Nuclear Magnetic Resonance Studies of the Binding of Substrate Analogs and Coenzyme to Dihydrofolate Reductase from *Lactobacillus casei*†

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ABSTRACT: The interactions of the coenzyme, NADPH, and of *p*-aminobenzoyl-L-glutamate, a fragment of the substrate, with *Lactobacillus casei* dihydrofolate reductase have been studied by ^1H nuclear magnetic resonance spectroscopy. The aromatic proton resonances of *p*-aminobenzoyl-L-glutamate shift upfield in the presence of the enzyme by 0.41 and 0.58 ppm (protons ortho and meta to the glutamate moiety, respectively); the binding constant is 1.05×10^3 M^{-1}. Similar chemical-shift changes on binding are observed with *p*-nitrobenzoyl-L-glutamate, but with *p*-aminobenzoyl-D-glutamate, no changes in chemical shift of the aromatic protons are observed, although it binds to the same site with a binding constant of 0.34×10^3 M^{-1}. In the presence of NADPH, all three compounds bind approximately 3.5-fold more tightly, and the shifts of the aromatic protons of *p*-aminobenzoyl- and *p*-nitrobenzoyl-L-glutamate are somewhat smaller. All three compounds produce significant changes in the histidine C-2-H, aromatic, and methyl regions of the nmr spectrum of the protein. The changes in the histidine resonances are particularly marked, at least three and probably four of the six histidine residues being affected in chemical shift and/or pK by the binding of *p*-aminobenzoyl-L-glutamate. The binding of NADPH, which is shown to have a stoichiometry of 1 mol of NADPH/mol of enzyme, produces substantial changes throughout the nmr spectrum of the protein, notably the appearance of three additional methyl resonances upfield of −4 ppm (from dioxane). The changes produced in the aromatic region on adding *p*-nitrobenzoyl-L-glutamate to the enzyme–NADPH complex are very similar to those observed on addition to the enzyme alone, but the changes in the methyl region are quite different.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP oxidoreductase, EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate. This reaction is of considerable importance in one-carbon metabolism, notably in thymidylate biosynthesis. Dihydrofolate reductases are also the target of a potent and important group of inhibitors of considerable chemotherapeutic interest (Blakley, 1969; Hitchings and Burchall, 1965). In order to improve our understanding of the mode of action of these folate antagonists at the molecular level, we are undertaking a detailed study of ligand binding to dihydrofolate reductase from a methotrexate-resistant strain of *Lactobacillus casei*.

We have begun by studying the binding of *p*-aminobenzoyl-L-glutamate (NH_2BzGlu),[1] a fragment of the substrate molecule, by high-resolution nmr spectroscopy. Both dihydrofolate and inhibitors such as methotrexate bind very tightly to dihydrofolate reductase ($K_a \simeq 10^8$–10^{10} M^{-1}) and are thus in slow exchange on the nmr time scale. Fragments such as L-NH_2BzGlu, however, bind much more weakly, are in rapid exchange, and can thus be studied much more conveniently. The low molecular weight of *L. casei* dihydrofolate reductase (17,500; Dann *et al.*, 1974) has made it possible to study the nmr spectrum of the protein as well as the ligand in some detail.

Materials and Methods

Enzyme. The isolation and purification of dihydrofolate reductase from *L. casei* MTX/R has been described elsewhere (Harding *et al.*, 1974; Dann *et al.*, 1974). The purified enzyme was homogeneous by the criteria of isoelectric focusing, sodium dodecyl sulfate gel electrophoresis, and gel chromatography (Dann *et al.*, 1974). The enzyme was obtained from the final step in the purification as a solution in 10 mM potassium phosphate–100 mM KCl (pH 6.5). This solution was lyophilized and stored at −10° until required.

Other Materials. NADPH, L-NH_2BzGlu and -NO_2BzGlu were obtained from the Sigma Chemical Co. D-NH_2BzGlu was prepared by hydrogenation of *p*-nitrobenzoyl-D-glutamate (prepared from D-glutamic acid and *p*-nitrobenzoyl chloride; Landsteiner and Van der Scheer, 1934; Van der Scheer and Landsteiner, 1935) using a Raney-nickel catalyst. D-NH_2BzGlu was isolated by crystallization from water at pH 3.0–3.5 and characterized by nmr spectroscopy. D_2O (99.85 atom % D) was from Norsk Hydroelektrisk, and DCl and NaOD (both >99 atom % D) from CIBA (ARL) Ltd. All other chemicals were of reagent grade.

Enzyme concentration was determined by spectrophotometric assay and by fluorimetric titration with methotrexate under conditions of stoichiometric binding (for details see Dann *et al.*, 1974).

Nmr Spectroscopy. The enzyme was lyophilized twice from D_2O to remove exchangeable protons, and then dissolved in a volume of D_2O equivalent to one-fifth of that of the original solution, giving an enzyme concentration of 0.8–1.4 mM and a salt concentration of 50 mM potassium phosphate–500 mM KCl, readjusted to a pH (meter reading) of 6.5. All ligands were dissolved in a D_2O buffer solu-

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[1] Abbreviations used are: nmr, nuclear magnetic resonance; L- and D-NH_2BzGlu, *p*-aminobenzoyl-L-(or D-)glutamate; L-NO_2BzGlu, *p*-nitrobenzoyl-L-glutamate.

tion of the same concentration, and volumes of up to 50 μl added to 2.0 ml of enzyme solution, using Hamilton microsyringes.

