Ptitsyn, O. B., and Finkelstein, A. V. (1970), *Biofizika 15*, 757.
Robson, B., and Suzuki, E. (1976), *J. Mol. Biol. 107*, 327-356.
Schiffer, M., and Edmundson, A. B. (1967), *Biophys. J. 7*, 121-135.
Tanaka, S., and Scheraga, H. A. (1976), *Macromolecules 9*, 142-159.

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

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

ABSTRACT: Dihydrofolate reductase labeled with [*guanidino*-^{13}C]arginine has been purified from *Streptococcus faecium* and ^{13}C nuclear magnetic resonance spectra of the enzyme and its complexes with various ligands have been recorded. Resonances of the eight residues are resolved into 4 to 6 peaks with chemical shifts over a range of 1.2 ppm. There appear to be two classes of residues: those with chemical shifts very close to that of free [*guanidino*-^{13}C]arginine (class 1); and those with significantly different shifts (class 2). Spin-lattice relaxation times (T_1), measured in H_2O, for residues of class 1 are approximately 50% greater than the values for residues of the second class. In D_2O the T_1 values for both classes of residues are essentially the same and approximately twice the values obtained in H_2O for residues of class 1. The temperature-dependent behavior of T_1 for residues of class 2, together with the small nuclear Overhauser enhancement values, and the difference in line width in H_2O vs. D_2O are consistent with the assumption that the internal motion of these residues is slow relative to the overall rotational motion of the protein. An overall rotational correlation time for the protein of 20 ns has been estimated from the data for these immobilized residues. Class 1 residues appear to have a significant degree of internal motion and are probably accessible to solvent, whereas class 2 residues are probably inaccessible. Of the immobilized residues, one is not shifted by ligand binding. Another at lower field is shifted both by ligand binding and by temperature and is apparently monitoring conformational changes which are produced by the combined interactions of the 2′- and 5′-phosphate groups with the enzyme. The third immobilized residue is shifted by binding of NADPH, 2′,5′-ADP, or 2′-AMP and seems to be monitoring conformational changes which are triggered by interaction of the 2′-phosphate group with a basic side chain, and which are prevented by the nicotinamide ring of bound $NADP^+$ or by bound methotrexate. Some of the mobile residues are also affected by ligand binding, probably reflecting direct interaction of ligand with arginine residues.

Increasing use is being made of nuclear magnetic resonance (NMR) methods to study the structure and function of dihydrofolate reductase as well as other enzymes. Reports of studies using the magnetic resonance of ^{19}F (Kimber et al., 1977), ^{1}H (Birdsall et al., 1977; Feeney et al., 1977), and ^{13}C (Blakley et al., 1978; Cocco et al., 1977) have recently appeared. Although ^{19}F does prove to be a sensitive probe, the problem of possible structural alteration due to the introduction of fluorine-labeled residues into the protein must be considered. In ^{1}H studies extensive deuterium substitution must frequently be employed to simplify the spectrum, although certain resonances can be observed in unsubstituted proteins. The use of ^{13}C-labeled amino acids has neither of these disadvantages and the commercial availability of several ^{13}C-labeled amino acids makes this an attractive technique.

Dihydrofolate reductase is of interest in that it is the target of anti-folate drugs such as methotrexate (Blakley, 1969), but in addition its relatively low molecular weight also makes the catalytic mechanism of this NADPH-linked enzyme more amenable to investigation by NMR methods than is the case with most other dehydrogenases.

In this paper we report ^{13}C NMR data for dihydrofolate reductase of *Streptococcus faecium* obtained by in vivo incorporation of [*guanidino*-^{13}C]arginine. Spin-lattice relaxation data have been used to obtain information about the relative mobility of different arginine residues, and some tentative conclusions about the effects of ligand binding on arginine residues have been reached from ^{13}C NMR spectra of enzyme complexes.

Experimental Procedure

The experimental methods are as previously reported (Blakley et al., 1978) with the following exceptions.

Limiting Arg Medium. The Met limiting medium was modified as follows (quantities in g for 58 L of medium): [*guanidino*-^{13}C]arginine (90% enriched, KOR Isotopes, Cambridge, Mass.), 1.0; Met, 8.2.

Enzyme Preparation. Cells were grown on a 58-L scale and extracted as previously described, but the protamine sulfate treatment was omitted in order to avoid introduction of unla-

† 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 March 23, 1978.* 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 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 (N.A.M.) 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 during the period in which this work was carried out.

