridine Substrates and Inhibitors on Chemical Shifts. The substrate dihydrofolate caused only slight changes in the ^{13}C NMR spectrum (Figure 2), although small shifts of one or two resonances have clearly occurred in the central and upfield region. 5-Formyltetrahydrofolate produced a rather similar spectral change, whereas folate itself caused more marked changes with movement of resonances in the central region of the spectrum and partial resolution of a peak shifted upfield to 14.31 ppm (Figure 2).

The ligands producing by far the most numerous changes in the ^{13}C NMR spectrum were the 2,4-diamino heterocycle inhibitors methotrexate, aminopterin, and 2,4-diaminopyrimidine (Figure 2, Table I). In the case of methotrexate and aminopterin the shifts appear to be mostly (perhaps exclusively) upfield, with one resonance moving to 14.16–14.12 ppm. Probably three resonances shift upfield in the case of these two ligands. Diaminopyrimidine also causes one resonance to move upfield from the main envelope, but, in contrast to methotrexate and aminopterin, diaminopyrimidine also produces a marked downfield shift of one resonance. The movements of resonance peaks which are evoked by binding of methotrexate and aminopterin but not by diaminopyrimidine are the apparently smaller movements in the central part of the spectrum which result in partial resolution of "new" peaks at 14.80 and 15.16 ppm (at 25 °C) in the enzyme-methotrexate spectrum.

Unlike methotrexate and aminopterin, dichloromethotrexate produces no pronounced upfield shift, and shifts within the main envelope did not result in the resolution of additional peaks.

Chemical Shifts in Ternary Complexes. When the spectrum of the abortive complex enzyme-dihydrofolate-NADP$^+$ (Figure 3) is compared with that of the binary complex (Figure 2), it is clear that additional movements of resonances have occurred due to the binding of NADP$^+$, with the range of chemical shifts increased to 0.78 ppm from 0.64 ppm for the binary complex (Table I). Nevertheless, the changes produced by NADP$^+$ and dihydrofolate are certainly not additive. In particular, the large downfield shift to 17.08 ppm produced by NADP$^+$ in the enzyme-NADP$^+$ complex does not occur in the ternary complex, whereas in the ternary complex a peak appears at higher field (14.62 ppm) than in either of the binary complexes (Table I).

When spectra for enzyme-methotrexate-NADP$^+$ (Figure 3) and enzyme-methotrexate (Figure 2, Table I) complexes are compared, the general effect of NADP$^+$ binding is much the same as in the previous case: no downfield movement of a resonance to 17 ppm occurs, but a number of smaller movements of several resonances are seen, resulting in greater spectral spread toward high fields with the result that the positions of six of the seven methionine resonances can now be identified. In the spectrum of the enzyme-methotrexate-NADPH complex (Figure 3, Table I) the resonances are

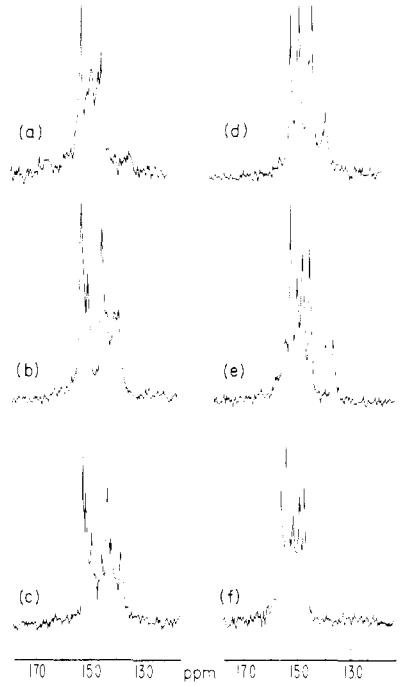

FIGURE 3: ^{13}C NMR spectra of ternary complexes of dihydrofolate reductase labeled with [*methyl*-^{13}C]methionine. Spectra were recorded at 15, 20, or 25 °C as indicated, and in the latter two cases a correction for lock shift has been applied. For other details, see Table I. (a) enzyme-dihydrofolate-$NADP^+$, 15 °C; (b) enzyme-methotrexate-$NADP^+$, 25 °C; (c) enzyme-methotrexate-NADPH, 25 °C; (d) enzyme-methotrexate-PADPR, 25 °C; (e) enzyme-diaminopyrimidine-NADPH, 20 °C; (f) enzyme-dichloromethotrexate-NADPH, 25 °C.

spread over a similar range of chemical shifts (1.4–1.5 ppm), but in this case all seven methionine resonances are partially resolved at 25 °C. At least four of the resonances have moved from their positions in the spectrum of the native enzyme and most, if not all, of these movements are upfield. Since $NADP^+$ causes no upfield movements in its binary complex and NADPH appears to cause only one in its binary complex, the upfield movement produced by both nucleotides in their ternary complexes is somewhat unexpected but again illustrates that ligand effects are not simply additive.

When PADPR replaced NADPH in the ternary complex formed with enzyme and methotrexate, a spectrum was obtained with considerably fewer resolved peaks and a slightly smaller range of chemical shifts (1.3 ppm, Figure 3, Table I). Nevertheless, examination of the spectrum of the ternary complex indicates that this nucleotide, which differs from NADPH only in the lack of the nicotinamide ring, probably also produced upfield shifts of at least three resonances.