^{1}H nmr spectra (100 MHz) were obtained using a Varian XL-100-15 spectrometer equipped with Fourier transform facilities controlled by a VDM 620*i* computer. For most experiments 500 transients were accumulated with an acquisition time of 1.00 sec, and the free induction decay was multiplied before Fourier transformation by an exponential weighting function with a time constant of 0.5–0.7 sec. In all experiments the large resonance due to the residual water protons was irradiated selectively and continuously with a second radiofrequency field to eliminate the dynamic range problem associated with large solvent resonances. The field-frequency lock of the spectrometer was obtained from the deuterium in the solvent. Spectra (270 MHz) were obtained under closely similar conditions (but without irradiation of the water peak) on the instrument of the Oxford Enzyme group. All samples contained 3 mM dioxane as a chemical-shift reference (the dioxane resonance is 3.71 ppm downfield from dimethylsilapentane-5-sulfonate). The sample temperature (±0.5°) for each experiment is indicated in the appropriate figure caption.

Data Analysis. In the equilibrium

$$E + I \rightleftharpoons EI$$

if the rate of exchange of the ligand (I) between the free and bound states is rapid (that is, if the lifetime $\tau \ll 1/2\pi\Delta\nu$, where $\Delta\nu$ (hertz) is the chemical-shift difference between the two states) then a single, averaged, resonance is observed, whose chemical shift is given by

$$\delta_{obsd} = \frac{[EI]}{I_t}\delta_{EI} + \frac{[I]}{I_t}\delta_I \quad (1)$$

where [EI] and [I] represent the concentrations of complex and free ligand, respectively, $I_t = [EI] + [I]$, and δ_{EI} and δ_I are the chemical shifts of a ligand nucleus in the complex and in the free ligand, respectively. Equation 1 can be rearranged to give

$$\frac{[EI]}{I_t} = \frac{(\delta_{obsd} - \delta_I)}{(\delta_{EI} - \delta_I)} \quad (2)$$

In addition we can define an association constant

$$K_a = [EI]/[I][E] \quad (3)$$

Most previous nmr experiments of this sort have been carried out under conditions such that $I_t \gg E_t$ and therefore $[EI] \ll [I]$ and $[I] \simeq I_t$. This allows a convenient linearization of eq 2 and 3 to give

$$\frac{1}{(\delta_{obsd} - \delta_I)} = \left\{\frac{1}{K_a} + I_t\right\}\frac{1}{E_t(\delta_{EI} - \delta_I)} \quad (4)$$

so that the parameters of interest, δ_{EI} and K_a, can be determined from a plot of $1/(\delta_{obsd} - \delta_I)$ against I_t. However, the condition $I_t \gg E_t$ implies that the *observed* change in chemical shift, $|\delta_{obsd} - \delta_I|$, is much less than the total change, $|\delta_{EI} - \delta_I|$; in many cases only 5–10% of the total change is observed. Thus, a considerable extrapolation is required to obtain δ_{EI}, and the value is consequently imprecise. In the present experiments ratios of I_t/E_t have been used such that up to 40% of the ligand is bound to the enzyme (at still lower ratios, the resonances of the ligand are obscured by those of the protein). Although these experimental conditions lead to much more precise estimates of δ_{EI} and K_a, they do not allow the linearization of eq 2 and 3 to give eq 4. Consequently, we have used the iterative method introduced by Nakano *et al.* (1967). Equations 2 and 3 can be combined to give

$$\frac{E_t}{\delta_{obsd} - \delta_I} = \frac{1}{\delta_{EI} - \delta_I}(I_t + E_t - [EI]) + \frac{1}{K_a(\delta_{EI} - \delta_I)} \quad (5)$$

This equation forms the basis of an iterative procedure in which $E_t/(\delta_{obsd} - \delta_I)$ is initially plotted against $(I_t + E_t)$, giving an approximate value for $(\delta_{EI} - \delta_I)$. From this, an estimate of [EI] is obtained (using eq 2), and the data are replotted against $(I_t + E_t - [EI])$. The iteration is continued until two successive replots yield essentially the same line. This procedure has been programmed for a VDM 620-L computer; at each stage in the iteration a linear least-squares fit of the data was performed. The iteration was terminated when successive estimates of K_a, $(\delta_{EI} - \delta_I)$, and the variance of the data points about the line changed by less than 0.5%.

Since the plot used in eq 5 (like that in eq 4) involves the reciprocal of the observed shift, the data will be weighted in a way unrelated to their actual precision. Therefore, the estimates of δ_{EI} and K_a obtained from the procedure outlined above were used as initial input to a program which refined these estimates iteratively by comparison with the data in the simple δ_{obsd} *vs.* I_t form (again using the least-squares criterion). The second procedure led to relatively small changes in the estimates of δ_{EI} and K_a, but substantial reductions in their standard errors.

pH titration curves were analyzed in a somewhat analogous manner, as described by King and Roberts (1971).

