Histidine Residues of *Lactobacillus casei* Dihydrofolate Reductase: Paramagnetic Relaxation and Deuterium-Exchange Studies and Partial Assignments†

P. Wyeth,‡ A. Gronenborn,§ B. Birdsall, G. C. K. Roberts,* J. Feeney, and A. S. V. Burgen

ABSTRACT: The exchange of the C2 protons of the histidine residues of *Lactobacillus casei* dihydrofolate reductase for deuterium has been followed by NMR spectroscopy. In the course of these experiments, the C2 proton resonance of the seventh histidine residue indicated by the amino acid sequence, but which had not previously been observed, has been identified. This residue has a high pK value (>8.5), its C2 proton resonance is at unusually low field, and its C2 proton exchanges very slowly for deuterium (no detectable exchange after 3 months). In the enzyme–trimethoprim complex, the order of exchange of the C2 protons is H_C, $H_E > H_F \gg H_A > H_D \gg H_B > H_G$. Methotrexate specifically retards the exchange of the C2 proton of H_F. The effects of a paramagnetic anion, $Cr(CN)_6^{3-}$, and its diamagnetic analogue, $Co(CN)_6^{3-}$, on the histidine C2 proton resonances have been investigated. Resonances C and F are the most affected. Competition experiments show that the effect on H_C arises from anion binding to a site overlapping that for the coenzyme, while that on H_F arises from binding to a site overlapping that for methotrexate. The results of these and earlier experiments are discussed in relation to the structure of the enzyme–methotrexate–NADPH complex determined crystallographically [Matthews, D. A., et al. (1978) *J. Biol. Chem. 253*, 6946]. Firm assignments of two of the histidine resonances, $H_C \equiv$ His-64 and $H_F \equiv$ His-28, are proposed, and partial assignments of the others are discussed.

In earlier work (Roberts et al., 1974; Birdsall et al., 1977a), we showed that the C2 proton resonances of four of the histidine residues of *Lactobacillus casei* dihydrofolate reductase were perturbed by the binding of substrates or inhibitors. The recent determination of the three-dimensional structure of the enzyme–methotrexate–NADPH complex (Matthews et al., 1978, 1979) reveals that two of the histidine residues are directly involved with ligand binding, histidine-28 forming an ion pair with the γ-carboxyl group of the glutamate moiety of methotrexate and histidine-64 binding to the 2′-phosphate group of the coenzyme.

Matthews (1979) has recently proposed assignments for the six histidine C2 proton resonances observed by Roberts et al. (1974) and by Birdsall et al. (1977a), based on the three-dimensional structure of the ternary complex (Matthews et al., 1978, 1979) and the chemical shift changes produced by the formation of the binary substrate or inhibitor complexes (Birdsall et al., 1977a). Because such changes in chemical shift can arise either from a direct interaction between the histidine residue in question and the ligand or from conformational changes, assignments made in this way cannot be unambiguous. We now report experiments directed toward the assignment of the histidine C2 proton resonances which depend upon properties of the histidine residues which can more easily be determined from the crystal structure. Deuterium exchange of the C2 proton will depend on the solvent accessibility of the imidazole ring, while line broadening produced by a paramagnetic anion depends simply on the distance between the C2 proton and the anion.

In our earlier studies (Roberts et al., 1974; Birdsall et al., 1977a), we observed only six histidine resonances. Subsequent determinations of the amino acid sequence of the enzyme (Freisheim et al., 1978; Morris, 1979) showed that it contained seven histidine residues. We have now identified a seventh histidine C2 proton resonance whose unusual characteristics led to its being overlooked in our earlier work.

Experimental Section

Dihydrofolate reductase was isolated and purified from *L. casei* MTX/R as described by Dann et al. (1976); its concentration was determined by fluorometric titration with methotrexate (Dann et al., 1976). Methotrexate (Nutritional Biochemical Corp.), trimethoprim (Wellcome Laboratories), adenosine 2′-phosphate, and NADPH were obtained commercially and used without further purification. Guanidinium chloride was deuterated by several cycles of dissolution in ^2H_2O followed by lyophilization. Potassium chromicyanide, $K_3[Cr(CN)_6]$, was prepared from chromium(III) acetate by the method of Bigelow (1946). The product was purified by precipitation from water with ethanol; it was then washed with ethanol and ether and dried in vacuo. $K_3[Co(CN)_6]$, prepared similarly, was recrystallized from water and dried in vacuo over phosphorus pentoxide. Methotrexate was esterified with methanol–HCl by a modification of the method of Pfiffner et al. (1947).

Fluorometric methods for determination of binding constants have been described in detail previously (Birdsall et al., 1978, 1980; Dunn et al., 1978).

^{31}P NMR spectra were obtained at 40.5 MHz by using a Varian XL-100 spectrometer operating in the Fourier transform mode. The samples consisted of 1.5 mL of a solution of ~1 mM enzyme in ^2H_2O containing 1 mM EDTA, 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol] chloride, and 500 mM KCl, pH* 7.03 (±0.02). (The notation pH* indicates a pH meter reading uncorrected for the isotope effect on the glass electrode.) In addition, the samples contained 1 mM inorganic phosphate as a chemical shift reference. Sample temperature was 10 (±1) °C.

^1H NMR spectra were obtained at 270 MHz by using a Bruker WH-270 spectrometer operating in the Fourier

† From the Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom. *Received December 31, 1979.*

‡ Present address: Department of Chemistry, University of Southampton, Southampton, England. Recipient of a fellowship from the Medical Research Council.