NADPH also causes upfield movements of resonances when combined with enzyme and diaminopyrimidine (Figure 3, Table I) and the additional displacements for the extreme downfield and upfield peaks must be at least 0.37 and 0.50 ppm, respectively. In this ternary complex the range of chemical shifts is greatest, 1.77 ppm. Although *p*-aminobenzoylglutamate did not cause significant inhibition at 1 mM, it still seemed possible that it might bind weakly to the catalytic site, particularly in view of the report of Birdsall et al. (1977) that dihydrofolate reductase from *Lactobacillus casei* forms a complex with *p*-aminobenzoylglutamate with a dissociation constant of 1 mM. In fact, addition of *p*-aminobenzoylglutamate to the enzyme-diaminopyrimidine-NADPH ternary complex did cause some minor changes in resonance positions (spectrum not shown) which were not due to resonances from the natural abundance ^{13}C in the *p*-aminobenzoylglutamate itself. The spectrum of the quaternary enzyme-*p*-aminobenzoylglutamate-diaminopyrimidine-NADPH complex did not approximate very closely that of the enzyme methotrexate-NADPH complex, but this may be the result of many rather small differences in chemical shifts, since in general the two spectra were spread over about the same range of chemical shifts.

In the enzyme-dichloromethotrexate-NADPH spectrum, NADPH did not cause the upfield shifts that it produced on combining with other enzyme-inhibitor complexes (Figure 3, Table I) and, in fact, one rather pronounced downfield shift occurred. This illustrates again that the introduction of the two chlorine atoms into methotrexate causes a greatly changed interaction of the inhibitor with the active site.

Effect of Temperature on Chemical Shifts. Most of the spectra were recorded at 15 °C, since the enzyme itself was stable for prolonged periods in the buffer-KCl solution at this temperature. Although the enzyme in the absence of ligands was not as stable at higher temperatures and some precipitation occurred after several hours at 25 °C, the inhibitor complexes were considerably more stable, particularly the ternary complexes containing NADPH. Since the enzyme-methotrexate-NADPH complex was especially stable at temperatures above 15 °C and at 25 °C gave the best resolution seen in any complex, the effect of temperature on the chemical shifts of resonances in this spectrum was examined at several temperatures. The relative changes in chemical shift between spectra obtained at 15 and 25 °C were all less than 0.1 ppm. The difference between spectra obtained at different temperatures was mainly in the extent of resolution of the peaks, which was generally better at 25 °C. For this reason, several of the spectra in Figures 2 and 3 were run at 25 °C. The shifts have been corrected for the temperature dependence of the lock (0.5 Hz per degree) so that the spectra can all be compared directly. The actual temperature dependence for each of the resonances was difficult to determine because not all peaks were resolved at each of the temperatures examined. In the enzyme-methotrexate-NADPH complex, at least two peaks show significant downfield shifts at higher temperature toward the urea denatured enzyme position. The changes in line width of single resonances over the temperature range studied were too small to be measured with accuracy.

Relaxation Measurements

Relaxation data obtained with the free enzyme, the binary enzyme-methotrexate complex, and the ternary enzyme-methotrexate-NADPH complex are summarized in Table II. In a few cases where individual resonances are fairly well resolved, line width values are given, with the errors dependent on the resolution. In all cases, the relatively sharp peak exhibiting a shift close to that observed for the urea-denatured enzyme (~15.34 ppm) gave the longest T_1, indicating the greatest degree of internal motion as discussed in the following section. The line width obtained for this resonance is the most accurate, since it is relatively well resolved.

An important question which must be considered in the present study is the extent to which resonance intensity is proportional to the number of contributing carbons, i.e., the possibility of differential NOE values. Studies of the accuracy of pulsed NOE experiments have indicated that in order to obtain reliable results it is necessary to have a nondecoupling delay of $\geq 9T_1$ (Harris and Newman, 1976; Opella et al., 1976). In the gated NOE experiments, interpulse delays of 4.0 s for the free enzyme and binary enzyme-methotrexate sam-

ples and 6.0 s for the enzyme-methotrexate-NADPH sample were used. These delays are sufficient to ensure accurate values for all but the sharpest peak for which the measured NOE must be considered a *minimum* value. In many cases, the poor resolution and low signal/noise inherent in the very long measurement periods that were required made a determination of the NOE values for individual resonances difficult. For this reason, only average values are stated in most cases in Table II. However, the appearance of the spectra was qualitatively very similar in the gated and continuously decoupled spectra, so that it is clear that no significant differences in NOE for the various resonances exist. The only exception to the above statement is the sharp resonance at ~15.34 ppm which has a significantly larger NOE in all cases. In general, the NOE values obtained are very close to the values of 1.5 and 1.7 obtained by Jones et al. (1976) for the two methionine C^{ϵ} resonances of sperm whale myoglobin.