0006-2960/78/0417-4285$01.00/0

TABLE I: Chemical Shifts of the Guanidino Carbon of Arginine in Dihydrofolate Reductase.[a]

complex	peak position					
	1	2	3a	3b	3c	4
E	158.24 (1)	157.86 (1)		157.46 (2)	157.40 (3)	157.17 (1)
E·NADPH	158.10 (1)	157.84 (1)		157.51 (2)	157.38 (4)	
E·PADPR	158.13 (1)	157.85 (1)		157.50 (2)	157.39 (4)	
E·2′,5′-ADP	158.14 (1)	157.85 (1)		157.50 (2)	157.38 (4)	
2′-AMP	158.29 (1)	157.90 (1)		157.47	157.42	
3′,5′-ADP	158.29 (1)	157.90 (1)		157.47	157.42	157.28
E·NADH	158.17 (1)	157.85 (1)		157.46 (2)	157.38 (3)	157.16 (1)
E·NADP$^+$	158.11 (1)	157.81 (1)		157.48 (2)	157.40 (3)	157.18 (1)
E·DHF	158.29 (1)	157.84 (1)		157.45 (5)		157.16 (1)
E·folate	158.28 (1)	157.89 (1)		157.48 (4)	157.38 (1)	157.17 (1)
E·amin	158.40 (1)	157.85 (1)	157.52 (2)	157.45 (2)	157.33 (1)	157.20 (1)
E·NADPH·amin	158.30 (1)	157.84 (1)	157.55 (2)	157.48 (2)	157.33 (1)	157.20 (1)
E·PADPR·MTX	157.32 (1)	157.82 (1)	157.55 (2)	157.48 (2)	157.37 (2)	
denatured					157.38	

[a] Peak positions are given in ppm downfield from Me_4Si. The number in parentheses after the chemical shift value is the normalized integrated intensity estimated by a cut and weigh technique.

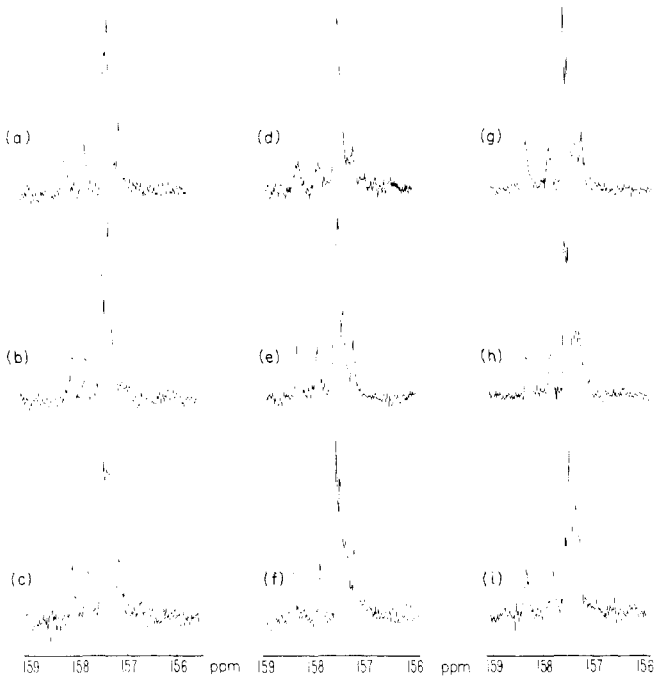

FIGURE 1: ^{13}C NMR spectra of [*guanidino*-^{13}C]arginine-labeled dihydrofolate reductase, alone (a), and in its complexes with NADPH (b), with NADP$^+$ (c), with dihydrofolate (d), with folate (e), with aminopterin (f), with NADPH and aminopterin (g), with NADPH and methotrexate (h), and with PADPR and methotrexate (i).

beled arginine in extraneous protein. Precipitation with ammonium sulfate was carried out as described by Nixon & Blakley (1968) and the remainder of the procedure was as described by Blakley et al. (1978). Solutions of extraneous proteins were dialyzed, concentrated by rotary evaporation, and acid hydrolyzed, and the labeled arginine was recovered from the hydrolysate. Approximately half of the arginine added to the medium was recovered without dilution of isotope.

