Effects of Coenzyme Binding on Histidine Residues of *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The effects of coenzyme binding on the seven histidine C2 proton resonances of *Lactobacillus casei* dihydrofolate reductase have been determined. Binary complexes containing $NADP^+$, NADPH, and their hypoxanthine, thionicotinamide, and acetylpyridine analogues, together with ternary complexes containing the inhibitors trimethoprim or methotrexate, have been examined. Four of the histidine residues are affected by coenzyme binding. The largest effect—a marked upfield shift (0.85 ppm) of the C2 proton resonance—is seen for His-64. The hypoxanthine analogue of the coenzyme was found to produce a smaller upfield shift and, in addition, a decrease in the p*K* of His-64. The effects on this residue are discussed in the light of the crystal structure [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], and it is concluded that His-64 is close to a carboxyl group in the free enzyme and that the hypoxanthine ring binds in a somewhat different orientation to the adenine ring. The effects on histidine resonances A, E, and G are significantly different for oxidized and reduced coenzymes. The changes in p*K* of the histidines giving rise to resonances A and E (probably His-22 and His-18) are discussed in terms of ligand-induced conformational changes, which differ for $NADP^+$ and NADPH.

Dihydrofolate reductase is one of the smallest known pyridine nucleotide-linked dehydrogenases, the enzyme from *Lactobacillus casei* having 162 residues (Bitar et al., 1977). The binding of the coenzyme to this enzyme is of interest not only in its own right but also because it substantially increases the binding constants of the therapeutically important inhibitors methotrexate and trimethoprim (Birdsall et al., 1980). The binding of NADPH[1] in the presence of methotrexate has been characterized crystallographically (Matthews et al., 1979), and we have studied the binding of a number of coenzyme analogues by ^1H and ^{31}P NMR (Feeney et al., 1975; Hyde et al., 1980a,b). The latter experiments showed clearly that the mode of binding varies significantly from one coenzyme analogue to another and is also altered on addition of substrate or inhibitors. To obtain further information on the nature and extent of these structural differences, we have now extended our earlier ^1H NMR studies of the histidine residues of the enzyme (Birdsall et al., 1977) to examine the effects of coenzyme binding to the enzyme and enzyme–inhibitor complexes.

Materials and Methods

Dihydrofolate reductase was isolated and purified from *L. casei* MTX/R as described by Dann et al. (1976). The enzyme was lyophilized twice from D_2O to remove readily exchangeable protons. In studies of histidine C2-H resonances, it is important to remove the interfering resonances from slowly exchanging amide protons. To achieve this, we dissolved the enzyme sample in D_2O to a concentration of ~0.5 mM and left it at room temperature for 40 h. After a further lyophilization, the enzyme was finally taken up in D_2O to give a protein concentration of ~1 mM in 50 mM potassium phosphate, 500 mM KCl, 1 mM EDTA, pH* 6.5 (pH* indicates a pH meter reading uncorrected for the isotope effect on the glass electrode). All samples also contained 1 mM dioxane as a chemical shift reference (the dioxane ^1H resonance is 3.71 ppm downfield from DSS). The enzyme concentration was determined by assaying its activity, by measuring its absorbance at 280 nm, and by fluorometric titration with methotrexate, using procedures described by Dann et al. (1976). $NADP^+$, NADPH, and the coenzyme analogues[1] $NHDP^+$, NHDPH, $APADP^+$, and $TNADP^+$ were obtained from Sigma Chemical Co. Ltd. (London). $APADP^+$ and $TNADP^+$ were converted to their reduced forms by using isocitrate and isocitrate dehydrogenase as outlined by Dunn et al. (1978) and concentrated on a column of diethylaminoethylcellulose, followed by lyophilization. PADPR-OMe was prepared by the calf spleen NAD^+ glycohydrolase catalyzed methanolysis of $NADP^+$ (Pascal & Schuber, 1976) and purified on a column of diethylaminoethylcellulose (Whatman DE52). Methotrexate (Nutritional Biochemical Corp.), trimethoprim (Wellcome Laboratories), and folic acid (Sigma) were used without further purification.