TABLE II: Summary of Relaxation Parameters for C^{ϵ} Resonances of Methionine Residues in Dihydrofolate Reductase.[a]

Complex	Chemical shift[b] (ppm)	T_1 (ms)	NOE	ν^c (Hz)
Enzyme alone	15.35	590	1.8	3.2 ± 0.5
	14.99	470	1.6	*d*
	14.66	500		*d*
Enzyme-methotrexate	15.37	720	2.2	2.4 ± 0.5
	15.24 (s)	400		3.0 ± 1.0
	14.95	410		3.2 ± 1.0
	14.74 (s)	330	1.5	4.4 ± 1.0
	14.57	460		3.2 ± 1.0
	14.18	370	1.6	5.0 ± 0.5
Enzyme-methotrexate-NADPH	15.36	710	2.1	2.6 ± 0.5
	15.24	560		3.0 ± 1.0
	15.03 (s)	470		3.6 ± 1.0
	14.68 (s)	350	1.6	4.4 ± 1.0
	14.44	480		2.8 ± 1.0
	14.31 (s)	410		4.6 ± 1.0
	13.96	420		5.0 ± 0.5

[a] All measurements made in samples containing 5% D_2O for the lock at 15 °C in the first two cases and at 25 °C in the last. The shifts have been corrected in the last case for lock shift. [b] Resonances correspond to resolvable peaks; only in the enzyme-methotrexate-NADPH spectrum are seven peaks corresponding to the seven methionine residues observed. Peaks labeled s appear only as shoulders; T_1 values for these resonances have a somewhat larger error (15%) than for resolved peaks (10%). [c] Line widths stated were deduced from simulations of the spectra as a sum of Lorentzians. [d] Not determinable.

Discussion

^{13}C Relaxation Measurements. In general, the relaxation behavior of the various amino acids incorporated into proteins can be divided into three classes: (1) residues which appear to be sufficiently immobilized so that internal motion occurs over a time scale which is slow relative to overall molecular rotation. This class includes several of the arginine residues of dihydrofolate reductase which we have studied (Cocco et al., 1977). For typical proteins exhibiting correlation times $\gtrsim 2 \times 10^{-8}$ s, the relaxation parameters indicate only the effects of very slow motion, e.g., an NOE value close to 1.1 and spin-lattice relaxation times which decrease with increasing temperature, all of which have been observed for the guanido carbon of the immobilized arginine residues in dihydrofolate reductase (Cocco et al., in press). (2) A second class of residues exhibit sufficiently free internal motion such that the effect of slow macromolecular tumbling is minimal. This class would include the C^{ϵ} of lysine which typically exhibits narrow line widths or the methyl resonances of the *N*-trimethyllysine residue of cytochrome *c* (Eakin et al., 1975; Wilbur and Allerhand, 1977) which exhibits an NOE of 3.0. For such highly mobile residues, chemical-shift inequivalence resulting from folding of the protein into its native conformation will probably be negligible as suggested by Oldfield et al. (1975). (3) A third class of residues exhibit an intermediate degree of motional restriction so that the relaxation behavior must be described by at least two correlation times. The methyl resonances of the methionine residues of dihydrofolate reductase fall into categories 2 and 3, exhibiting intermediate NOE values and line widths consistent with a substantial degree of internal motion.

It should be noted that the above conclusions are based on the assumed dominance of the ^{13}C-^{1}H dipolar interaction with the directly bonded methionine protons. Calculations indicate that this assumption, generally valid for a variety of small molecules, should be particularly good in the case of proteins, since the effectiveness of this relaxation mechanism is enhanced due to the longer rotational correlation time of the protein. The relaxation mechanism most likely to be of secondary importance is the dipolar interaction with traces of paramagnetic ions in solution. Addition of EDTA to one of the protein solutions produced no observable narrowing of the resonances, however. Furthermore, paramagnetic ion contamination is most likely to be significant for solvent-accessible residues. As noted above, such residues would most likely exhibit shifts close to that of the denatured enzyme, i.e., ~15.3 ppm. Thus, significant paramagnetic contamination should selectively broaden and shorten the T_1 values of the peak at 15.3 ppm, in contrast with the observed pattern.

The simplest model involving partially restricted motion of the methionine methyl groups is the assumption of very rapid rotation about the methyl axis and negligible internal motion about any other bond. This assumption can be tested by comparing the measured spin-lattice relaxation rates with calculations based on the overall molecular correlation time of 2×10^{-8} s deduced from experiments with [*guanido*-^{13}C]arginine-labeled enzyme. Assuming very rapid methyl rotation so that the calculated T_1 value is a factor of nine greater than that in the absence of such rotation, NT_1 values of ~650 ms are obtained, substantially shorter than the observed values (Table II). It is unlikely that the overall correlation time used in the calculation is significantly in error in that it is quite typical for a protein of molecular weight 20 000 [e.g., $\tau_0 = 2 \times 10^{-8}$ s for sperm whale myoglobin, 17 000 daltons (Visscher and Gurd, 1975); $\tau_0 = 2.7 \times 10^{-8}$ s for tryptophan synthetase, 29 000 daltons (Browne et al., 1973b); $\tau_0 = 1.7 \times 10^{-8}$ s for α-lytic protease, 20 000 daltons (Hunkapiller et al., 1973a,b)]. The above discrepancy suggests the importance of additional rapid internal motion which would lead to larger calculated values for T_1. A similar conclusion can be drawn from the observed line widths. For a completely immobilized methyl group, ν = 89 Hz, rapid methyl rotation reduces the value to 9.9 Hz, still substantially broader than the values obtained for the resolved peaks (Table II). As above, a further decrease in line width would be predicted to result from additional internal motion. Finally, it is worth noting that Jones et al. (1976) have been unable to adequately describe the frequency dependence of the T_1 values for the methyl-labeled methionines in sperm whale myoglobin using a model assuming rapid methyl rotation as the only internal motion. It must be noted, however, that the