Efficiency of Incorporation of Labeled Arginine. A 1-L culture of bacteria was grown on the limiting arginine medium containing [*guanidino*-^{14}C]arginine instead of [*guanidino*-^{13}C]arginine. The cells were broken by sonication and the dihydrofolate reductase was partially purified (specific activity ~20 units/mg). At least 85% of the arginine in the medium was incorporated into the bacterial protein. Comparison of the specific radioactivity of the arginine in the medium and that of a hydrolyzed sample of the reductase showed that there was less than 5% dilution of the label upon incorporation.

Spectra were recorded as previously described (Blakley et al., 1978) with the following exceptions. A sweep width of 250 Hz was employed for all spectra. Enzyme was present in 20% or 99% D_2O containing 50 mM potassium phosphate buffer, pH 7.3, and 0.5 M KCl. Samples of the enzyme complexes were prepared by adding sufficient ligand, calculated from the appropriate dissociation constants (Blakley et al., 1978), to convert at least 95% of the enzyme to the complex. Peak positions were determined by computer examination of the final Fourier transformed spectrum. All measurements unless otherwise noted were made at 15 °C. NOE[1] values were determined as previously described (Blakley et al., 1978).

Results

Chemical Shifts. Dihydrofolate reductase from *S. faecium* contains eight arginines and five resonances can be resolved in the proton-decoupled ^{13}C NMR spectrum of the uncomplexed enzyme (Cocco et al., 1977). A comparison of the proton-decoupled ^{13}C spectra obtained in D_2O (Figure 1) with those obtained in H_2O (Cocco et al., 1977) indicates significantly better resolution in the former, as expected (Oldfield et al., 1975a).

The chemical shifts of the guanidino carbons for the enzyme and the enzyme–ligand complexes in D_2O are collected in Table I. In the spectrum of the uncomplexed enzyme the resonances have a chemical shift range of 1.2 ppm and integrated intensities of 1:1:2:3:1 (in the order, low to high field). Resonances 3b and 3c have chemical shifts similar to that of free arginine, and when the protein is denatured with [^{12}C-99.999%] urea all the resonances collapse to a single line near the position of peak 3c.

The binding of NADPH to the enzyme markedly alters the appearance of the spectrum (Figure 1b), peak 1 shifting upfield and peaks 3b and 4 downfield. Nearly identical changes in the spectrum are caused by the binding of 2′-phosphoadenosine 5′-diphosphoribose (PADPR) and 2′,5′-ADP. The binding of 2′-AMP also results in the shift of peak 4 into peak 3, while 3′,5′-ADP causes peak 4 to shift about half as much. In both cases the remaining peaks are essentially unchanged. The

[1] Abbreviations used: PADPR, 2′-phosphoadenosine 5-diphosphoribose; amin, aminopterin; NOE, nuclear Overhauser enhancement.

TABLE II: Summary of T_1 Values (in s) of the Guanidino Carbon of Arginine Residues in Dihydrofolate Reductase at 15 °C.

	H_2O				D_2O			
	1	2	3[a]	4	1	2	3[a]	4
enzyme	0.43	0.45	0.65	0.44	1.04	1.14	1.28	1.13
enzyme-NADPH	0.43	0.43	0.62		1.02	1.02	1.09	
			0.62				1.02	
enzyme-methotrexate	0.38	0.43	0.62	0.37				
enzyme-NADPH-methotrexate					0.95	0.95	1.27	0.88
enzyme-NADPH-aminopterin	0.39	0.41	0.61					

[a] Poor resolution of the components of peak 3 prevented determination of separate T_1 values for these resonances.

TABLE III: Effect of Temperature on T_1 Values (in s) of the Guanidino Carbons of Arginine Residues in the Enzyme-NADPH-Methotrexate Complex in D_2O.

peak	5 °C	15 °C	25 °C
1	1.14	0.96	0.91
2	1.21	1.06	0.85
3	1.14	1.37	1.31
4		0.94	0.80

spectrum of the $NADP^+$ complex (Figure 1c) is intermediate in appearance between that of the uncomplexed enzyme and the NADPH binary complex, peaks 1 and 2 having the same chemical shift in the latter whereas the shift of peak 4 is the same as that in the uncomplexed enzyme. The binding of NADH produces almost no effect on the spectrum; peak 1 is shifted upfield slightly, while the remaining peaks are unchanged.