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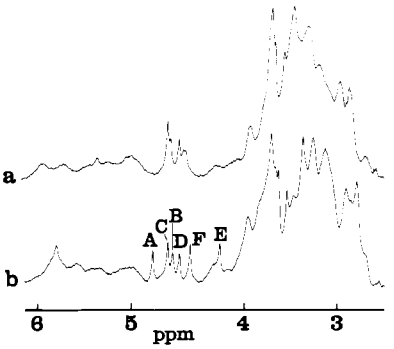

FIGURE 1: Aromatic region of the 270-MHz ^1H NMR spectra of (a) dihydrofolate reductase and (b) the dihydrofolate reductase–trimethoprim complex. In the latter spectrum the C2 proton resonances of the histidine residues are identified by using the nomenclature of Birdsall et al. (1977a).

transform mode. A spectral width of 4200 Hz was used, and the free induction decays were collected in either 4096 or 8192 points; before Fourier transformation the free induction decay was multiplied by an exponential function, giving a line broadening of 2 Hz, and, if 4096 data points had been collected, the data table was filled to 8192 points with zeroes. Between 2000 and 10 000 transients were averaged for each spectrum. The samples consisted of ~1 mM enzyme in 350 μL of ^2H_2O containing 50 mM potassium phosphate, 500 mM KCl, and 1 mM dioxane as the chemical shift reference (the ^1H resonance of dioxane is 3.71 ppm downfield of that of dimethylsilapentane-5-sulfonate). Ligands were added as microliter volumes of solutions in the ^2H_2O–phosphate buffer or as solids. The pH of the samples was adjusted with 0.1–1.0 M KO^2H or ^2HCl and measured to ±0.02 unit; the sample temperature, 10–40 °C, was maintained to ±1 °C. The pH dependence of the histidine C2 proton resonances was determined and analyzed as described by Birdsall et al. (1977a) and A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen (unpublished experiments).

Results

Figure 1 shows the low-field region of the 270-MHz ^1H NMR spectrum of *L. casei* dihydrofolate reductase, alone (Figure 1a) and in its complex with trimethoprim (Figure 1b), at pH* 6.5. The six histidine C2 proton resonances identified earlier at 100 MHz (Roberts et al., 1974; Birdsall et al., 1977a) are labeled A–F. Even at 270 MHz they are incompletely resolved in the spectrum of the free enzyme, but after addition of trimethoprim six separate signals can be seen between 4 and 5 ppm [see also Birdsall et al. (1977a)]. At lower field (between 5 and 6.5 ppm) there are a number of broad and ill-resolved resonances arising from NH protons which do not readily exchange with the ^2H_2O solvent. In addition, there is a relatively sharp resonance at 5.80 ppm visible in the spectrum of the enzyme–trimethoprim complex but not in that of the enzyme alone.

A sharp signal at a similar position (5.5–5.8 ppm) is observed in spectra of almost all the enzyme–ligand complexes studied to date. In each complex, the chemical shift of this resonance is independent of pH in the range pH* 5.0–7.2, shifting slightly (~0.1 ppm) upfield between pH* 7.2 and pH* 8.0 (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments).

The relatively narrow line at 5.8 ppm in Figure 1b contrasts with the very broad NH resonances in the same region of the spectrum, suggesting that it arises from a carbon-bound proton. On the other hand, it appears almost 1 ppm to low field of the position characteristic of C2 proton resonances of protonated histidine residues (e.g., resonance A in Figure 1b). No resonance at this position is seen in spectra of complexes of selectively deuterated dihydrofolate reductase, in which all the aromatic protons except the 2 and 6 protons of tyrosine (Feeney et al., 1977) were replaced by deuterium. The signal at 5.80 ppm must thus arise from an aromatic carbon-bound proton with an unusually low-field chemical shift.

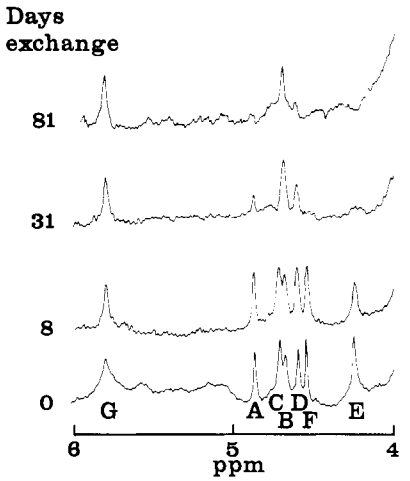

FIGURE 2: Histidine C2 proton resonances of the dihydrofolate reductase–trimethoprim complex as a function of the length of time of incubation in ^2H_2O at 37 °C.

Further evidence for the identity of this low-field resonance was obtained from deuterium-exchange experiments. Two groups of signals in the low-field region of protein ^1H NMR spectra would be expected to show effects of deuterium exchange at neutral pH: nitrogen-bound protons and the imidazole C2 protons of histidine residues [e.g., Meadows et al. (1968) and Markley (1975)]. The rate of deuterium exchange depends markedly on the extent to which the individual residues are accessible to the solvent, and this information can be very helpful in assigning the histidine C2 proton resonances on the basis of estimates of accessibility made from the crystal structure.

The enzyme–trimethoprim complex was incubated in ^2H_2O–phosphate buffer, pH* 6.5, at 37 °C for several months, and ^1H NMR spectra were obtained at intervals during this period. Representative spectra are shown in Figure 2. A few days incubation suffices to remove all the broad NH resonances between 5 and 6 ppm, while the intensities of the histidine C2 proton signals A–F are only slightly reduced, C and E being most affected. The resonance at 5.80 ppm (labeled G in Figure 2) is also undiminished in intensity; in the absence of the background of NH signals, resonance G can be seen to be comparable in intensity and line width (8–10 Hz) to signals A–F (Figure 2).

After 31 days of incubation, appreciable exchange of the histidine C2 protons for deuterium has occurred, and this exchange is selective, since the C2 proton resonances of the different histidine residues differ markedly in intensity. Resonances E and F have completely disappeared, and resonances A and D have decreased to about half their original intensity. The intensity of resonance C has also decreased markedly; although no separate resonances can be seen, a small residual signal could be masked by the neighboring resonance B. Resonances B and G do not appear to have decreased significantly in intensity. On further incubation in ^2H_2O, for a total of 81 days, the exchange of the C2 protons giving resonances A and D is completed, so that only signals B and G remain. There were no detectable changes over this period in other regions of the spectrum, indicating that the complex