^1H NMR spectra were obtained at 270 MHz by using a Bruker WH270 spectrometer. The sample (0.35 mL) was maintained at 11 (±1) °C. A spectral width of 4.2 kHz was used, and either 4096 or 8192 data points were collected (pulse interval 0.5–1.0 s) by using quadrature detection; 500 transients were averaged. Before Fourier transformation, the free induction decay was multiplied by an exponential function corresponding to a line broadening of 2 Hz, and, if 4096 data points had been collected, the data table was filled to 8192 data points with zeros to give a final digital resolution of 1 Hz/point. ^{31}P NMR spectra were obtained at 40.5 MHz by using a Varian XL-100 spectrometer. The sample (1.4 mL) was maintained at 11 (±1) °C. Noise-modulated proton decoupling was employed for all spectra, which were acquired in the block-averaging mode; typically 300 blocks of 200 spectra were averaged. An acquisition time of 0.5 s was used

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[1] Abbreviations used: $NHDP^+$, nicotinamide hypoxanthine dinucleotide phosphate; $APADP^+$, acetylpyridine adenine dinucleotide phosphate; $TNADP^+$, thionicotinamide adenine dinucleotide phosphate; corresponding abbreviations (NHDPH, etc.) for the reduced forms; PADPR-OMe, β-methyl riboside of 2′-phosphoadenosine 5′-diphosphoribose; EDTA, ethylenediaminetetraacetic acid; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate.

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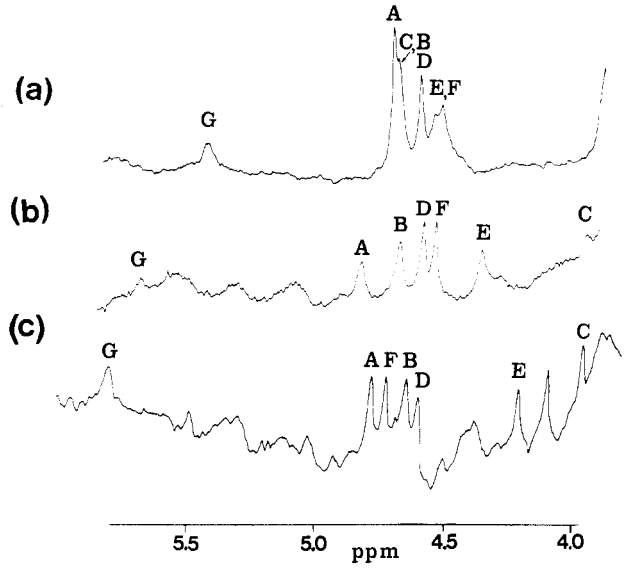

FIGURE 1: Histidine C2 proton resonance region of the 270-MHz ^1H NMR spectrum of (a) *L. casei* dihydrofolate reductase, (b) dihydrofolate reductase–$NADP^+$ complex, and (c) dihydrofolate reductase–$NADP^+$–methotrexate complex.

for a spectral width of 2 kHz. Before Fourier transformation, the free induction decay was multiplied by an exponential function corresponding to a line broadening of 1.6 Hz. ^{31}P chemical shifts are expressed relative to inorganic phosphate at pH* 8.0 (this is 2.84 ppm downfield from H_3PO_4); downfield shifts are positive.

The pH of the samples was adjusted by addition of microliter volumes of 0.1–1.0 N KOD or DCl [>99 atom % D; CIBA (ARL) Ltd.] and measured by using a combination glass/reference electrode and a Radiometer Model 26 pH meter. The pH* was checked after each spectrum, and the spectrum was accepted only if this was within 0.04 unit of the reading before the spectrum was acquired.