Competition Experiments. The binding constant of D-NH_2BzGlu was measured by competition with L-NH_2BzGlu or L-NO_2BzGlu. Two types of competition experiments were used. In the first, the binding curve of the "indicator ligand" (L-NH_2BzGlu or L-NO_2BzGlu) was obtained in the presence of a fixed concentration of D-NH_2BzGlu, while in the second a fixed concentration of "indicator ligand" was progressively displaced from the enzyme by increasing concentrations of D-NH_2BzGlu. Both methods gave the same value for the binding constant of D-NH_2BzGlu, but since they depend on an accurate knowledge of the binding parameters of the "indicator ligand," the values are less accurate than those determined directly.

Results

Changes in Ligand Spectra. The aromatic protons of L-NH_2BzGlu form an AA′BB′ spin system, giving rise to a deceptively simple spectrum consisting of two doublet resonances at 3.11 ppm (protons ortho to the amino group) and 3.92 ppm (protons meta to the amino group) from the dioxane reference. In the presence of dihydrofolate reductase, both resonances are shifted upfield (Figure 1). The dependence of the observed shift on L-NH_2BzGlu concentration, at constant enzyme concentration, is shown in Figure 2. Data from experiments of this type can be analyzed to give values of Δ (where $\Delta = (\delta_{EI} - \delta_I)$, the difference in chemical shift of the protons between free L-NH_2BzGlu and the complex) and K_a (the binding constant), as described in the Materials and Methods section; the values obtained are given in Table I.

The excellent fit of the data to the theoretical line (Figure 2) is presumptive evidence that L-NH_2BzGlu binds to a single site on the enzyme. Addition of methotrexate at a molar concentration equal to 1.05 times that of the enzyme

TABLE I

Compd	Without NADPH			With NADPH		
	$10^{-3}K_a$[a] M^{-1}	Δ[b] (ppm) Ortho	Meta	$10^{-3}K_a$[a] M^{-1}	Δ[b] (ppm) Ortho	Meta
L-NH_2BzGlu	1.05 ± 0.15	−0.41 ± 0.02	−0.58 ± 0.02	3.57 ± 0.35	−0.30 ± 0.02	−0.35 ± 0.02
L-NO_2BzGlu	0.47 ± 0.06	−0.36 ± 0.02	−0.67 ± 0.02	1.51 ± 0.02	−0.31 ± 0.02	−0.45 ± 0.02
D-NH_2BzGlu	0.34 ± 0.08	<0.05	<0.05	1.4 ± 0.3	<0.05	<0.05

[a] Binding constant. [b] $\Delta = \delta_{EI} - \delta_I$. Values are given for the protons ortho and meta to the glutamate moiety. Negative values denote upfield shifts.

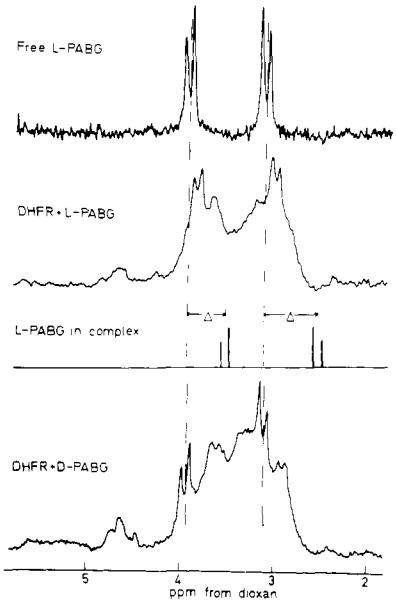

FIGURE 1: The aromatic region of the 100-MHz ^1H nmr spectra of, top to bottom, 5 mM L-NH_2BzGlu, 4.5 mM L-NH_2BzGlu + 1.38 mM dihydrofolate reductase, and 4.5 mM D-NH_2BzGlu + 1.38 mM dihydrofolate reductase. The positions of the resonances of L-NH_2BzGlu in the fully-formed complex (calculated from data such as that in Figure 2) are shown diagrammatically, and the definition of Δ ($=\delta_{EI} - \delta_I$) is indicated; sample temperature, 20 ± 0.5°.

caused the aromatic resonances of L-NH_2BzGlu to shift back to within less than 0.5 Hz of their position in the absence of enzyme. This indicates that L-NH_2BzGlu binds to a single site, overlapping the methotrexate binding site.[2]

We can therefore tentatively conclude that L-NH_2BzGlu binds to the same site as the corresponding moiety of methotrexate (and, presumably, of the substrate). L-NH_2BzGlu also binds to the binary dihydrofolate reductase · NADPH complex to give a ternary complex; chemical-shift data are shown in Figure 2, and the derived values of Δ and K_a are in Table I. The binding constant is approximately 3.5-fold higher than that in the absence of NADPH. Furthermore, the changes in chemical shift are smaller, showing that the magnetic environment of the aromatic ring of L-NH_2BzGlu is significantly different in the binary and ternary complexes. Additional evidence that L-NH_2BzGlu binds at the active site comes from the observation (J. Dann and G. C. K. Roberts, unpublished results) that the binding constant of L-NH_2BzGlu determined from its inhibition of enzyme activity is essentially identical with that found by nmr for the reductase · NADPH complex.