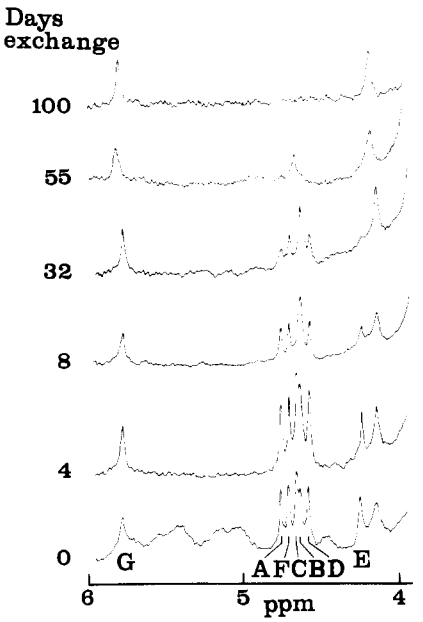

FIGURE 3: Histidine C2 proton resonances of the dihydrofolate reductase–methotrexate complex as a function of the length of time of incubation in ^2H_2O at 37 °C. The resonance at 4.14 ppm is that of the 7 proton of bound methotrexate.

remained in its native state. From the complete time course of the deuterium exchange of the enzyme–trimethoprim complex at pH* 6.5, the rates of histidine C2 proton exchange can be placed in the order C, E > F ≫ A > D ≫ B with signal G remaining readily detectable after 3 months in ^2H_2O at 37 °C.

The deuterium exchange of the histidine C2 protons in the enzyme–methotrexate complex is illustrated in Figure 3. After the initial exchange of NH protons, the first two resonances to be affected are C and E (compare 0 and 8 days). Resonance F clearly exchanges much more slowly. It is still readily observable when resonance E has disappeared completely (32 days) and decreases at a rate comparable to the rate of disappearance of A and D. After 55 days of incubation at 37 °C, only signals B and G remain (the resonance at 4.14 ppm is that of the 7 proton of bound methotrexate; Feeney et al., 1977, and unpublished work), and after 100 days B too has disappeared, leaving G as the only resonance below 4.2 ppm; the other regions of the spectrum were unaffected. For the methotrexate complex, the rates of exchange of the histidine C2 protons are in the order E, C > A > D, F ≫ B, signal G showing no sign of a decrease in intensity even after 3 months of incubation at 37 °C (compare its intensity to that of the resonance at 4.14 ppm from the nonexchangeable 7 proton of methotrexate in Figure 3). Compared to trimethoprim, methotrexate thus leads to a selective decrease in the rate of exchange of the C2 proton of histidine F.

The final product of the deuterium-exchange experiments on both complexes is an enzyme sample in which the C2 protons of histidines A–F have been replaced by deuterium, while the proton giving rise to resonance G remains unaffected. If signal G arises from a histidine C2 proton, this should be apparent in these samples by comparing its chemical shift in the *denatured* enzyme with that of histidine itself.

After denaturation of the deuterium-exchanged enzyme–methotrexate complex (which gave the spectrum of Figure 4a) by addition of guanidinium chloride to a concentration of 6 M, we obtained the spectrum shown in Figure 4b. This contains only two signals, of approximately equal intensity, to low field of 4 ppm. The higher field of the two (4.79 ppm)

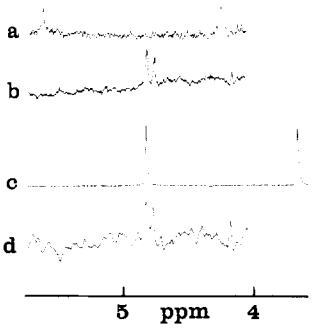

FIGURE 4: Low-field region of the ^1H NMR spectrum of (a) the dihydrofolate reductase–methotrexate complex, after deuterium exchange at 37 °C for 100 days, (b) the same sample after addition of 6 M guanidine hydrochloride, (c) histidine in 6 M guanidine hydrochloride, and (d) an unexchanged sample of dihydrofolate reductase–methotrexate complex in 6 M guanidine hydrochloride.

arises from the 7 proton of *free* methotrexate (released from the complex on denaturation). The other resonance has exactly the same chemical shift (4.86 ppm) as the C2 proton resonance of histidine in the same solvent (Figure 4c). The spectrum of an enzyme–methotrexate complex dissolved in 6 M guanidinium chloride *without* prior deuterium exchange of the histidine C2 protons (Figure 4d) shows the same two resonances at low field, indicating that all the histidine C2 protons resonate at 4.86 ppm in the denatured enzyme. Similar results were obtained on denaturation of the deuterium-exchanged trimethoprim complex.

This shows clearly that resonance G does indeed arise from the C2 proton of a histidine residue.

Paramagnetic Relaxation Studies. As noted in the introduction, chemical shift changes produced by ligand binding are an unreliable basis for resonance assignment, since they may be caused by conformational changes remote from the binding site. However, if a rapidly exchanging paramagnetic ligand is used, its line-broadening effects will be greatest for residues near its binding site(s) and, provided that these binding sites can be identified in the crystal structure, assignments can thus be made with a greater degree of confidence. Since the substrates and coenzyme of dihydrofolate reductase contain a number of negatively charged groups, we have investigated the effects of the binding of chromicyanide [$Cr(CN)_6^{3-}$], a simple spherical, substitution-inert, paramagnetic anion.

Fluorometric experiments showed that this anion and its diamagnetic analogue, $Co(CN)_6^{3-}$, bind to dihydrofolate reductase competitively with etheno-NADP$^+$, with a binding constant of the order of 10^3 M^{-1}. This suggests that these anions bind to the site(s) normally occupied by the 2′-phosphate and/or pyrophosphate of the coenzyme. Binding to the 2′-phosphate site was confirmed by competition experiments with adenosine 2′-phosphate. In a solution containing 1.14 mM enzyme and 1.20 mM adenosine 2′-phosphate at pH* 7.03, the ^{31}P resonance of the ligand is 0.34 ppm to low field of its position in the free ligand [see Birdsall et al. (1977b)]. Addition of $K_3Co(CN)_6$ (1–14 mM) led to a progressive upfield shift of the ^{31}P resonance toward the free position; the dependence of the ^{31}P chemical shift on cobalticyanide concentration was consistent with a simple competition with adenosine 2′-phosphate. These experiments do not, of course, exclude binding to other sites. The ^1H NMR experiments below demonstrate that there is, in fact, binding to several sites,