In the spectrum of the dihydrofolate complex, peak 3c is shifted downfield relative to its position in the spectrum of the uncomplexed enzyme and coalesces completely with peak 3b, while peak 1 is shifted downfield only slightly (Figure 1d). The effects produced by the binding of folate are very similar with one exception: the resonances of 2 of the 3 carbons contributing to peak 3c are shifted into peak 3b (Figure 1e). The binding of the inhibitor, aminopterin, gives spectral changes similar to those of folate but more pronounced (Figure 1f). Thus peak 1 is shifted downfield and 2 of the 3 carbons contributing to peak 3c are shifted to position 3a.

In the ternary complex enzyme-NADPH-aminopterin, it appears that the binding of aminopterin reverses some of the effects of NADPH binding (Figure 1g) so that the spectrum resembles that of the uncomplexed enzyme. Peak 1 is shifted downfield (relative to the enzyme-NADPH spectrum) and peak 4 has been shifted upfield away from peak 3c. The spectrum of the ternary complex enzyme-PADPR-aminopterin is nearly identical with that of enzyme-NADPH-aminopterin, except that peak 4 has not been shifted upfield (Figure 1i).

Relaxation Behavior. Spin-lattice relaxation times were measured in both H_2O and D_2O for the uncomplexed enzyme, the enzyme-NADPH complex, and for the enzyme-NADPH-methotrexate complex (D_2O) or the enzyme-NADPH-aminopterin complex (H_2O), Table II. In H_2O peak 3 has a T_1 longer than those for the remaining peaks. In D_2O differences in the T_1 values are less pronounced except for peak 3 in the enzyme-NADPH-methotrexate complex, which has a slightly longer T_1.

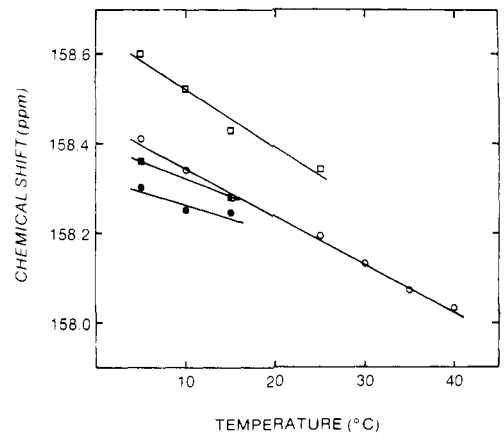

FIGURE 2: Temperature dependence of peak 1 in the ^{13}C NMR spectrum of dihydrofolate reductase (●) and in spectra of its complexes with folate (■), with methotrexate (□), and with NADPH and methotrexate (○).

T_1 values for the enzyme-NADPH-methotrexate complex were measured at several temperatures (Table III). Two trends are evident: the T_1 for peak 3 increases with increasing temperature, while the values for the remaining peaks decrease.

An important consideration in the interpretation of the proton decoupled ^{13}C spectra is the possibility of different NOE values for the different arginine residues. The temperature dependence of T_1 for the resolved peaks 1, 2, and 4 is consistent with complete immobilization of the corresponding arginine residues in a slowly tumbling enzyme with a rotational correlation time of 2×10^{-8} s (see below), parameters which are consistent with a negligible NOE (Doddrell et al., 1972). NOE values were determined by comparing the intensities of proton coupled and decoupled spectra, and by doing a pulsed NOE experiment as described previously (Blakley et al., 1978). The latter was performed on the 80% H_2O–20% D_2O sample so that a delay of 4 s gave a delay/T_1 ratio of ~10 for peak 1, and ~6 for peak 3. NOE values thus determined are 1.13 for peak 1 and an average value of 1.26 for the remaining peaks. Values in D_2O are expected to be less than or equal to those determined in H_2O, thus confirming the above conclusions. The integrated intensity of peak 3 corresponds to five times that of any of the resolved peaks, indicating a negligible NOE for the peak 3 resonances also.

Temperature Dependence of the Chemical Shifts. Spectra at several temperatures were obtained for the uncomplexed enzyme and for the enzyme-folate, enzyme-methotrexate, and enzyme-NADPH-methotrexate complexes. In all cases, resonance 1 shifts toward the large central envelope at higher temperature and is the only resonance to exhibit any significant temperature dependence (Figure 2).