TABLE III: Calculated Relaxation Parameters for a Model with Two Free Internal Rotations.[a]

$D_1 \times 10^{-9}$ (s^{-1}) (CH_2-S)	$D_2 \times 10^{-9}$ (s^{-1}) (S-CH_3)	T_1 (ms)	NOE	ν (Hz)
7	50	610	1.99	2.3
5	50	560	2.06	2.3
1	50	320	2.39	2.3
5	40	530	2.11	2.4
4	40	500	2.16	2.4
3	30	430	2.28	2.5
8	20	470	2.21	2.4

[a] In the above calculation, $\tau = 1/(6D_0) = 2 \times 10^{-8}$ s; bond angles: CH_2-S-CH_3 = 100°, S-C-H_3 = 109.5°.

T_1 values obtained in that case differ substantially from those obtained for dihydrofolate reductase.

In order to obtain a better fit of the data, a calculation using two free, uncorrelated internal rotations superimposed on overall isotropic motion was carried out. The calculation of T_1 and T_2 values (Wallach, 1967; Levine et al., 1973) as well as NOE values (London and Avitabile, 1976) corresponding to such a model have been described in the literature. In addition to methyl rotation, the internal motion assumed most likely to be significant is that about the CH_2-S bond. The relevant angles entering the calculation are then the C-S-C bond angle of 100° (Mathieson, 1952; Torii and Itaka, 1973) and the S-C-H_3 bond angles of 109.5°. Three diffusion coefficients, D_0, D_1, and D_2 describing the overall (isotropic) protein motion, internal rotation about the CH_2-S bond, and internal methyl rotation,respectively, are utilized. Several representative calculations are included in Table III. In these calculations, the value of $D_0 = 1/(6\tau_0)$ was fixed at 8.33×10^6 s^{-1}, the value of D_2 is typical for a methyl group and D_1 was assumed to be lower than D_2. The best fit obtained is for the sharp peak at 15.34 ppm. T_1, line width, and NOE values of 610 ms, 2.3 Hz, and 2.0 corresponding to $D_1 = 7 \times 10^9$ s^{-1} and $D_2 = 5 \times 10^{10}$ s^{-1} are in reasonable agreement with the results for this peak in enzyme alone. Although this approach leads to spin-lattice relaxation times closer to the measured values (Table II), the predicted line widths are somewhat narrower than the measured values and the calculated NOE's are substantially larger than the measured values. Furthermore, it is not possible to explain the differences in T_1 and NOE observed for the various methyl resonances by varying D_1 and/or D_2. Increasing the rates of internal diffusion leads to longer T_1 values but to lower NOE values, whereas experimentally it is found that the peaks exhibiting the longest T_1 also had the largest NOE. The behavior of the calculated NOE reflects the fact that as the rate of internal motion increases the spectral density is dominated by the slower overall tumbling rate which corresponds to a reduced NOE.

The most likely explanation of the discrepancies noted above is that the internal rotation about the CH_2-S bond is not free but highly restricted, so that the methyl group oscillates through a relatively narrow angular range, significantly less than 360°. Differences in the relaxation parameters for the various methionines would then reflect differences in the accessible angular range for diffusion about this bond, with the larger range corresponding to the longer T_1 values, narrower line widths, and larger NOE values. As a rough approximation to this behavior, a model has recently been constructed in which the range of motion is limited. This model is more qualitatively consistent with the data obtained here and suggests that the best interpretation of the methionine relaxation data involves free internal rotation about the methyl axis and significant but highly restricted motion about the CH_2-S bond, the latter varying significantly for the different methionine residues in the protein. Using this model (London and Avitabile, 1978), the diffusion about the CH_2-S bond is limited to a range of ~90° for the broadest lines. For example, for $\tau = 2 \times 10^{-8}$ s, $D_1 = 4 \times 10^{10}$ s^{-1} and $D_2 = 8 \times 10^{10}$ s^{-1}, restriction of the motional (D_1) range to 90° leads to the relaxation parameters $T_1 = 344$ ms, $\nu = 5.9$ Hz, and NOE = 1.36, in rough agreement with the parameters for the broadest lines. Increasing the range to 180° and using the same diffusion coefficients gives $T_1 = 738$ ms, $\nu = 2.5$ Hz, and NOE = 1.62, closer to the values for the sharpest peaks. We note, however, that the uniqueness of these parameters has not been determined.

It should be noted in the above discussion that the internal motion considered is rapid compared with the rotational correlation time of the protein. Even highly immobilized residues such as the aromatic rings of phenylalanine or tyrosine appear to undergo abrupt 180° jumps sufficient to eliminate shift inequivalence of the two ortho (or two meta) protons (Campbell et al., 1975; Wurthrich and Wagner, 1975; Gelin and Karplus, 1975; Feeney et al., 1977). It is similarly probable that over some slower time scale free rotation about the CH_2-S bond is possible, but that rapid motion is highly restricted.

The above results indicate that a significant degree of internal motion is not necessarily incompatible with chemical shift inequivalence of the resonances as suggested elsewhere (Oldfield et al., 1975). In fact, including the large downfield shift observed in the enzyme-$NADP^+$ complex, the range of methionine methyl shifts is greater than the range of shifts observed for the guanido carbons of [*guanido*-^{13}C]arginine-labeled dihydrofolate reductase despite the much greater immobilization of the guanido group in the enzyme (Cocco et al., 1977).