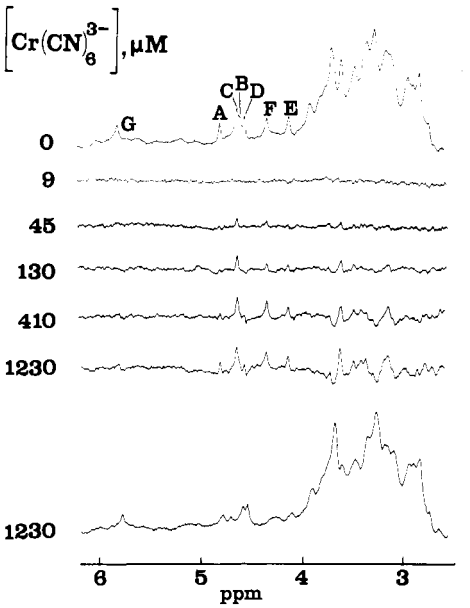

FIGURE 5: Effects of potassium chromicyanide on the aromatic region of the ^1H NMR spectrum of the dihydrofolate reductase–trimethoprim complex. Normal spectra are shown for 0 and 1230 μM chromicyanide; the remainder are difference spectra obtained by subtracting the spectrum obtained in the presence of the indicated concentration of chromicyanide from that obtained in the absence of chromicyanide.

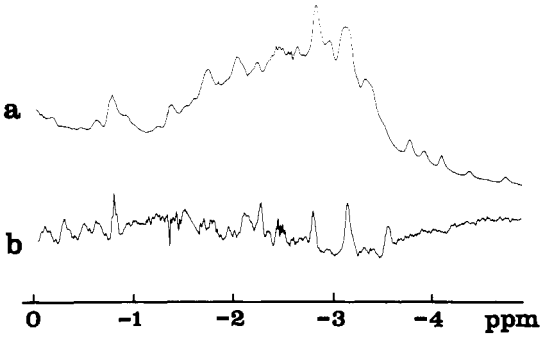

FIGURE 6: (a) The aliphatic region of the ^1H NMR spectrum of the dihydrofolate reductase–trimethoprim complex. (b) The corresponding region of the difference spectrum obtained by subtraction from (a) of the spectrum obtained on addition of 50 μM chromicyanide.

so that accurate estimates of the anion binding constants cannot be obtained from these competition experiments.

The effects of chromicyanide on the ^1H NMR spectrum of the enzyme–trimethoprim complex are shown in Figures 5 and 6. As the chromicyanide concentration is increased, its line-broadening effects progressively increase, indicating that exchange between the free and bound states is rapid on the NMR time scale. Since the observed spectrum is the average of those from enzyme molecules with and without chromicyanide bound, resonances which are most affected by the chromicyanide will appear in the difference spectrum at the lowest chromicyanide concentrations (i.e., when only a small fraction of the enzyme has chromicyanide bound).

In the aromatic region of the spectrum (Figure 5), the largest effects are seen on the histidine resonances. As the chromicyanide concentration is increased, the first histidine C2 proton signals to appear in the difference spectrum are C and F; at rather higher concentrations A and E appear, while the line widths of B, D, and G are unaffected except at the highest concentrations. In the difference spectrum at 1230 μM chromicyanide, resonance D shows a positive–negative feature indicative of a slight upfield shift *without* line broadening. The same shift (together with a smaller shift of

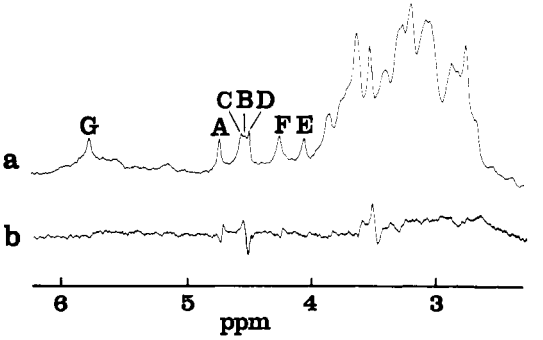

FIGURE 7: (a) The aromatic region of the ^1H NMR spectrum of the dihydrofolate reductase–trimethoprim complex. (b) The corresponding region of the difference spectrum obtained by subtraction from (a) of the spectrum obtained in the presence of 1.6 mM potassium cobalticyanide.

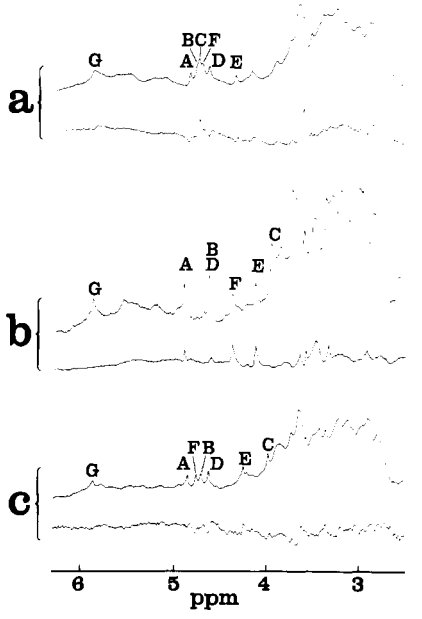

FIGURE 8: Effects of chromicyanide (400 μM) on the aromatic region of the ^1H NMR spectra of complexes of dihydrofolate reductase with (a) methotrexate, (b) trimethoprim plus NADPH, and (c) methotrexate plus NADPH. In each case the normal spectrum in the presence of chromicyanide and the difference spectrum obtained by subtraction from a control spectrum are shown.

resonance A) is produced by the diamagnetic anion cobalticyanide (Figure 7). Closely similar line-broadening effects were produced by chromicyanide addition to the free enzyme, but the poorer resolution of the C2 proton resonances (cf. Figure 1) made them harder to follow in detail.

A relatively small number of other resonances in the spectrum are affected by chromicyanide. Resonances of approximately 6 protons in the aromatic region and 16–18 in the aliphatic region appear in the difference spectra of Figures 5 and 6; of these, several resonances close to 3.5 ppm are most probably those of histidine C4 protons. Possible assignments of the C4 proton resonances are discussed by Feeney et al. (1980) and A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen (unpublished work). In the aliphatic region, the sharp triplet at −0.80 ppm, which is a reproducible feature of all the chromicyanide difference spectra (Figure 6), most probably arises from the C_ϵ-methylene protons of a lysine or the C_δ-methylene protons of an arginine residue. The three-dimensional structure of the enzyme–methotrexate–NADPH complex (Matthews et al., 1978, 1979) shows that arginine residues 43, 44, and 57 are involved in binding the anionic groups of the ligands.