The titration curves were analyzed by nonlinear regression methods. In most cases, the curves were fit to the Henderson–Hasselbalch equation, with δ_{HA} (the C2 proton chemical shift of the protonated imidazole), δ_A (the shift of the unprotonated species), and the pK as variables. When only partial titration curves could be obtained, $\delta_{HA} - \delta_A$ was constrained to the range 0.9–1.0 ppm. The titration curves of resonances A and C of the free enzyme and that of resonance C in the enzyme–methotrexate complex were not satisfactorily fit by the Henderson–Hasselbalch equation (see text); they were described in terms of two independent ionizations by using a five-parameter (pK_1, pK_2, δ_{H_2A}, δ_{HA}, and δ_A) least-squares fit.

Results

Enzyme Alone. The region of the 270-MHz ^1H NMR spectrum of dihydrofolate reductase containing the C2-H resonances of the histidine residues is shown in Figure 1, where the resonances are labeled as in our earlier papers (Birdsall et al., 1977; Wyeth et al., 1980). In previous studies at 100 MHz (Roberts et al., 1974; Birdsall et al., 1977), only the six resonances A–F were recognized. We have recently described the identification of the seventh resonance, G, at very low field (5.86 ppm) in the enzyme–methotrexate and enzyme–trimethoprim complexes (Wyeth et al., 1980). With the development of improved procedures for the exchange of amide protons (see Materials and Methods), we can now observe a broad signal at 5.53 ppm in the spectrum of the enzyme alone (Figure 1a). On addition of increasing amounts of *p*-aminobenzoyl-L-glutamate, a weakly binding inhibitor which interacts with the enzyme in a way similar to the corresponding part of methotrexate (Birdsall et al., 1977; Feeney et al., 1977; Roberts et al., 1977), this resonance shifts progressively downfield from 5.53 to 5.80 ppm, confirming that it is the histidine C2-H signal G identified in the methotrexate complex.

With the improved resolution obtained at 270 MHz, it became apparent that the pH dependence of the chemical shifts of resonances A and C in the spectra of the free enzyme could not be satisfactorily described by the simple Henderson–Hasselbalch equation. In both cases, the data were much better fit by using two pK values, one of ~7.5, responsible for 80% of the chemical shift change, and another of ~5.2, responsible for the remaining 20%. Because resonances A and C overlap with others at some pH values and because the instability of the enzyme at pH* ≥8 prevents us from obtaining complete titration curves for these residues, we have not attempted to fit the data with specific models [see, for example, Cohen & Shindo (1975) and Markley & Finkenstadt (1975)]. The analysis in terms of two independent pK values is intended only as a convenient description of the data. As will be seen below, resonances A and C both give normal Henderson–Hasselbalch titration curves in the complexes with coenzyme.

Enzyme–Coenzyme Complexes. The histidine C2-H resonances of the enzyme–$NADP^+$ and enzyme–methotrexate–$NADP^+$ complexes are shown in Figure 1b,c. In these complexes, *two* histidine resonances are found to have unusual chemical shifts. Resonance G can be identified at low field; although it remains broad in the binary complex, it sharpens markedly on addition of methotrexate. A comparison of the pH dependence of the spectrum of the enzyme–methotrexate–$NADP^+$ complex (see below) with that of the enzyme–methotrexate complex (Birdsall et al., 1977) shows that the second resonance missing from the "normal" region of 4.5–5.0 ppm in Figure 1c is resonance C. A sharp resonance with the pH dependence characteristic of a histidine C2-H signal can be seen at 3.94 ppm in Figure 1c. The identification of this as resonance C was confirmed by the transfer of saturation experiments shown in Figure 2.