Discussion

The range of chemical shifts observed for the guanidino carbons in arginine-labeled dihydrofolate reductase is somewhat larger than that observed in other proteins (Oldfield et al., 1975b). The arginine residues in the reductase can be divided into two classes based on their chemical shifts: the first class gives rise to the components of peak 3 (Table I), while peaks 1, 2, and 4 are produced by residues of the second class. Residues of the first class appear to be solvent-accessible since their chemical shifts are close to those for free arginine and for arginine in the denatured enzyme. On the other hand, those resonances outside the central envelope correspond to residues

TABLE IV: Summary of Line Widths[a] (in Hz) of the Resolved Guanidino Carbons of Arginine Residues in Dihydrofolate Reductase.

	H_2O			D_2O		
	1	2	4	1	2	4
enzyme	3.8	5.1	3.8	3.1	1.8	2.0
enzyme-aminopterin	3.8	4.6	3.8	2.8	2.0	2.0
enzyme-aminopterin-NADP-H	4.0	3.8	4.0	2.0	2.0	1.8

[a] Estimated error is ±25%.

subject to significant environmental perturbations such as proximity to the ring currents of aromatic residues, and possibly involvement in salt bridges, hydrogen-bonding interactions, as well as inaccessibility to solvent. Further, as noted below, this class of resonances exhibits significantly different spin-lattice relaxation rates consistent with greater immobilization in the protein compared with the residues corresponding to peak 3. It is possible that the same factors which immobilize these residues produce the chemical shift perturbations.

Spin-Lattice Relaxation Times. A significant feature of the T_1 values obtained for peaks 1, 2, and 4 is the inverse temperature dependence (Table III). This behavior, coupled with the very small NOE values and the line widths shown in Table IV, indicates that the motion of these residues falls into the slow tumbling range. It should be noted that this conclusion is valid regardless of the relative importance of dipolar interactions with ^1H, ^{14}N, or ^2H which may all be significant for the arginine guanidino carbon (Oldfield et al., 1975a) since the position of the T_1 minimum as a function of τ is similar in all cases. The T_1 values for peaks 1, 2, and 4 can therefore be used to calculate the rotational correlation time of the enzyme. One approach is to measure the difference in relaxation rates observed in D_2O and H_2O which can be attributed primarily to dipolar interaction with the five exchangeable guanidino protons. Although intermolecular dipolar interactions with solvent may also contribute, this effect is negligible due to the shorter correlation time for this interaction. The rate attributable to the exchangeable guanidino protons, $1/T_{1\text{eff}}$, is related to the measured values by

$$\frac{1}{T_{1\text{eff}}} = \frac{1}{T_{1,H_2O}} - \frac{1}{T_{1,D_2O}}$$

This rate can then be related to the rotational correlation time using the relation:

$$\frac{1}{T_{1\text{eff}}} = \frac{5\gamma_C^2\gamma_H^2\hbar^2}{10r_{CH}^6}[J(\omega_C - \omega_H) + 3J(\omega_C) + 6J(\omega_C + \omega_H)]$$

where $r_{CH} = 2.05$ Å for each of the five guanidino protons. When the above approach is applied to peaks 1, 2, and 4, it leads to values of 0.66–0.74 s for $T_{1\text{eff}}$. Due to the double-valued nature of the T_1 curve as a function of τ, these values lead to τ values of about 7×10^{-10} or 2×10^{-8} s.[2] One means of choosing between these values is based on a comparison of the line widths obtained in H_2O and in D_2O (Table IV). Using an approach analogous to that employed in T_1, above,[3] a contribution due to 4 exchangeable protons is calculated to be ~0.4 Hz for the shorter correlation time, and 2.7 Hz for the longer one. The mean observed line-width difference of 1.9 Hz (Table IV) is thus more consistent with a correlation time of 2×10^{-8} s. This value is similar to that observed for a number of proteins with molecular weights close to that of dihydrofolate reductase (mol wt = 20 000: Allerhand et al., 1973; Browne et al., 1973; Hunkapillar et al., 1973; Cozzone et al., 1975; Vischer & Gurd, 1975).

In contrast to the data for peaks 1, 2, and 4, the longer T_1 values and the complex temperature dependence for the T_1 of peak 3 imply significant internal motion, so that several correlation times are needed to provide a minimal description of the motion. Furthermore, the marked T_1 increase between 5 and 25 °C is consistent with some motion in the extreme narrowing limit. Oldfield et al. (1975a) have concluded that the arginine resonances of hen egg white lysozyme exhibit a considerable degree of internal motion. The T_1 values observed in that case cover the range observed in dihydrofolate reductase but consist primarily of values similar to those of peak 3 in the present study.