Table I: Effects of Methotrexate and Its Dimethyl Ester on the C2 Proton Chemical Shifts[a] and pK Values of the Histidine Residues of *L. casei* Dihydrofolate Reductase

complex	H_A		H_B		H_C		H_D		H_E			H_F			H_G
	δ_{HA}	pK^b	δ_{HA}	pK^b	δ_{HA}	pK^b	δ_{HA}	pK^b	δ_{HA}	$\Delta\delta$	pK	δ_{HA}	$\Delta\delta$	pK	δ_{HA}
enzyme alone[c]	4.85	7.28[d]	4.80	7.41	4.88	7.11[d]	4.65	7.89	4.92	1.0	6.57	4.91	1.0	6.67	5.51
enzyme-methotrexate[c]	4.89	7.46	4.78	7.35	4.81	7.49	4.66	7.74	4.95	1.0	6.21	4.78	0.9	7.81[b]	5.87
enzyme-methotrexate dimethyl ester	4.92	7.37	4.78	7.25	4.88	7.22	4.66	7.83	4.97	1.0	6.24	5.01	1.0	5.96	5.89

[a] δ_{HA}, chemical shift (±0.01 ppm) in the protonated form (ppm from dioxane); $\Delta\delta$, chemical shift difference between protonated and unprotonated forms. [b] Complete titration curves could not be obtained, so these pK values are only estimates (±0.1 unit). [c] From A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen (unpublished experiments) and Birdsall et al. (1977a). [d] In the free enzyme, the titration curves of H_A and H_C cannot be adequately described by the Henderson-Hasselbalch equation; A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen (unpublished experiments).

To associate the line-broadening effects of chromicyanide on individual histidine resonances with its binding to a particular site, we have examined its effects on a number of other complexes[1] (Figure 8). Addition of a moderate concentration of chromicyanide (400 μM) to the enzyme–methotrexate complex leads to a broadening of histidine resonance C, but resonance F is not affected (Figure 8a). In the enzyme–trimethoprim–NADPH complex the behavior is reversed, resonance F but not resonance C being broadened (Figure 8b). When both methotrexate and NADPH are present, in the ternary complex (Figure 8c), there is little effect at this chromicyanide concentration, although at higher concentrations resonances A and E show the same slight broadening observed in the spectrum of the trimethoprim complex in Figure 5. These experiments thus show that the broadening of histidine resonance C is due to the binding of chromicyanide to a site overlapping that for NADPH, while the broadening of resonance F is due to binding to a site overlapping with that for methotrexate. The broadening of resonances A and E, which persists in the ternary complex, must then arise from chromicyanide binding to a site (or sites) outside the "active site" of the enzyme (or perhaps from nonspecific "collision complex" effects).

Binding of Methotrexate Dimethyl Ester. We suggested earlier (Birdsall et al., 1977a) that resonance F might arise from a histidine residue which interacts with the glutamate carboxylate group(s) of the ligand, and the results of the chromicyanide experiments are fully consistent with this. As a further test of this hypothesis, we have studied the effects of the binding of the dimethyl ester of methotrexate on the histidine residues.

Binding of the diester, like that of methotrexate, leads to quenching of the tryptophan fluorescence of the protein. At pH 6.5, the binding of methotrexate is stoichiometric at all usable enzyme concentrations (Dann et al., 1976; Hood & Roberts, 1978). The diester, however, binds less tightly, so that the binding constant could readily be determined; a value of 3 (±2) × 10^6 M^{-1} was obtained. Comparison of this with the estimated binding constant of 2 × 10^9 M^{-1} for methotrexate under the same conditions [from the results of Hood & Roberts (1978)] demonstrates that the glutamate carboxyl groups of methotrexate make a significant contribution to its binding.

The pH dependence of the chemical shifts of the histidine C2 proton resonances in the enzyme–methotrexate dimethyl ester complex (formed by using an enzyme/ligand ratio of 1:1) is shown in Figure 9. The chemical shifts in the protonated and unprotonated states and the pK values are summarized

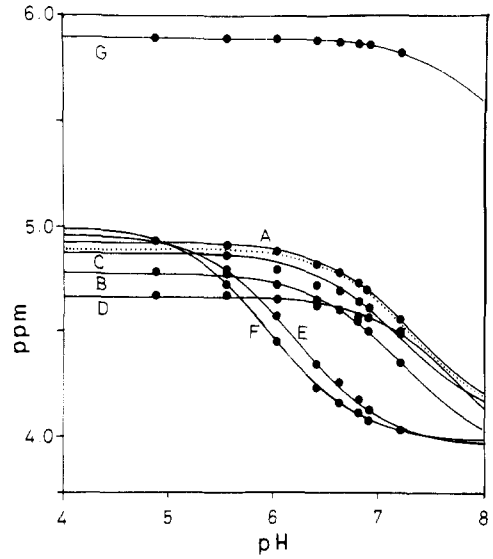

FIGURE 9: The pH dependence of the chemical shift of the histidine C2 proton resonances of dihydrofolate reductase in its complex with the dimethyl ester of methotrexate. The solid lines are "best-fit" Henderson–Hasselbalch titration curves. The dotted line is the titration curve of residue H_F in the methotrexate complex.

in Table I, along with the corresponding values for the free enzyme and the enzyme–methotrexate complex (Birdsall et al., 1977a; A Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) for comparison.[2]

Like methotrexate, the dimethyl ester has clear effects on the resonances of four histidine residues. Signal G sharpens markedly, appearing at 5.88 (±0.01) ppm in both complexes, and signal A shows a downfield shift of 0.1 ppm at pH* ~6.5 (the shape of the partial titration curve of this resonance[2] makes it difficult to establish whether this shift arises from a change in pK or simply from a change in C2 proton shielding in the protonated state). The pK of the histidine residue giving rise to resonance E, residue H_E, is decreased from 6.54 to 6.22 when the dimethyl ester binds, precisely the same effect as that in the methotrexate complex. Resonances A, E, and G are thus affected in the same way by the binding of either methotrexate or its dimethyl ester; these two compounds have markedly different effects, however, on resonance F. The binding of methotrexate leads to a marked increase in the pK

[1] The identification of the histidine resonances in these complexes is discussed by Birdsall et al. (1977a) and A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen (unpublished work).