Chemical Shifts. The chemical shifts observed for the C^ϵ resonances ranged between 17.1 and 13.7 ppm downfield from tetramethylsilane (Table I). This range includes the shifts reported for the methionine C^ϵ resonances of cytochrome *c* (Eakin et al. 1975) and those for myoglobin in most of its forms (Jones et al., 1976).

The urea-denatured enzyme gave a single resonance centered at 15.32 ppm (Table I). This resonance is distinctly downfield of the free methionine methyl resonance which appears at 15.04 ppm. This difference in chemical shifts is unequivocal, as evidenced by the two peaks observed in the spectrum of a mixture of free methionine and the denatured enzyme. These results demonstrate that the methyl chemical shift is about 0.3 ppm downfield in a peptide relative to free methionine, in contrast to the results of Keim et al. (1974) who report a single chemical shift for C^ϵ of both free methionine and of Gly-Gly-Met-Gly-Gly. The 0.3-ppm shift difference is also supported by the observation that free methionine is carbamylated at 60° in urea and this derivative gives the same chemical shift as the denatured enzyme.

The single resonance for the urea-denatured enzyme has the same shift as the relatively sharp resonance in the spectra of the native enzyme and its complexes (Table I). This resonance also has the longest T_1 and largest NOE (Table II). These observations are consistent with the idea that the sharp resonance is due to a highly mobile residue at the surface of the enzyme where it is readily accessible to solvent. Since this sharp resonance has been observed at the same position in all of the complexes which have been examined, it must be unaffected by enzyme conformational transitions or by direct effects of substrates or inhibitors. Most of the resonances in the complexes examined are upfield of this very sharp peak. This

contrasts with the arginine-labeled enzyme (Cocco et al., 1977) in which there are peaks on both sides of the resonance representing surface residues. This effect could result from the orientation of the methionines with respect to aromatic rings; i.e., the methionine is never on the edge of a ring. Alternatively, the upfield shifts may reflect the inaccessibility of the internal residues to solvent.

Changes in Chemical Shift due to Nucleotide Binding. The changes in chemical shifts of methionine C^{ϵ} resonances caused by ligand binding to the catalytic center of an enzyme may be produced in three ways. (1) The bound ligand may directly cause a change in the magnetic field experienced by methionines at the catalytic site. An example would be the effect of ring currents in the pteridine or benzene rings of methotrexate or the nicotinamide ring of $NADP^+$ on the C^{ϵ} of a methionine at the catalytic site. This would be particularly likely for a methionine involved in binding the ligand by hydrophobic interaction with a ring. (2) The bound ligand might alter the electronic microenvironment of C^{ϵ} of a methionine at the catalytic site, for example by formation of a charge-transfer complex. (3) The ligand binding may induce conformational changes that cause the magnetic or electronic environment of methionine residues to change due to relative movement of other amino acid residues in their vicinity. This could affect methionine residues remote from the catalytic site as well as those actually forming part of the site. The problem of distinguishing between these types of ligand effects on chemical shifts has been discussed by Birdsall et al. (1977), Feeney et al. (1977), and Kimber et al. (1977). One of the criteria proposed is that if different types of ligand all change the chemical shift of the same resonance then it is likely that conformational changes mediate the effects. It is not presently possible to apply this test to the [*methyl*-^{13}C]methionine-labeled reductase because of the incomplete resolution of resonances for the enzyme in absence of ligands. However, the test is in any case of uncertain validity both because the binding sites might overlap enough for both pteridine and nucleotide ligands to influence directly the same residue and also because one kind of ligand might have a direct effect on a given residue and the other might effect it indirectly through conformation changes. Additionally, a given ligand could exert both a direct and indirect effect on a particular chemical shift.

The large downfield shift produced by the binding of $NADP^+$ is so great (1.7 ppm, Table I) that it is difficult to explain solely by an "edge" effect of the electron current in the nicotinamide ring. On the other hand, NADPH causes no downfield shift at all, though it would be expected to produce rather similar conformational effects to those induced by $NADP^+$. Perhaps the most plausible explanation of the downfield shift produced by $NADP^+$ is that it is due to a combination of direct magnetic and electronic effects of the nicotinamide ring on a methionine residue at the nucleotide binding site. One possible type of electronic effect on the C^{ϵ} nucleus is a transfer of electron density from the methionine sulfur to the positively charged nicotinamide ring. That a positive charge on the sulfur causes a major shift of the C^{ϵ} resonance is demonstrated by the fact that the C^{ϵ} of *S*-carboxymethylmethionine was found to have a chemical shift of 24.3 ppm. The possibility of such a charge transfer between the nicotinamide ring and a methionine sulfur is suggested by the known interaction of the sulfur of a cysteine residue and the 4 position of the nicotinamide ring in glyceraldehyde phosphate dehydrogenase (Moras et al., 1975).

The inability of $NADP^+$ to produce a marked downfield shift in ternary complexes (Table I) may result from altered orientation of the methionine residue relative to the nicotinamide ring when methotrexate is bound in the active site. Both the ring-current effect and the occurrence of a charge transfer would depend critically on the distance of the methionine side chain from and its orientation with respect to the ring. Certainly the pteridine portion of methotrexate or DHF must bind ix close proximity to the nicotinamide moiety of NADPH.