Finally, we note that changes in T_1 coincident with the binding of ligands are negligible for the H_2O solutions and generally within experimental error for the D_2O solutions (±15%). Although the T_1 values for peak 3 are somewhat reduced in the enzyme–NADPH complex in D_2O, six residues including peak 4 contribute to this resonance making specific conclusions on the possible effects of ligands impossible.

Temperature Dependence of the Chemical Shifts. The temperature dependence of the peak 1 chemical shift (Figure 2) is remarkable given the relatively large shift (0.4 ppm) observed over a range of only 35 °C. Although the data are insufficient for the determination of hydrodynamic parameters describing the effect, some evaluation is possible subject to a simplifying assumption. In particular, if the data are assumed to reflect equilibrium between two stable conformational states, A and B, thermodynamic parameters can be calculated if the limiting shifts corresponding to both states are known. However, it is not possible to lower the temperature sufficiently to observe the system completely in state A (because of freezing) or to raise the temperature enough to obtain the system completely in state B (because of denaturation). Since at high temperature the resonance appears to move toward the position of peak 3, i.e., the denatured resonance shift, the most likely model is that at low temperature the shift is perturbed by some residue interaction in state A and that in the high temperature state, B, this interaction is eliminated. Taking the shift for state B equal to that of the denatured enzyme, we have then estimated that the shift of peak 1 in state A is approximately 2 ppm downfield. This is a reasonable order of magnitude given the typical perturbations and the total arginine chemical shift range observed. A fit of the temperature dependent data then leads to the parameters $\Delta H = 5$ kcal/mol and $\Delta S = 1.9 \times 10^{-2}$ kcal/(mol deg). The significant result is the rough equality of ΔH and $T\Delta S$. If, on the other hand, the ΔS term is neglected, it becomes necessary to assume that the shift difference $\Delta\nu = \nu_A - \nu_B$ is ≥12 ppm—clearly an un-

[2] Since the "H_2O" sample actually contains 20% D_2O, there are only 4 exchangeable protons, and, if the calculation is corrected for this, the value of τ_c is decreased. On the other hand, correction for the viscosity difference between H_2O and D_2O based on the assumption that $T_1 \propto \tau \propto \eta$ in the slow tumbling limit increases the value of $T_{1\text{eff}}$ and almost exactly offsets the former correction. Thus the uncorreeted calculation gives the same value of τ within experimental error as the corrected calculation.

[3] In contrast to the T_1 viscosity correction, the assumption that $\nu \propto 1/T_2 \propto \tau \propto \eta$ implies an increased line width in D_2O. The value of 2.7 Hz given in the text includes the effect of the 4 exchangeable protons. The viscosity correction will further reduce this value, making it closer to the measured mean of 1.9 Hz.

reasonable assumption.

Ligand-Induced Changes in Chemical Shift. The relatively large displacements of peaks 1, 2, and 4 (solvent-inaccessible residues) from the position for denatured enzyme are most likely due to charged residues or ring currents in aromatic residues which are in close proximity to the corresponding arginine side chains. Salt linkages presumably contribute to the immobilization of the arginine residues in this microenvironment. Peak 2 is essentially unaffected by addition of any ligand yet studied, so that the microenvironment of this residue is unaffected by whatever conformational changes are induced in the enzyme as ligands bind and is therefore probably remote from the active site. Peak 1 is the most interesting one of this class, shifting upfield upon complexation of the enzyme with NADPH, downfield upon complexation of inhibitor, and being the only peak for which the chemical shift is temperature dependent. Since the rate of change of chemical shift with temperature is essentially the same for free enzyme and for the enzyme–methotrexate, enzyme–folate, and enzyme–NADPH–methotrexate complexes, it seems likely that the position of peak 1 is monitoring a conformational change. This is consistent with the presence of a charged residue or an aromatic ring in the vicinity of this side chain, since the chemical shift should be sensitive to small changes in the position of an arginine residue with respect to these residues.