[2] The limited pH range accessible (governed by the stability of the enzyme; Birdsall et al., 1977a) means that the pK values quoted for the residues corresponding to resonances A–D are only estimates. In addition, the 270-MHz data on the free enzyme (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) show that the partial titration curves of histidines A and C are not well described by the Henderson–Hasselbalch equation.

of the histidine corresponding to resonance F, H_F, from 6.67 to ~7.8. The titration curve of H_F in the enzyme–methotrexate complex is shown in Figure 9 as the dotted line. No such increase in pK is produced by the dimethyl ester; in fact, this compound produces a decrease in the pK of H_F to 5.96. This is clearly consistent with the suggestion (Roberts et al., 1974; Birdsall et al., 1977a) that H_F interacts with a glutamate carboxylate group of methotrexate, its pK being increased as a result of ion-pair formation.

Discussion

In our earlier studies of the histidine C2 proton resonances of *L. casei* MTX/R dihydrofolate reductase at 100 MHz (Roberts et al., 1974; Birdsall et al., 1977a), we observed only six resonances. Although this was consistent with the original amino acid analyses (Dann et al., 1976), the subsequent determination of the amino acid sequence of the *L. casei* MTX/R enzyme (Morris et al., 1974; Batley & Morris, 1977; Morris, 1979), as well as that of the enzyme from another, independently isolated, methotrexate-resistant strain of *L. casei* (Bitar et al., 1977; Freisheim et al., 1978), showed clearly that there are seven histidine residues. We have now detected the C2 proton signal from the seventh histidine residue. The intensity, line width, "singlet" character, and, above all, chemical shift in the denatured state of this resonance all support its assignment as a histidine C2 proton resonance. However, it does have a number of unusual characteristics which led to its being overlooked in our earlier work. In particular, the resonance is substantially to low field of the position expected for a histidine C2 proton signal: 0.83 ppm downfield of that of Gly-His-Gly (Markley, 1975). Its chemical shift is virtually independent of pH in the accessible range, indicating that this histidine has a p$K > 8.5$. The C2 proton is strikingly resistant to exchange with the solvent. These observations all suggest that the histidine residue giving rise to this resonance is in an unusual and solvent-inaccessible environment in the protein. The identity of this residue is discussed further below. Resonance G is not readily observed in spectra of the enzyme in the absence of ligands (cf. Figure 1). Unless it is more than 2 ppm to higher field in the free enzyme than in the complexes, and thus lies under the main aromatic envelope, it must be very broad. A resonance at 5.5 ppm, with a line width of 25 Hz, has been observed in samples of the free enzyme in which at least the majority of the slowly exchanging NH protons have been exchanged for deuterium (B. Birdsall and A. Gronenborn, unpublished work), but its assignement to a histidine C2 proton (rather than to a very slowly exchanging NH) has not been established with certainty. In a small protein such as this, such marked line broadening would most likely arise from an exchange process—for example, a relatively slow exchange of this histidine residue between two or more conformational states. ^1H and ^{19}F resonances from other aromatic residues of the protein (Feeney et al., 1977; Kimber et al., 1977; Roberts et al., 1977) show an analogous, though less marked, broadening in the free enzyme as compared to the complexes.

The recent determination of the three-dimensional structure of the ternary complex of *L. casei* dihydrofolate reductase with methotrexate and NADPH (Matthews et al., 1978, 1979) provides the opportunity to relate the characteristics of the individual histidine residues deduced from the crystal structure to those revealed by NMR in solution and thus to assign the proton resonances to individual residues. In making this comparison, we have used atomic coordinates (kindly provided by Dr. D. Matthews) obtained from a 2.5-Å electron density map calculated with isomorphous replacement phases and subjected to one cycle of real-space refinement. It is recognized that such coordinates may contain inaccuracies, and the arguments used to assign the histidine resonances must be based on the general environment of these residues rather than on their precise position. However, a number of different kinds of information about the environment of the histidines in solution are now available from our NMR experiments: (1) effects of ligand binding on the pK values and C2 proton chemical shifts (Roberts et al., 1974; Birdsall et al., 1977a; A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished results; present work); (2) paramagnetic relaxation effects (present work)—related to the proximity to anion binding sites; (3) deuterium-exchange rates and effects of ligands on them (present work)—related to solvent accessibility and pK; (4) photo-CIDNP effects in the presence and absence of ligands (Feeney et al., 1980)—related to the accessibility of the imidazole ring to a flavin dye molecule. Matthews (1979) has proposed tentative assignments of the histidine C2 proton singals based only on information of type 1. Although none of the above methods can give unequivocal assignments, the results taken together can be relied on with more confidence than those of one method alone. Our assignments based on these experiments are consistent with those of Matthews (1979).

Histidine Resonance F. The pK of the histidine residue giving rise to this resonance, H_F, is increased on the binding of folate or methotrexate but not trimethoprim (Birdsall et al., 1977a). This increase in pK is associated with the binding of a glutamate carboxylate group, as indicated by the observation that it is also produced by the binding of (*p*-aminobenzoyl)-L-glutamate (Roberts et al., 1974) but not by the binding of the dimethyl ester of methotrexate. Such an increase in pK is just what would be expected if one of these carboxylate groups formed an ion pair with the protonated imidazole ring. However, a conformational effect of ligand binding cannot be ruled out on this evidence alone. In both photo-CIDNP (Feeney et al., 1980) and deuterium-exchange experiments, methotrexate binding produces a striking and selective decrease in the accessibility of H_F. The clearest evidence that histidine H_F is *close* to one or both of the glutamate carboxylate groups of methotrexate in its complex with the enzyme comes from the effects of chromicyanide binding. Resonance F is one of the two signals most affected by the binding of this paramagnetic anion, and it is clearly affected by chromicyanide binding to some part of the methotrexate binding site, presumably that part normally occupied by the anionic carboxylate groups. (Chromicyanide is relatively large and may well span both carboxylate binding subsites.) The present data do not permit a quantitative estimate of the chromicyanide–histidine distance, but the fact that broadening of signal F is readily detectable at chromicyanide/enzyme ratios of 0.04, coupled with the inverse sixth power distance dependence of paramagnetic line-broadening effects, suggests that the histidine is very close to the anion binding site.