The aromatic resonances of *p*-nitrobenzoyl-L-glutamate (L-NO_2BzGlu) show very similar changes to those of L-NH_2BzGlu on formation of the binary and ternary complexes; the binding is about a factor of two weaker than for L-NH_2BzGlu in both cases (Table I).

The behavior of the D isomer of NH_2BzGlu is, however, quite different. D-NH_2BzGlu binds to a site which is closely similar to that for L-NH_2BzGlu, as indicated by the findings that (a) D-NH_2BzGlu inhibits enzyme activity (J. Dann and G. C. K. Roberts, unpublished results), (b) both L- and D-NH_2BzGlu produce very similar changes in the protein spectrum (see below), and (c) D-NH_2BzGlu binds competitively with L-NH_2BzGlu and L-NO_2BzGlu. However, addition of reductase to a solution of D-NH_2BzGlu produces no observable shifts of the aromatic resonances of the inhibitor (Figures 1 and 3; an upper limit to Δ of 0.05 ppm can be calculated). Thus, the orientations of the aromatic rings of L- and D-NH_2BzGlu when bound to the enzyme must be quite different, insofar as the aromatic protons experience markedly different magnetic environments. On the other hand, the binding constant of D-NH_2BzGlu (Table I), determined by competition with L-NH_2BzGlu or

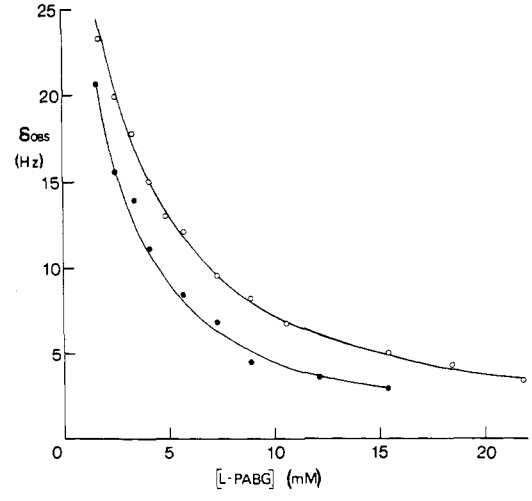

FIGURE 2: The dependence of the chemical shift of the resonance of the meta protons of L-NH_2BzGlu on the concentration of L-NH_2BzGlu in the presence of 1.38 mM dihydrofolate reductase. The chemical shift is expressed relative to that of the same resonance in the absence of enzyme: (○) dihydrofolate reductase + L-NH_2BzGlu; (●) dihydrofolate reductase + 1.1 equiv of NADPH + L-NH_2BzGlu; lines are theoretical curves calculated from the parameters in Table I.

[2] The chemical shift of the L-NH_2BzGlu protons in the presence of enzyme + methotrexate can be taken as a good measure of δ_I, since it includes any bulk susceptibility effects of the enzyme. Under the present conditions, such effects are clearly small.

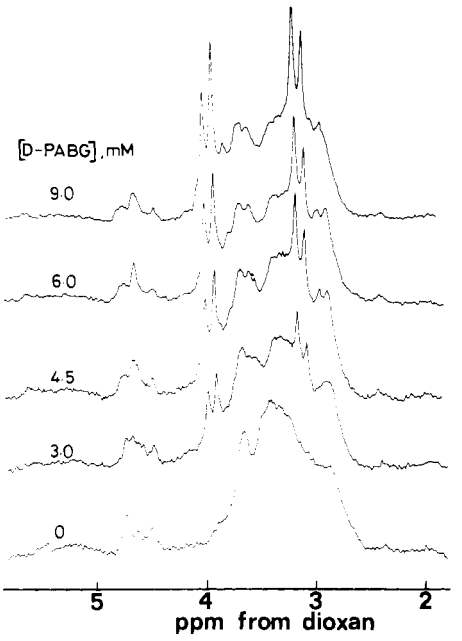

FIGURE 3: The aromatic region of the 100-MHz ^{1}H nmr spectrum of dihydrofolate reductase (0.85 mM) alone (bottom) and in the presence of various concentrations of D-NH_2BzGlu; sample temperature, 20 ± 0.5°.

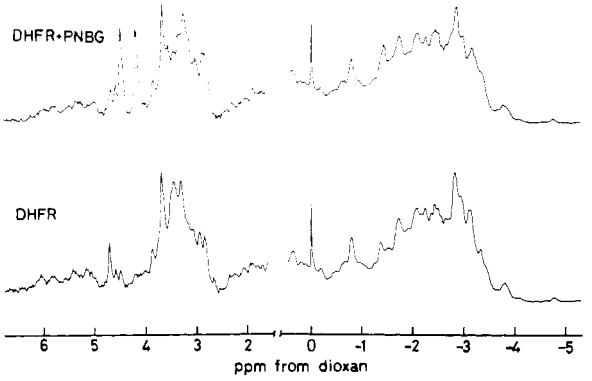

FIGURE 4: The 270-MHz ^{1}H nmr spectrum of dihydrofolate reductase (0.9 mM) alone and in the presence of 5 mM L-NH_2BzGlu. The region of the spectrum immediately around the large HDO resonance has been omitted, and the vertical gain for the aromatic region of the spectrum is substantially greater than that for the aliphatic region; sample temperature, 18 ± 0.5°.