Peak 4 is sensitive to the binding of NADPH, which shifts this resonance downfield so that it coalesces with peak 3c. Since 2′,5′-ADP and PADPR also produce this downfield shift, it is caused by the interaction of the ADP moiety of NADPH with the enzyme. When a series of related nucleotides was investigated, it was found that 2′-AMP, 3′,5′-ADP, and NADH were all weak inhibitors, competitive with NADPH, with K_i values of 2.75, 0.76, and ~1 mM, respectively. They are therefore all capable of binding at the NADPH site, but the relative weakness of the binding compared with 2′,5′-ADP indicates that both the phosphate groups of the latter interact strongly with the enzyme, presumably by electrostatic bonds to arginine or lysine residues. The association constants of 2′-AMP, 3′-,5′-ADP, and NADH are sufficiently great that the enzyme can be converted almost quantitatively to the nucleotide complexes at suitable nucleotide concentrations. When the NMR spectra of these complexes were examined, 2′-AMP was found to shift peak 4 into peak 3, in the same way as 2′,5′-ADP, PADPR, and NADPH, so that it is specifically the interaction of the 2′-phosphate with the enzyme that perturbs the peak 4 arginine. This is further substantiated by the spectrum of the 3′,5′-ADP complex where peak 4 is shifted only part of the way downfield toward peak 3. Apparently the 3′-phosphate is near enough to the position occupied by the 2′-phosphate to produce a partial effect similar to the 2′-phosphate. Neither 2′-AMP nor 3′,5′-ADP produce the upfield movement of peak 1 that is caused by 2′,5′-ADP, PADPR, or NADPH, so that both the 2′- and 5′-phosphates are necessary for the conformational change induced by NADPH binding.

Although the position of peak 4 is clearly affected by the presence on the enzyme of a ligand with a 2′-phosphate, it is unlikely that the 2′-phosphate group interacts *directly* with the peak 4 arginine. This is indicated by the observation that the enzyme–$NADP^+$ complex does not show the downfield shift of peak 4; yet the 2′-phosphate groups of $NADP^+$ and NADPH interact with the enzyme in the same way. Evidence for the latter was obtained by Feeney et al. (1975) who showed that the chemical shift of the 2′-phosphate is the same in the ^{31}P NMR spectra of the binary complexes of $NADP^+$, NADPH, and 2′-AMP. A more likely interpretation is that the 2′-phosphate interacts with a lysine residue or one of the peak 3 arginines (causing a very small change in its chemical shift) but that this interaction in turn causes a conformation change that affects a rather large part of the active site and adjacent areas. It is this conformation change which causes the changes in position of peak 1, peak 4, and perhaps some of the individual resonances underlying peak 3. Examples of such a widespread conformation change resulting from the binding of a single ligand group can be found in the literature. Thus the binding of a glycyltyrosine to carboxypeptidase A and particularly the interaction of its terminal carboxylate group with Arg-145 cause significant movement of a considerable portion of the peptide backbone, a large (12 Å) movement of the side chain of Tyr-248 and significant movements of the side chains of Arg-145 and Glu-270 (Hartsuck & Lipscomb, 1971).

The presence of the fully unsaturated nicotinamide ring in $NADP^+$ must produce some additional conformational effects, one result of which is to nullify the effect of the 2′-phosphate-induced conformational transition on the peak 4 arginine. Other evidence for differences in the interaction of the nicotinamide rings of $NADP^+$ and NADPH with the enzyme has been obtained from NMR studies of reductase labeled with [*methyl*-^{13}C]methionine (Blakley et al., 1978). Similarly, the conformational effects of methotrexate binding must also prevent the conformational transition caused by the 2′-phosphate group from affecting peak 4 arginine in the usual way, since there is no downfield shift of peak 4 in the spectrum of the enzyme–NADPH–methotrexate complex.

The small displacements of resonances in peak 3 from the position of the denatured enzyme resonance are perhaps explicable in terms of salt linkages with other residues, although other effects, such as ring currents of adjacent aromatic residues, cannot be ruled out. The positions of resonances within peak 3 are changed by the binding of either the nucleotides or dihydrofolate, folate or aminopterin. There is presently no direct evidence as to whether these peak movements are caused by direct interaction of ligand residues with the corresponding arginine side chains, or to the conformational transitions induced by ligand binding, or both. However, it has been argued above that some (or all) of these arginines of peak 3 are accessible to the solvent, so that any surface arginines which interact with ligands probably have resonances in this group. At least some of the changes occurring on ligand binding may therefore be the result of direct interaction between ligand and arginine side chains.