As noted in the introduction, the crystal structure of the enzyme–methotrexate–NADPH binary complex (Matthews et al., 1978, 1979) shows that His-28 forms an ion pair with the γ-carboxylate group of the bound methotrexate. Such an interaction would lead to the observed effects of methotrexate on the pK and solvent accessibility of this histidine (the effects of methotrexate on the histidine pK are the same in the presence and absence of NADPH; A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments). Therefore, our additional results strongly support the assignment of the C2 proton resonance to histi-

dine-28, as proposed by Matthews (1979).

This His-28–γ-carboxyl ion pair and that between the α-carboxyl and Arg-57 (Matthews et al., 1978) appear to contribute substantially to the binding of methotrexate, as judged by the ~700-fold weaker binding of the dimethyl ester. Johns et al. (1973) have observed much weaker binding of the dimethyl ester than of methotrexate to the reductase from L1210 cells, and a number of earlier experiments [e.g., Baker et al. (1964), Greenberg et al. (1966), and Plante et al. (1967)] also indicated an important role for the glutamate moiety in substrate analogue binding. The difference in binding energy to the *L. casei* enzyme between methotrexate and the dimethyl ester, 3.9 kcal/mol, is closely similar to the 4.0 kcal/mol binding energy of (*p*-aminobenzoyl)-L-glutamate (Birdsall et al., 1978), whose binding is probably determined largely by the glutamate (Roberts et al., 1974).

Histidine Resonance C. The chemical shift of this signal is not affected by the binding of substrates or inhibitors [Birdsall et al., 1977a; the only exception is the binding of (*p*-aminobenzoyl)-L-glutamate to a site which does not overlap with that for methotrexate]. Histidine H_C is also substantially accessible to the solvent in the free enzyme and in binary complexes with substrate or inhibitors, as shown by both the photo-CIDNP (Feeney et al., 1980) and deuterium-exchange experiments. This residue thus appears to be remote from the substrate binding site. Resonance C is broadened by low concentrations of chromicyanide and must come from a residue which is close to the coenzyme-competitive binding site(s) of this anion (cf. Figure 8). The binding of NADPH, $NADP^+$, and a series of structural analogues to the enzyme leads, in each case, to a substantial upfield shift of resonance C (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) and to a marked decrease in the accessibility of H_C (Feeney et al., 1980). These results strongly suggest that resonance C should be assigned to histidine-64, which forms an ion pair with the 2′-phosphate of the coenzyme and also forms part of the adenine ring binding site (Matthews et al., 1978, 1979). Matthews (1979) also proposed this assignment, on the assumption that the secondary binding site for (*p*-aminobenzoyl)-L-glutamate was that normally occupied by the adenine ring of the coenzyme.

Histidines-28 and -64 are at present the only two histidine residues whose C2 proton signals can be assigned with confidence. Some further partial assignments can be tentatively made on the basis of estimates of solvent accessibility. The remaining C2 proton signals can be divided into groups according to the accessibility of the corresponding histidine residues (H_A, H_B, H_D, H_E, and H_G), as judged by the photo-CIDNP (Feeney et al., 1980) and deuterium-exchange experiments. Both techniques agree in showing that H_B and H_G are essentially inaccessible, while H_A is quite significantly accessible, in the enzyme–trimethoprim complex. H_D has an exchange rate comparable to that of H_A but in the photo-CIDNP experiment gives no signal, suggesting very low accessibility. This difference most probably reflects the difference in size of the "probe" molecules used by the two methods; H_D appears to be accessible to a water molecule but not to the flavin (accessibility to different *parts* of the imidazole may also be involved). Similarly, H_E shows only slightly greater accessibility than H_A in the photo-CIDNP experiments but much faster deuterium exchange. An additional factor here is the difference in p*K* value between the two residues; the pH dependence of deuterium exchange (Markley, 1975) is such that H_E, with a p*K* ~1 unit lower (Birdsall et al., 1977a), would show substantially faster exchange than H_A even if their accessibilities were identical. In the enzyme–trimethoprim complex, therefore, the order of solvent accessibility obtained by combining the results of both methods is H_E, $H_A > H_D > H_B$, H_G.

Table II: Environments of the Histidine Residues in the *L. casei* Dihydrofolate Reductase–Methotrexate–NADPH Complex[a]

residue	nonhydrogen atoms within 6 Å of ring center	nearest neighbor	nearest charge[b]
18	30	Lys-15 Cβ	Asp-15 (5.8)
22	12	Pro-24 Cδ	*d*
28	15/10[c]		methotrexate Glu-γ-COO^- (3.0)
64	24/5[c]		NADPH 2′-phosphate (3.3)
77	22	Gln-65 O	Glu-66 (4.4)
89	42	Ile-38 Cδ	Asp-91 (7.8)
153	39	Asp-25 Oδ	Asp-25 (5.3)

[a] From the crystallographic work of Matthews et al. (1978). [b] In parentheses is the distance (Å) from ring center to charge, calculated as the average distance to the two carboxyl oxygens. [c] The first figure includes all atoms; the second includes only those of the protein, not those of the ligands. [d] None within 9 Å.

A precise specification of what is meant by "accessibility" for these two methods is not possible [see Feeney et al. (1980)]. We have therefore estimated the solvent accessibility of the histidine residues in the crystallography determined structure of the enzyme–methotrexate–NADPH ternary complex very simply by counting the number of nonhydrogen atoms (other than those of the histidine itself) within 6 Å of the center of the imidazole ring of each histidine. The results of these calculations are shown in Table II, together with an indication of the nature of the imidazole environment.