No information is yet available from x-ray diffraction studies on whether any methionine residues are in close proximity to the nicotinamide ring of bound $NADP^+$, but from the results of Matthews et al. (1977) and on the assumption of homologous structures for *S. faecium* and *E. coli* reductases Met-1 and -163 seem unlikely to be close to the nucleotide binding site. Since Met-5, -28, and -50 probably interact with methotrexate (see below) and since the carboxymethylation of Met-28 and -50 does not affect the resonance shifted downfield by $NADP^+$ (unpublished results), it is unlikely that Met-5, -28, or -50 is the residue interacting with $NADP^+$. Although Met-36 and -42 seem likely to be in the general region of the binding site, there is no information on whether they are affected by or contribute to nucleotide binding. However, it is noteworthy that Met-42 occurs in a highly conserved region of the sequence and is invariant in three sequences compared by Stone and Philips (1977), though it is not conserved in the *L. casei* sequence (Bitar et al., 1977).

The many small upfield shifts produced by both $NADP^+$ and NADPH when they bind to the enzyme–methotrexate complex (Table I) may be indirect effects mediated through induced conformation changes. This view is consistent with the observation that in the enzyme–dichloromethotrexate–NADPH complex such upfield shifts are not apparent and with the temperature sensitivity of the chemical shifts for the enzyme–methotrexate–NADPH complex.

Changes in Chemical Shift due to Pteridine Binding. Since binding of methotrexate to the enzyme causes obvious changes in the chemical shifts of two or more resonances (Figure 2, Table I) and since the movements are probably all upfield, it seems plausible to suggest that these upfield shifts are due to π electron currents in the pteridine and benzene rings. The magnitude of these upfield shifts is 0.17 to 0.5 ppm, which is consistent with the assumption that they are caused by ring currents.

Some idea of the methionine residues which are likely to interact directly with methotrexate can be gained from the structural information reported for the methotrexate complex of *Escherichia coli* dihydrofolate reductase on the basis of x-ray diffraction studies by Matthews et al. (1977). These authors report that side chains of Ile-5 and Leu-28 are among those in van der Waals contact with the pteridine ring. They also state that side chains of Leu-28 and Ile-50 contribute to the formation of a hydrophobic pocket in which the aromatic ring of the *p*-aminobenzoyl portion of methotrexate binds. In the sequence of the *S. faecium* reductase, positions 5, 28, and 50 are all methionine residues (Gleisner et al., 1974, 1975; Peterson et al. 1975b) and it is reasonable to assume that these play similar roles in the binding of methotrexate. Independent evidence that Met-28 and -50 are involved in the binding of methotrexate by the *S. faecium* enzyme is provided by the fact that carboxymethylation of these residues decreases the association constants for combination of aminopterin or DHF with the enzyme and that aminopterin protects these residues from carboxymethylation (Gleisner and Blakley, 1975a; Gleisner and Blakley, 1975b). Direct ring-current effects of methotrexate on the C^{ϵ} resonances of these three residues therefore appear to be possible. This conclusion is also consistent with preliminary data obtained with [*methyl*-^{13}C]-methionine-labeled dihydrofolate reductase after carboxy-

methylation.

Since the binding of folate causes fewer and smaller upfield shifts (Figure 2, Table I), it must be concluded that there are significant differences in the way in which methotrexate and folate bind to the active site. A similar conclusion has been reached from a study of the effects of the binding of these ligands to *L. casei* dihydrofolate reductase as monitored by NMR of histidine protons (Birdsall et al., 1977), tyrosine protons (Feeney et al., 1977), and fluorine of fluorotyrosine and fluorotryptophan (Kimber et al., 1977). 5-Formyltetrahydrofolate (leucovorin) produces very small upfield shifts (Figure 2, Table I) and in the spectrum of the dihydrofolate–enzyme complex none can be discerned at all. This may be interpreted as indicating that in addition to the different mode of binding of 2-amino-4-oxopteridines the diminished ring current of the pteridine ring caused by its partial hydrogenation significantly decreases the shifts resulting from the interaction of the pteridine ring with methionine residues.

The spectra for dichloromethotrexate and 2,4-diaminopyrimidine complexes (Figure 2, Table I) are difficult to interpret along the lines above. The binary complex of the former gives a spectrum with no observable upfield shift changes, yet the pteridine ring current should be normal even though the bulky chlorine atoms might hinder interaction of methionine residues with the benzene ring. A possible explanation may be that the chlorines also sterically interfere with the interaction of methionine residues with the pteridine ring. Diaminopyrimidine has no benzene ring at all, yet it produces one large upfield shift ($\geq$0.4 ppm) as well as one large downfield shift ($\geq$0.5 ppm). If these are direct ring-current effects then they must be due to the pyrimidine ring, and the largest upfield shift produced by methotrexate would, by analogy, be attributable to the interaction of a methionine with the pyrimidine portion of the inhibitor. The downfield shift of a resonance due to diaminopyrimidine binding could conceivably arise due to "edge" ring-current effects on a methionine that normally interacts with the pyrazine portion of the pteridine ring.

References

Ben-Hayyim, G., Hockman, A., and Avron, M. (1967), *J. Biol. Chem. 242,* 2837–2839.