L-NO_2BzGlu, is only a factor of three less than that of L-NH_2BzGlu. Like L-NH_2BzGlu and L-NO_2BzGlu, D-NH_2BzGlu is bound about 3.5-fold more tightly to the reductase · NADPH complex than to reductase alone, but the aromatic proton resonances still show no observable shift.

Changes in the Protein Spectrum. The 270-MHz ^{1}H spectrum of reductase is shown in Figure 4. The spectrum is very complex, and clearly consists, as one would expect, of a large number of overlapping absorption bands. A small number of single resonances can, however, be resolved. At this pH, the C-2-H resonances of the six histidine residues can be seen (partially overlapping) between 4.7 and 5.0 ppm, while at high field the resonances of approximately five methyl groups can be seen above −3.5 ppm (the small resonance due to a single methyl group at −4.9 ppm being particularly distinct).

On addition of ligands such as NH_2BzGlu, changes are seen in the histidine C-2-H, aromatic, and methyl regions of the spectrum. All three ligands examined (L- and D-NH_2BzGlu and NO_2BzGlu) produce at least qualitatively the same changes in the protein spectrum. The effects on the aromatic and methyl regions are shown in Figure 4. No detailed description of these changes is possible at present; clearly, however, one or more resonance(s) in the aromatic region have been shifted upfield, while in the methyl region the changes center around −3.2 ppm. The high-field methyl resonances are not shifted, nor do any new resonances appear above −3.5 ppm. In the experiment shown in Figure 4, L-NO_2BzGlu was used as the ligand, since its aromatic resonances do not obscure the aromatic region of the protein spectrum; similarly, to examine the effects on the histidine C-2-H resonances in detail one must use NH_2BzGlu. Figure 5 shows the effect of adding excess L-NH_2BzGlu on these resonances at various pH values; it is clear that the positions of three of the six histidine C-2-H resonances are changed when L-NH_2BzGlu binds to the enzyme. The titration curves of the histidine residues of dihydrofolate reduc-

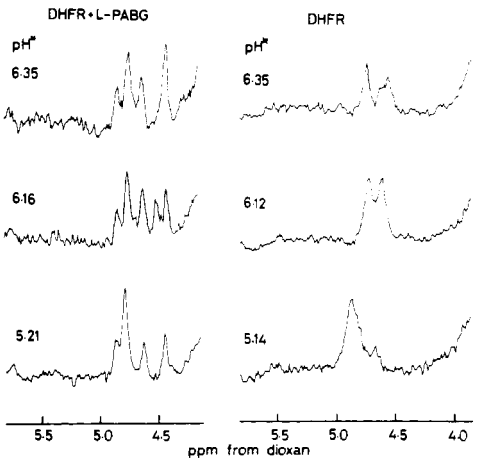

FIGURE 5: The histidine C-2-H resonances (100 MHz) of dihydrofolate reductase (0.9 mM) at various pH values in the presence and absence of 18 mM L-NH_2BzGlu; sample temperature, 10 ± 0.5°.

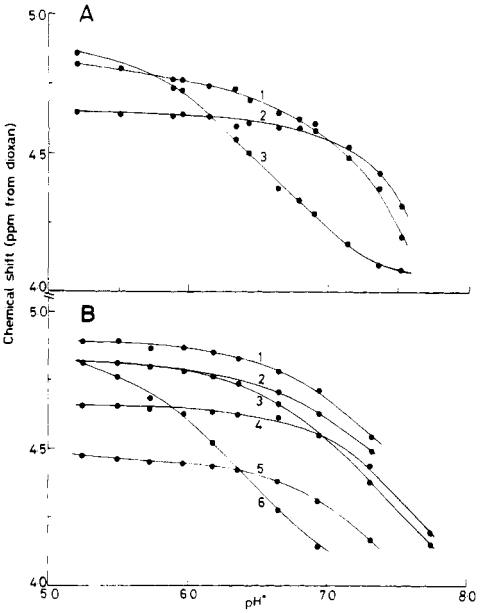

FIGURE 6: The chemical shifts of the histidine C-2-H resonances of dihydrofolate reductase (0.9 mM) as a function of pH*, in the absence (A) and presence (B) of 18 mM L-NH_2BzGlu. The points are experimental; the lines are Henderson-Hasselbalch titration curves (*cf.* Table II and text); sample temperature 10 ± 0.5°.

TABLE II: Chemical Shifts and pK Values of the Histidine Residues of Dihydrofolate Reductase.

Curve No.	Area of resonance (No. of Protons)	δ_{HA} (ppm)	pK	δ_A (ppm)
		Dihydrofolate Reductase		
1	3	4.82	>7.3[a]	
2	1	4.65	>7.3[a]	
3	2	4.87	6.51 ± 0.02	4.02
		Dihydrofolate Reductase + L-NH_2BzGlu		
1	1	4.90	>7.3[a]	
2	1	4.82	>7.3[a]	
3	1	4.82	>7.3[a]	
4	1	4.66	>7.3[a]	
5	1	4.48	>7.3[a]	
6	1	4.87	6.37 ± 0.02	3.95

[a] Assuming ($\delta_{HA} - \delta_A$) $\simeq$ 1.0 ppm, these pK values are in the range 7.3–7.7; accurate estimates are not possible.