The sample used for this experiment contained 1 mM enzyme, 1 mM methotrexate, and 0.5 mM $NADP^+$ and thus consisted of a 1:1 mixture of the enzyme–methotrexate and enzyme–methotrexate–$NADP^+$ complexes. Irradiation at 4.7 ppm, the position of resonance C in the binary enzyme–methotrexate complex (Birdsall et al., 1977), led to a marked decrease in the intensity of the resonance at 3.94 ppm. This arises from a transfer of saturation from the binary to the ternary complex as the two are interconverted by the exchange of $NADP^+$ on and off the enzyme. Although all the five histidine resonances near 4.7 ppm are affected by the irradiation shown in Figure 2, systematic low-power irradiation at a series of frequencies in this region of the spectrum showed that the maximum effect on the signal at 3.94 ppm was obtained by irradiation at 4.7 ppm, the position of resonance C. In the presence of $NADP^+$, then, histidine resonance C is shifted substantially (~0.8 ppm) upfield. The remaining histidine resonances in the spectra of the binary and ternary coenzyme complexes can be readily identified by comparison with the spectra of the enzyme alone and of enzyme–inhibitor binary complexes, respectively.

In order to characterize the effects of coenzyme binding on the histidine residues, we have determined the pH dependence of the C2-H resonances for a series of nine binary and six ternary complexes. A typical set of titration curves for the

Table I: Chemical Shifts[a] and pK Values of Histidine Residues of Dihydrofolate Reductase in Its Complexes with Coenzymes

	resonance														
	A		B		C		D		E			F			G
coenzyme	δ_{HA}	pK[b]	δ_{HA}	pK[b]	δ_{HA}	pK[b]	δ_{HA}[c]	pK[b]	δ_{HA}	$\Delta\delta$[d]	pK[b]	δ_{HA}	$\Delta\delta$[d]	pK[d]	δ_{HA}
none	5.0[e]	5.1[e]	4.80	7.4	5.0[e]	5.2[e]	4.65	7.9	4.91	1.00	6.67	4.94	1.00	6.57	5.51
	4.80[e]	7.5[e]			4.78[e]	7.7[e]									
NADP$^+$	4.92	7.65	4.82	7.25	4.04	7.55	4.66	7.85	4.94	1.00	6.35	4.94	0.94	6.59	5.70
NHDP$^+$	4.90	7.8	4.80	7.3	4.44	6.75	4.65	7.85	4.98	0.93	6.18	4.92	1.00	6.62	*f*
TNADP$^+$	*f*		4.85	7.25	4.06	7.7	4.65	7.9	4.94	0.90	6.37	4.94	0.90	6.57	*f*
APADP$^+$	*f*		4.80	*f*	4.02	*f*	4.65	*f*	4.9	1.0[g]	6.4	4.9	1.0[g]	6.4	*f*
PADPR-OMe	4.86	7.6	4.78	7.5	4.03	7.8	4.65	7.8	4.93	0.92	6.50	4.91	0.90	6.53	5.56
NADPH	4.90	7.9	4.80	7.3	4.03[c]	7.7	4.66	7.8	4.92	0.94	6.66	4.89	0.89	6.55	5.53
NHDPH	4.92	7.9	4.82	7.3	4.45	6.9	4.65	7.9	4.92	1.00	6.89	4.94	1.00	6.75	5.48
TNADPH	4.90	7.85	4.77	7.45	4.02[c]	7.7	4.64	7.9	4.88	0.9[g]	6.57	4.88	0.9[g]	6.57	5.47
APADPH	4.90	7.9	4.78	7.4	4.04[c]	7.6	4.65	7.9	4.87	0.95	6.76	4.87	0.95	6.76	5.56

[a] δ_{HA}, chemical shift of C2 proton resonance of protonated imidazole (ppm from dioxane ± 0.03 ppm unless otherwise noted). [b] pK values derived from incomplete titration curves by constraining $\delta_{HA} - \delta_A$ to 0.9–1.0 ppm; ±0.1 unit. [c] Clearly resolved at low pH; ±0.01 ppm. [d] $\Delta\delta$ ($=\delta_{HA} - \delta_A$) and pK obtained from complete titration curve; ±0.05 ppm and ±0.05 unit, respectively. [e] Titration curve does not fit Henderson–Hasselbalch equation; characterized in terms of two independent pK values (see text) with the total chemical shift change $\Delta\delta$ ($=\delta_{H_2A} - \delta_A$) constrained to 1.0 ppm. [f] Line broadened by exchange effects; reliable value could not be obtained. [g] $\Delta\delta$ fixed since exchange broadening made it difficult to follow the full titration curve.