Histidine Resonances B and G. These two signals clearly arise from the two most "buried" histidine residues, and in the crystal structure these are His-89 and His-153. Although H_B and H_G differ appreciably in C2 proton chemical shift and p*K*, it is not at present possible to decide which is His-89 and which is His-153. Matthews (1979) assigned H_B as His-89 and the "unobserved" histidine (now H_G) as His-153, largely on the basis of the "unusual" environment of His-153. This is the more completely "buried" of the two and has a carboxylate group nearby, which would tend to increase its p*K*; both these facts favor assignment of H_G to His-153. On the other hand, the C2 proton signal of His-89 would be expected to experience a significant downfield shift from the ring current of Tyr-85, while that of His-153 would be affected to a negligible extent by its nearest aromatic residue, Tyr-155. Yet it is resonance G, not B, which has an unusually low-field position, so the distinction between the two is not unambiguous.

Histidine Resonance A. Although in the enzyme–trimethoprim complex H_A and H_E show comparable degrees of accessibility, photo-CIDNP experiments on the enzyme–methotrexate–$NADP^+$ complex (Feeney et al., 1980) show that H_A is by far the most accessible histidine residue in this ternary complex. Assuming that this ternary complex is similar to that containing NADPH[3] —at least in this respect—examination of the crystal structure of the latter strongly suggests that H_A is His-22 (see Table II). The C2 proton chemical shift of H_A is affected similarly by the binding of almost all ligands, suggesting that the shift is conformational in origin (Birdsall

[3] Complexes containing NADPH could not be studied by the photo-CIDNP method, owing to a photochemical reaction between NADPH and the flavin dye (Feeney et al., 1980).

Table III: Proposed Assignments of the Histidine C2 Proton Resonances of *L. casei* Dihydrofolate Reductase

resonance	residue[a]
A	His-22 (18 or 22)
B	His-89 or His-153 (89)
C	His-64 (64)
D	His-18 or His-77 (77)
E	His-18 or His-77 (18 or 22)
F	His-28 (28)
G	His-89 or His-153 (153)

[a] Residue numbers in parentheses indicate the assignments proposed by Matthews (1979).

et al., 1977a), and the involvement of H_A in a ligand-induced conformational change has been conclusively demonstrated by the observation that its accessibility to the flavin *increases* on methotrexate binding (Feeney et al., 1980). Matthews et al. (1978, 1979) have proposed that the loop of residues 12–22 may change conformation on ligand binding, in agreement with the NMR observations and with this tentative assignment.

Histidine Resonances D and E. By elimination, these must be assigned to His-18 and His-77, residues which are largely, though not totally, inaccessible in the ternary complex (Table II). Matthews (1979) assigned H_D as His-77 and H_E as His-18 or His-22, noting that His-77, being hydrogen bonded to the backbone carbonyl of Gln-65 and close to the carboxylate of Glu-66, should have a high pK value. Histidine H_D does indeed have a pK value of the order of 7.8 (Birdsall et al., 1977a; A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished results; only H_G has a higher pK). However, there is a carboxylate group almost as close to His-18 (Table II), and, without detailed calculations (Shire et al., 1974; Botelho et al., 1978) it is not possible to make a firm assignment on this basis, even though the pK values of H_D and H_E differ by more than 1 unit.

The partial assignments made in the present paper are summarized in Table III; given the ambiguity in assignment between pairs of residues, they are consistent with those of Matthews (1979), as indicated in Table III. Chemical evidence for the sites of deuterium exchange (Markley, 1975) will be required to remove these ambiguities.

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References

Baker, B. R., Santi, D. V., Almaula, P. L., & Werkheiser, W. C. (1964) *J. Med. Chem. 7*, 24.

Batley, K., & Morris, H. R. (1977) *Biochem. Biophys. Res. Commun. 75*, 1010.

Bigelow, J. H. (1946) *Inorg. Synth. 2*, 203.

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

Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1977b) *FEBS Lett. 80*, 313.

Birdsall, B., Burgen, A. S. V., Rodrigues de Miranda, J., & Roberts, G. C. K. (1978) *Biochemistry 17*, 2102.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980) *Biochemistry* (in press).

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

Botelho, L. H., Friend, S. H., Matthew, J. B., Lehman, L. D., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochemistry 17*, 5197.

Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V., & Harding, N. G. L. (1976) *Biochem. J. 157*, 559.

Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356.

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

Feeney, J., Roberts, G. C. K., Kaptein, R., Birdsall, B., Gronenborn, A., & Burgen, A. S. V. (1980) *Biochemistry* (in press).

Freisheim, J. H., Bitar, K. G., Reddy, A. V., & Blankenship, D. T. (1978) *J. Biol. Chem. 253*, 6437.

Greenberg, D. M., Tam, B.-D., Jenny, E., & Payes, B. (1966) *Biochim. Biophys. Acta 122*, 423.

Hood, K., & Roberts, G. C. K. (1978) *Biochem. J. 171*, 357.

Johns, D. G., Farquar, D., Wolpert, M. K., Chabner, B. A., & Loo, T. L. (1973) *Drug. Metab. Dispos. 1*, 580.

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.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70.

Matthews, D. A. (1979) *Biochemistry 18*, 1602.

Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946.

Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N., & Kraut, J. (1979) *J. Biol. Chem. 254*, 4144.

Meadows, D. H., Jardetzky, O., Epand, R., Ruterjans, H., & Scheraga, H. A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 766.

Morris, H. R. (1979) *Philos. Trans. R. Soc. London, Ser. A 293*, 39.

Morris, H. R., Batley, K. E., Harding, N. G. L., Bjur, R. A., Dann, J. G., & King, R. W. (1974) *Biochem. J. 137*, 409.

Pfiffner, J. J., Binkley, S. B., Bloom, E. S. & O'Dell, B. L. (1947) *J. Am. Chem. Soc. 69*, 1476.

Plante, L. T., Crawford, E. J., & Friedkin, M. (1967) *J. Biol. Chem. 242*, 1466.

Roberts, G. C. K., Feeney, J., Burgen, A. S. V., Dann, J. G., Yuferov, V., & Bjur, R. A. (1974) *Biochemistry 13*, 5351.

Roberts, G. C. K., Feeney, J., Birdsall, B., Kimber, B. J., Griffiths, D. V., King, R. W., & Burgen, A. S. V. (1977) in *Nmr in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) p 95, Academic Press, London.

Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974) *Biochemistry 13*, 2967.