tase in the presence and absence of L-NH_2BzGlu are shown in Figure 6. *L. casei* dihydrofolate reductase is unstable at pH >7.5, and though the experiments shown in Figures 5 and 6 were performed at 10° in an attempt to improve the stability, reliable data could not be obtained above this pH. Within the pH range shown in Figure 6, all the titration shifts were wholly reversible. Since most of the titration curves are incomplete, and since in addition in the absence of ligands several of the C-2-H resonances overlap over the whole accessible pH range, accurate pK values for most of the histidine residues cannot be obtained at present. The chemical shifts of the protonated histidines, δ_{HA}, and estimates of the pK values are given in Table II. On the other hand, in the presence of an excess of L-NH_2BzGlu (Figure 6B), six separate titration curves can be resolved. Curve 4 in Figure 6B corresponds closely to curve 2 in Figure 6A, and the peak giving rise to these curves has an area of one proton in both cases. However, while curves 1 in Figures 6A and 3 in Figure 6B are also closely similar, the corresponding resonance has an area of three protons in dihydrofolate reductase alone but of only one proton in the presence of L-NH_2BzGlu. Thus, of these three residues, only one is entirely unaffected by L-NH_2BzGlu binding; most probably one corresponds to curve 2 (Figure 6B), its environment having been only slightly altered, and the third must correspond to either curve 1 or curve 5, which have no counterparts in Figure 6A. Addition of increasing concentrations of D-NH_2BzGlu at pH 6.5 (Figure 3; closely similar results were obtained with L-NH_2BzGlu or NO_2BzGlu) shows that a resonance at 4.70 ppm, corresponding to curve 1 (Figure 6A), shifts *upfield* as the ligand concentration is increased. This suggests that the resonance which "disappears" from curve 1 (Figure 6A) gives rise to curve 5 (Figure 6B) in the presence of the ligand. Analysis of the dependence of the chemical shift of this histidine resonance on ligand concentration gives an extrapolated total shift of 0.28 ± 0.05 ppm at pH 6.5—very close to the 0.31-ppm separation of curves 1 (Figure 6A) and 5 (Figure 6B) at this pH.

Again, while curve 3 in Figure 6A is similar to curve 6 in Figure 6B, the corresponding resonance has an area of two

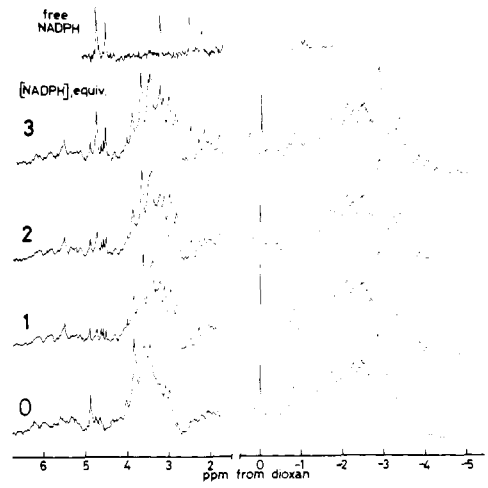

FIGURE 7: The 270-MHz ^{1}H nmr spectra of dihydrofolate reductase (0.9 mM) alone (bottom) and in the presence of 1, 2, and 3 molar equivalents of NADPH. The spectrum of 0.9 mM NADPH alone is shown at the top. The vertical gain for the aromatic region is substantially greater than for the aliphatic region; sample temperature, 18 ± 0.5°.

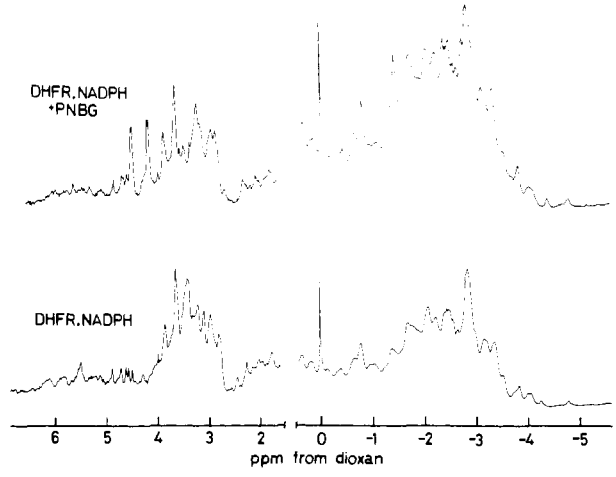

FIGURE 8: The 270-MHz ^{1}H nmr spectra of the 1:1 dihydrofolate reductase · NADPH complex (0.9 mM) in the presence and absence of 5 mM NH_2BzGlu. The vertical gain for the aromatic region is substantially greater than for the aliphatic region; sample temperature, 18 ± 0.5°.

protons in dihydrofolate reductase alone, and one proton in the presence of L-NH_2BzGlu. By elimination, the "missing" proton must correspond to curve 1, Figure 6B, and the pK of the corresponding histidine residue must have been increased by about 1 pH unit on ligand binding. In addition, there is a small but significant change in the pK of the remaining residue giving curve 6 (Table II).