Birdsall, B., Griffiths, D. V., Roberts, G. C. K., Feeney, J., and Burgen, A. (1977), *Proc. R. Soc. London, Ser. B 196,* 251–256.

Bitar, K. G., Blankenship, D. T., Walsh, K. A., Dunlap, R. B., Reddy A. V., and Freisheim, J. H. (1977), *FEBS Lett. 80,* 119–122.

Browne, D. T., Earl, E. M., and Otvos, J. D. (1976), *Biochem. Biophys. Res. Commun. 72,* 398–403.

Browne, D. T., Kenyon, G. L., Packer, E. L., Sternlicht, H., and Wilson, D. M. (1973a), *J. Am. Chem. Soc. 95,* 1316–1323.

Browne, D. T., Kenyon, G. L., Packer, E. L., and Wilson, D. M. (1973b), *Biochem. Biophys. Res. Commun. 50,* 42–47.

Campbell, I. D., Dobson, C. M., and Williams, R. J. P. (1975), *Proc. R. Soc. London, Ser B. 189,* 503–509.

Chaiken, I. M., Cohen, J. S., and Sokoloski, E. A. (1974), *J. Am. Chem. Soc. 96,* 4703–4705.

Cocco, L., Blakley, R. L., Walker, T. E., London, R. E., and Matwiyoff, N. A. (1977), *Biochem. Biophys. Res. Commun. 76,* 183–188.

Cocco, L., Blakley, R. L., Walker, T. E., London, R. E., and Matwiyoff, N. A. (1978), *Biochemistry* (in press).

Eakin, R. T., Morgan, L. O., and Matwiyoff, N. A. (1975), *Biochem. J. 152,* 529–535.

Feeney, J., Roberts, G. C. K., Birdsall, B., Griffiths, D. V., King, R. W., Scudder, P., and Burgen, A. (1977), *Proc. R. Soc. London, Ser. B 196,* 267–290.

Freeman, R., Hill, H. D. W., and Kaptein, R. (1972), *J. Magn. Reson. 7,* 327–329.

Gelin, B. R., and Karplus, M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2002–2006.

Gleisner, J. M., and Blakley, R. L. (1975a), *J. Biol. Chem. 250,* 1580–1587.

Gleisner, J. M., and Blakley, R. L. (1975b), *Eur. J. Biochem. 55,* 141–146.

Gleisner, J. M., Peterson, D. L., and Blakley, R. L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3001–3005.

Gleisner, J. M., Peterson, D. L., and Blakley, R. L. (1975), *J. Biol. Chem. 250,* 4937–4944.

Harris, R. K., and Newman, R. H. (1976), *J. Magn. Reson. 24,* 449–456.

Hunkapiller, M. W., Smallcombe, S. H., and Richards, J. H. (1975), *Org. Magn. Reson. 7,* 262–265.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., and Richards, J. H. (1973a), *Biochemistry 12,* 4732–4743.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., and Richards, J. H. (1973b), *J. Biol. Chem. 248,* 8306–8308.

Jones, W. C., Jr., Rothgeb, T. M., and Gurd, F. R. N. (1976), *J. Biol. Chem. 251,* 7452–7460.

Keim, P., Vigna, R. A., Nigen, A. M., Morrow, J. S., and Gurd, F. R. N. (1974), *J. Biol. Chem. 249,* 4149–4156.

Kimber, B. J., Griffiths, D. V., Birdsall, B., King, R. W., Scudder, P., Feeney, J., Roberts, G. C. K., and Burgen, A. S. V. (1977), *Biochemistry 16,* 3492–3500.

Levine, Y. K., Partington, P., and Roberts, G. C. K. (1973), *Mol. Phys. 25,* 497–514.

London, R. E., and Avitabile, J. (1976), *J. Chem. Phys. 65,* 2443–2450.

London, R. E., and Avitabile, J. (1978), submitted for publication to *J. Am. Chem. Soc.*

London, R. E., Gregg, C. T., and Matwiyoff, N. A. (1975), *Science 188,* 266–268.

Loo, T. L. (1965), *J. Med. Chem. 8,* 139.

Mathieson, A. McL. (1952), *Acta Crystallogr. 5,* 332–341.

Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M., and Hoogsteen, K. (1977), *Science 197,* 452–455.

Melville, D. B., Rachele, J. R., and Keller, E. B. (1947), *J. Biol. Chem. 169,* 419–426.

Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., and Rossmann, M. G. (1975), *J. Biol. Chem. 250,* 9137–9162.

Nixon, P. F., and Blakley, R. L. (1968), *J. Biol. Chem. 243,* 4722–4731.

Oldfield, E., Norton, R. S., and Allerhand, A. (1975), *J. Biol. Chem. 250,* 6368–6380.

Opella, S. J., Nelson, D. J., and Jardetzky, O. (1976), *J. Chem. Phys. 64,* 2533–2535.

Peterson, D. L., Gleisner, J. M., and Blakley, R. L. (1975a), *Biochemistry 14,* 5261–5267.

Peterson, D. L., Gleisner, J. M., and Blakley, R. L. (1975b), *J. Biol. Chem. 250,* 4945–4954.

Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235,* 3177–3181.

Stone, D., and Phillips, A. W. (1977), *FEBS Lett. 74,* 85–87.

Torii, K., and Iitaka, Y. (1973), *Acta Crystallogr. Sect. B 29,* 2799–2807.