Tentative Peak Assignments. The *S. faecium* reductase has eight arginine residues which occur at sequence positions 23, 29, 32, 44, 58, 102, 116, and 156 (Gleisner et al., 1975; Peterson et al., 1975). Two of these residues (Arg-44 and Arg-58) are conserved in three other dihydrofolate sequences (Stone et al., 1977; Bitar et al., 1977; Stone & Phillips, 1977). Arg-32 is conserved in the L1210 and *L. casei* sequences, but it is not clear whether Lys-32 or Arg-33 is the functionally corresponding residue in the *E. coli* sequence. Matthews et al. (1977) have reported data derived from X-ray crystallography on the three-dimensional structure of the methotrexate complex of *E. coli* dihydrofolate reductase. From examination of their results they conclude that Arg-57 is hydrogen bonded to the α-carboxyl group of the glutamic acid moiety of methotrexate. In one of the two complexes within the asymmetric unit, the γ-carboxyl group may be hydrogen bonded to Lys-32, though in the other complex it is hydrogen bonded to an exterior water molecule. It is possible that the corresponding residues in the sequences of *S. faecium* reductase are Arg-32 and Arg-58 and these two residues may be involved in binding the carboxyl groups of methotrexate, folate, and dihydrofolate

to the *S. faecium* reductase.

Which of the NMR peaks in Figure 1 correspond to arginine residues that are hydrogen bonded to the carboxyls of the bound substrate or inhibitor? The binding of folate or aminopterin shifts two of the three carbon resonances in peak 3c downfield into peak 3b. It has been argued above that these resonances correspond to solvent-accessible, mobile residues and they should therefore be able to hydrogen bond with substrate carboxyl groups. Consequently, it seems not unreasonable to conclude that the two resonances of peak 3c which are displaced by folate and aminopterin binding correspond to Arg-32 and Arg-58, the presumptive carboxyl sites. So far as one can tell from the backbone diagram provided by Matthews et al. (1977), Arg-32 and Arg-58 would be solvent-accessible and mobile as required by this hypothesis. When dihydrofolate binds to the reductase of *S. faecium*, all three resonances of peak 3c move downfield. Presumably the displacement of the third resonance in this case results from a conformational change.

Matthews et al. (1977) deduced from a comparison of the $(\beta\alpha\beta)_2$ fold of *E. coli* dihydrofolate reductase with the $(\beta\alpha\beta)_2$ fold of lactate dehydrogenase that Arg-44 of the reductase is likely to be the residue that hydrogen bonds to the 2′-phosphate of NADPH. This is one of the conserved arginine residues in the reductase sequence. Since the binding of NADPH, 2′,5′-ADP, 2′-phosphoadenosine 5′-diphosphoribose, and (to a smaller extent) $NADP^+$ to the *S. faecium* reductase causes a downfield shift of peak 3b (a two-carbon resonance), it seems likely that the resonance corresponding to Arg-44 lies within peak 3b.

Acknowledgment

We wish to thank Greg Milleville for his assistance in determining limiting concentrations for the defined bacterial medium and Greg Thorgaard for his assistance in determining several binding constants needed for this study.

References

Allerhand, A., Childers, R. F., & Oldfield, E. (1973) *Biochemistry 12,* 1335–1341.

Birdsall, B., Griffiths, D. V., Roberts, G. C. K., Feeney, J., & Burgen, A. (1977) *Proc. R. Soc. London, Ser. B 196,* 251–265.

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

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines,* Chapter 5, North-Holland Publishing Co., Amsterdam.

Blakley, R. L., Cocco, L., London, R. E., Walker, T. E., & Matwiyoff, N. A. (1978) *Biochemistry 17,* 2284–2293.

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

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

Cozzone, P. T., Opella, S. J., Jardetzky, O., Berthon, J., & Jolles, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 2095–2098.

Doddrell, D., Glushko, V., & Allerhand, A. (1972) *J. Chem. Phys. 56,* 3683–3689.

Feeney, J., Birdsall, B., Roberts, G. C. K., & Burgen, A. S. V. (1975) *Nature (London) 257,* 564–566.

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

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

Hartsuck, J. A., & Lipscomb, W. N. (1971) *Enzymes, 3rd Ed. 3,* 1–56.

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

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

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

Nixon, P. F., & Blakley, R. L. (1968) *J. Biol. Chem. 250,* 4722–4733.

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

Oldfield, E., Norton, R. S., & Allerhand, A. (1975b) *J. Biol. Chem. 250,* 6381–6402.

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

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

Stone, D., Phillips, A. W., & Burchall, J. J. (1977) *Eur. J. Biochem. 72,* 613–624.

Visscher, R. B., & Gurd, F. R. N. (1975) *J. Biol. Chem. 250,* 2238–2242.