On addition of an equimolar concentration of NADPH to dihydrofolate reductase, marked changes are observed throughout the enzyme spectrum (Figure 7). Particularly notable are the changes in the histidine C-2-H region and the appearance of three additional methyl resonances between −4.0 and −4.5 ppm. NADPH binds very strongly to dihydrofolate reductase ($K_a \simeq 10^8$ M^{-1}) and is thus in slow exchange; it is therefore not possible at present to identify the resonances of bound NADPH in the spectrum with any certainty. However, the stoichiometric binding of NADPH has the advantage that it is possible to determine the number of NADPH molecules bound to the enzyme in a straightforward manner. Addition of a second and third

equivalent of NADPH to dihydrofolate reductase (Figure 7) leads to no further changes in the protein spectrum, and the resonances of the additional NADPH appear at precisely the same frequencies as in free NADPH, clearly indicating 1:1 stoichiometry.

On addition of L-NO_2BzGlu to the 1:1 dihydrofate reductase · NADPH complex, further changes in the protein spectrum are observed (Figure 8). The changes in the aromatic region are similar to those produced by L-NO_2BzGlu in the absence of NADPH (Figure 4); indeed the aromatic regions of the difference spectra {reductase · NO_2BzGlu − reductase} and {reductase · NADPH · NO_2BzGlu − reductase · NADPH} are almost, though not completely, superimposable. In the methyl region, however, the pattern of changes is quite different. Of particular interest is the observation that a methyl resonance at −4.24 ppm, which is present in reductase · NADPH but not in reductase alone, is shifted still further upfield to −4.40 ppm on addition of 5 mM L-NO_2BzGlu. L-NO_2BzGlu itself produces no changes above about −3.5 ppm (Figure 4).

Discussion

A large number of studies of the specificity of the dihydrofolate binding site (particularly for inhibitors) have been reported (see Blakley, 1969; Baker, 1967). The majority of these studies have been concerned with the pteridine moiety of the substrate or inhibitor, though the binding of NH_2BzGlu has been recognized for a number of years (Baker *et al.*, 1966). While it is true that the major part of the very considerable binding energy of methotrexate and related compounds (ΔF° = −12 to −15 kcal/mol) is attributable to the pteridine ring, significant variations in affinity are seen with changes in the NH_2BzGlu moiety (Baker *et al.*, 1964; Blakley, 1969; Johns *et al.*, 1973).

The present work shows that L-NH_2BzGlu binds to a single site on *L. casei* dihydrofolate reductase, and that this site overlaps that occupied by methotrexate (and, presumably, since NH_2BzGlu inhibits the enzyme, that occupied by dihydrofolate). However, since methotrexate binds stoichiometrically to dihydrofolate reductase and produces large changes in the nmr spectrum of the protein, we have not yet been able to identify the aromatic resonances of bound methotrexate unequivocally. Thus, we do not know whether the *orientation* of the aromatic ring of L-NH_2BzGlu when bound is the same as that of the corresponding moiety of methotrexate. Both L-NH_2BzGlu and L-NO_2BzGlu appear to bind in very much the same orientation. At present, the nature of the interaction responsible for the changes in chemical shift of the aromatic protons of these compounds on binding to the enzyme is unknown, so the significance of the differences in shifts between L-NH_2BzGlu and L-NO_2BzGlu in structural terms cannot be precisely assessed. However, if one assumes for illustrative purposes that the shifts are due to the magnetic anisotropy of an aromatic ring on the protein, then a movement of only 0.2–0.5 Å would be sufficient to explain the observed differences (Johnson and Bovey, 1958). Such a difference in orientation could readily be produced by unfavorable steric interactions between the nitro group of NO_2BzGlu and groups on the enzyme, or alternatively by hydrogen bonding to the amino group; in either case the somewhat weaker binding of NO_2BzGlu would be expected.

The striking observation that the binding of D-NH_2BzGlu does not lead to any observable shift of its aromatic proton resonances does, however, imply a very significant difference in orientation for the aromatic fragment of the molecule. It is quite clear from the competition experiments that D- and L-NH_2BzGlu and L-NO_2BzGlu all bind to overlapping sites, but on the ring-current model used above, the aromatic ring of D-NH_2BzGlu would have to be at least 3 Å and probably 5 Å distant from the position of the same ring in the L-NH_2BzGlu complex. The orientation of the aromatic ring in the complex is thus governed by the stereochemistry of the glutamate residue, and by the latter's interactions with the enzyme. Examination of molecular models indicates that if D- and L-NH_2BzGlu bound to the enzyme in the same conformation, with their α- and γ-carboxylate groups occupying the same positions, then the position of their aromatic rings would differ by some 6 Å. The differences in chemical shift of the aromatic protons between L- and D-NH_2BzGlu in their complexes with dihydrofolate reductase reflect only a difference in *orientation* of the aromatic ring, and *not* its contribution to the binding energy. Indeed, the fact that the binding constant of D-NH_2BzGlu is only about a factor of 2.5–3 less than that of L-NH_2BzGlu indicates either that the interactions of the glutamate residue provide the major part of the binding energy, or, less probably, that the aromatic ring of D-NH_2BzGlu occupies a different "subsite" from that of L-NH_2BzGlu which provides a similar binding energy but produces no change in the magnetic environment of the aromatic protons.