Visscher, R. B., and Gurd, F. R. N. (1975), *J. Biol. Chem. 250,*

2238-2242.
Vold, R. L., Waugh, J. S., Klein, M. P., and Phelps, D. E. (1968), *J. Chem. Phys. 48,* 3831-3832.
Wallach, D. (1967), *J. Chem. Phys. 47,* 5258-5268.
Wilbur, D. J., and Allerhand, A. (1977), *FEBS Lett. 74,* 272-274.
Würthrich, K., and Wagner, G. (1975), *FEBS Lett. 50,* 265-268.

Ultraviolet and Visible Absorption Spectra of the Purple Membrane Protein and the Photocycle Intermediates†

Brian Becher, Fumio Tokunaga,‡ and Thomas G. Ebrey*

ABSTRACT: The visible and ultraviolet absorption spectra of purple membrane as well as the K, L, and M intermediates of the purple membrane photocycle are reported. The long wavelength absorbance by K (λ_{max} = 628 nm) and L (λ_{max} = 547 nm) strongly suggests that the Schiff base bond between retinal and the purple membrane protein is protonated while the position of the 412-nm band of M suggests that the Schiff base of this intermediate is unprotonated. The extinction of purple membrane at 280 nm is estimated to be ca. 75 000 L cm^{-1} mol^{-1} of which ca. 51 000 can be attributed to individual amino acids (in a hydrophilic environment), ca. 8000 due to retinal's absorption at 280 nm, and ca. 16 000 due to environmental (largely hydrophobic) effects on the aromatic amino acid transitions. There are very large decreases in the near-ultraviolet spectra on conversion to the photocycle intermediates L and M and on bleaching in hydroxylamine; their difference spectra have the shape of the protein itself. Decreasing the polarity of the membrane environment results in a decrease in the extinction changes. These results suggest that ca. 60% of the tryptophans and tyrosines in the protein move from a hydrophobic (interior) environment to a hydrophilic (exterior) environment either on bleaching or on conversion of purple membrane to L or M. Additional evidence of conformational changes during the photocycle includes the inability of hydroxylamine to bleach purple membrane except during the photocycle and the fact that when L or M is irradiated at −196 °C, a temperature at which protein conformational changes probably do not take place, they cannot be converted back to purple membrane until warmed to −100 °C. The conformational changes seen on photoconversion to L and M may well be involved in the mechanism of proton transport across the purple membrane which occurs on illumination.

The purple-colored membrane of *Halobacterium halobium* contains retinal bound to a single protein species by a Schiff base bond (Oesterhelt & Stoeckenius, 1971). When a photon is absorbed by a light-adapted purple membrane protein, a photochemical reaction cycle through a set of intermediates is initiated ending with the pigment returning to its original form (Lozier et al., 1975). The first three intermediates in the cycle are called K, L, and M. Accompanying this cycle is the pumping of protons across the purple membrane (Oesterhelt & Stoeckenius, 1973). Although the actual mechanism of the transfer of protons across the membrane is not known, reversible conformational changes in the protein during the purple membrane photocycle (possibly induced by retinal isomerization) may be involved.

In this study, new evidence of protein conformation changes in purple membrane on bleaching (chromophore loss) is discussed. This evidence includes an ultraviolet difference spectrum indicating large changes in extinction and resembling the shape of the protein absorption spectrum itself. A decrease in the difference spectrum extinction results when the polarity of the membrane environment is reduced indicating that buried aromatic amino acids are more exposed to the external polar media on bleaching. In addition, accurate visible absorption spectra of the purple membrane photocycle intermediates K, L, and M have been determined. The near-ultraviolet absorption and difference spectra of purple membrane and the K, L, and M intermediates have also been measured and indicate significant protein conformational change on conversion of purple membrane to L and M but not to K. Thus, it may be that the purple membrane photocycle effects the transfer of protons across the cell membrane by means of conformational changes in the purple membrane protein.

Materials and Methods

Cultures of *Halobacterium halobium* R_1 were grown and the purple membrane purified according to the procedures of Becher & Cassim (1975). The samples were buffered at pH 7.0 with a 0.02 M potassium phosphate buffer. Purple membrane samples were also placed in a buffered solution of 25% (w/w) NaCl with 1% Ammonyx LO detergent (65% dodecyldimethylamine oxide and 35% tetradecyldimethylamine oxide, Onyx Chemical Co.) to decrease light-scattering (Figure 1, curve 2).

A Cary 118 spectrophotometer was used to record all absorption spectra and difference spectra. Optically matched 0.2-cm pathlength quartz cells were used to record all absorption and difference spectra; these short pathlength cells were fixed within 2 cm of the photomultiplier tube in order to reduce light scattering artifacts by increasing the percentage of scattered light entering the photomultiplier.

The retinal chromophore of the purple membrane was removed by irradiating samples which were suspended in 0.5 M hydroxylamine titrated to pH 7.0 with 1 M NaOH immediately before sample irradiation. The suspensions were placed

† From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801. *Received October 12, 1977.* This work was supported by the National Institutes of Health (EY01323) and the National Science Foundation (PCM 76-82764). Brian Becher was the recipient of an Institutional National Research Service Award (EY07005) postdoctoral fellowship.

‡ Present address: Department of Biophysics, Kyoto University, Kyoto, Japan.

0006-2960/78/0417-2293$01.00/0