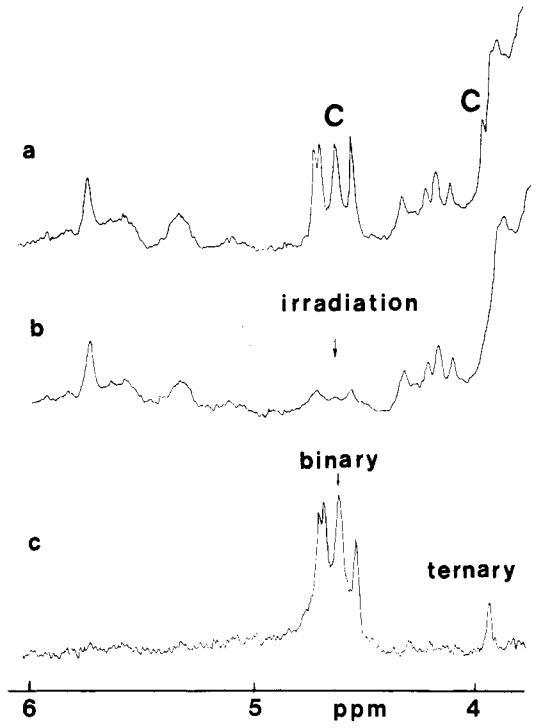

FIGURE 2: Histidine C2 proton resonance region of the 270-MHz ^{1}H NMR spectrum of a sample containing dihydrofolate reductase, methotrexate (1 mol/mol of enzyme) and NADP$^+$ (0.5 mol/mol of enzyme). (a) Control; (b) with irradiation at 4.65 ppm; (c) difference a – b.

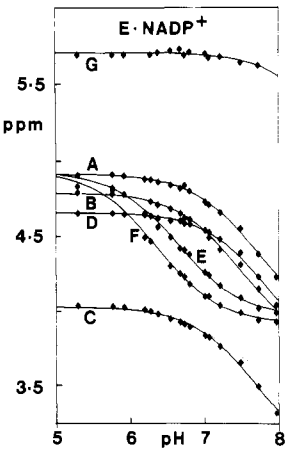

FIGURE 3: pH dependence of histidine C2 proton resonances of the dihydrofolate reductase–NADP$^+$ complex. The points are experimental and the lines the "best-fit" Henderson–Hasselbalch curves calculated by using the parameters in Table I. The *dotted* line shows the behavior of resonance C in the enzyme–NHDP$^+$ complex.

enzyme–NADP$^+$ complex is shown in Figure 3. For this and all other coenzyme complexes examined, all the data could be satisfactorily described by the Henderson–Hasselbalch equation. The limiting chemical shifts and pK values obtained by fitting the data for each histidine resonance to this equation are summarized in Tables I and II. The limited range of pH over which the enzyme is stable makes it impossible to obtain complete titration curves for most of the histidines. Incomplete titration curves, such as those of resonances A, B, C, and D in Figure 3, were analyzed by constraining $\delta_{HA} - \delta_A$, the chemical shift difference between protonated and unprotonated forms, to the range 0.9–1.0 ppm, observed for resonances E and F. The pH dependence of the chemical shift of resonance G was too small to permit any estimate of the pK value of the corresponding histidine other than a lower limit of ~8.0.

The *changes* in chemical shift or pK of the various histidine residues on coenzyme binding to form the binary complexes are summarized in Table III to facilitate a comparison of the effects of the various coenzyme analogues. Within experimental uncertainty, histidine resonances B, D, and F are unaffected by coenzyme binding. The histidine giving rise to resonance E, H_E, has a somewhat lower pK in the presence of oxidized coenzymes (6.18–6.4) than the enzyme alone (6.67) or the complexes with reduced coenzymes (6.57–6.89). Comparison of the behavior of resonances A and C is made more difficult by the change in the shape of their titration curves on ligand binding. However, as noted earlier for substrate and inhibitor binding (Birdsall et al., 1977), addition of coenzyme leads to a distinct downfield shift (~0.1 ppm) of resonance A at pH* 6.5. The pK of H_A also seems to be about 0.2–0.3 unit higher in complexes with reduced coenzymes than in those with oxidized coenzymes.