A number of earlier experiments have indicated the importance of the glutamate residue for the binding of substrate analogs to dihydrofolate reductase (Baker *et al.*, 1964, 1966; Morales and Greenberg, 1964; Greenberg *et al.*, 1966; Plante *et al.*, 1967). Recently Johns *et al.* (1973), have reported that the dimethyl ester of methotrexate (in which both carboxylate groups of the glutamate residue are blocked) has a strikingly lower affinity (almost 100-fold) for L1210 dihydrofolate reductase than does methotrexate itself.

At present, the changes observed in the protein spectrum on ligand binding cannot be interpreted in detail, though a number of interesting qualitative points emerge. Both L- and D-NH_2BzGlu (as well as L-NO_2BzGlu) produce closely similar changes in the protein spectrum, in the histidine C-2–H, aromatic, and methyl regions. As far as the histidine C-2–H resonances are concerned, the effects seem to be quantitatively as well as qualitatively the same. It was argued above that the aromatic rings of L- and D-NH_2BzGlu are in distinctly different positions in their respective complexes with dihydrofolate reductase; since the changes in the protein spectrum are the same, they must be produced primarily by the interactions of the glutamate moiety, either directly or by a conformational rearrangement of the protein on ligand binding. The instability of dihydrofolate reductase at pH values much above neutrality has so far prevented us from determining the pK values of most of the histidine residues, though two histidines have a pK of 6.52 (at 10°) in the absence of ligands. In discussing the environments of these histidine residues, it is useful to use pK and chemical-shift values for a completely solvent-accessible histidine residue as a basis for comparison. The choice of values is necessarily somewhat arbitrary; we shall follow King and Roberts (1971) and use those of His-105 in bovine pancreatic ribonuclease (pK = 7.1 (10°); δ_{HA} = 5.0; δ_A = 4.0 ppm from dioxane; Roberts *et al.*, 1968). On this basis, a pK of 6.5 is perhaps rather low; the other histidine residues have estimated pK values which are probably close

to normal. The single histidine residue giving rise to curve 2 in Figure 6A has a chemical shift in the protonated form which is significantly farther upfield than expected for a solvent-accessible histidine; this residue is apparently unaffected by the binding of NH_2BzGlu. Three (and perhaps four) histidine residues are, however, affected by NH_2BzGlu binding.

It is impossible to say from the very limited information available at present whether or not these are direct effects of the binding of the glutamate moiety close to these histidine residues, but the marked increase in pK seen for one histidine residue would be expected if it interacted directly with one of the carboxylate groups of the glutamate.

The binding of the coenzyme, NADPH, produces substantial changes throughout the spectrum of the protein. The binding constant of NADPH is such that exchange of NADPH molecules between the free and bound states is slow on the nmr time scale. As a result, we are not yet able to definitely identify the resonances of the NADPH protons in the complex. However, it is clear that *L. casei* dihydrofolate reductase binds only one molecule of NADPH per molecule of enzyme. (More strictly, if a second binding site exists, its affinity must be less than about 10^3 M^{-1}, while the major binding site has a binding constant of $\sim 10^8$ M^{-1} (R. Bjur, unpublished work.)) The recent reports by Williams *et al.* (1973) and Poe *et al.* (1974) that *Escherichia coli* dihydrofolate reductase binds two molecules of NADPH, if confirmed, point to a striking difference between these two enzymes. Other differences, in kinetic behavior (Dann *et al.*, 1974) and in amino acid sequence (Bennett, 1974; Morris *et al.*, 1973, and unpublished work), have been noted. One of the clearest effects of NADPH on the protein spectrum is the appearance of several additional methyl resonances to high field of −3.5 ppm. There is strong circumstantial evidence from other proteins, notably lysozyme (McDonald and Phillips, 1967; Sternlicht and Wilson, 1967), that these resonances arise from methyl groups positioned close to the face of aromatic rings—for example of tryptophan residues, or indeed the adenine ring of NADPH (in lactate dehydrogenase, the binding site of the adenine ring of NADPH is largely hydrophobic and includes as many as 12 methyl groups (Chandrasekhar *et al.*, 1973)).

The simultaneous binding of NADPH to give the ternary complex affects the binding of NH_2BzGlu in a number of ways. The binding constants of L- and D-NH_2BzGlu and L-NO_2BzGlu are all increased, and by approximately the same amount in each case (a factor of 3.2–3.5, corresponding to ~ -0.8 kcal/mol). The binding of methotrexate and related compounds to dihydrofolate reductase from a variety of sources has been shown to be increased in the presence of NADPH (see Blakley, 1969). In addition, the environment of the aromatic ring of L-NH_2BzGlu and NO_2BzGlu is altered by the binding of NADPH. Again using the ring-current shift model, for illustrative purposes only, a change in position of 0.5–1.0 Å is implied for both molecules. Alternatively, if NADPH binds in close proximity to the aromatic ring of L-NH_2BzGlu, the change in shift might be a direct effect of NADPH itself. Any change in the environment of the aromatic ring of D-NH_2BzGlu, however, is not sufficient to cause a shift of its aromatic protons.

The experiments reported here, though somewhat preliminary in nature, show that nmr will be capable of providing a substantial amount of information on the topography of the active site of dihydrofolate reductase, particularly when isotopic substitution experiments, presently in progress, permit the analysis of the protein spectrum in more detail.

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