By far the largest effect of coenzyme binding on any histidine resonance is that on resonance C. As shown in Figures 1–3, it is shifted upfield by 0.8 ppm (at low pH) on the binding of NADP$^+$, with little if any effect on the histidine pK. Essentially identical effects are seen with all the coenzyme analogues examined, with the notable exception of the hypoxanthine analogues NHDP$^+$ and NHDPH. As shown for

Table II: Chemical Shifts[a] and pK Values of Histidine Residues of Dihydrofolate Reductase in Its Ternary Complexes with Coenzymes and Inhibitors

		resonance														
		A		B		C		D		E			F			G
inhibitor	coenzyme	δ_{HA}	pK[b]	δ_{HA}	pK[b]	δ_{HA}	pK[b]	δ_{HA}[c]	pK[b]	δ_{HA}	$\Delta\delta$[d]	pK[d]	δ_{HA}	$\Delta\delta$	pK	δ_{HA}[c]
						5.0[f]	5.2[f]									
methotrexate	none	4.89	7.45	4.78	7.35	4.78[f]	7.5[f]	4.65	7.85	4.95	1.00	6.21	4.78	*b*	7.8[b]	5.87
	NADP$^+$	4.92	7.35	4.79	7.35	4.03[c]	7.5	4.65	7.9	5.00	1.00	6.00	4.79	*b*	7.8[b]	5.86
	NADPH	4.89	7.45	4.81	7.35	4.04[c]	7.65	4.65	7.85	4.99	1.10	6.12	4.80	*b*	7.8[b]	5.87
trimethoprim	none[e]	4.95	7.5	4.77	7.4	4.77	7.4	4.63	7.7	4.96	1.00	5.92	4.94	1.00[d]	6.53[d]	5.80
	NADP$^+$	4.97	7.35	4.78	7.45	4.01[c]	7.7	4.65	7.75	4.92	1.00	6.02	4.97	1.00[d]	6.46[d]	5.85
	NHDP$^+$	4.94	7.65	4.79	7.35	4.44[c]	6.8	4.64	8.0	4.91	0.95	6.10	4.97	0.95[d]	6.61[d]	5.85
	TNADP$^+$	5.00	7.4	4.78	7.45	4.05[c]	7.5	4.65	7.9	4.95	1.00	6.00	4.97	0.96[d]	6.53[d]	5.87
	NADPH	4.94	7.85	4.79	7.35	4.03[c]	7.65	4.66	7.8	4.91	1.00	6.05	4.97	1.00[d]	6.59[d]	5.87

[a] δ_{HA}, chemical shift of C2 proton resonance of protonated imidazole (ppm from dioxane ± 0.03 ppm unless otherwise noted). [b] pK values derived from incomplete titration curves by constraining $\delta_{HA} - \delta_A$ to 0.9–1.0 ppm; ±0.1 unit. [c] Clearly resolved at low pH; ±0.01 ppm. [d] $\Delta\delta$ ($=\delta_{HA} - \delta_A$) and pK obtained from complete titration curve; ± 0.05 ppm and ± 0.05 unit, respectively. [e] From Birdsall et al. (1977); resonance G from 270-MHz experiments. [f] Characterized in terms of two independent pK values (see text).

Table III: Changes in Chemical Shift or pK of Histidine Residues of Dihydrofolate Reductase on Forming Binary Coenzyme Complexes[a]

	resonance				
	A	C		E	G
coenzyme	ΔpK[b]	$\Delta\delta_{HA}$	ΔpK[b]	ΔpK	$\Delta\delta_{HA}$
NADP$^+$	−0.05	−0.85	−0.15	−0.3	+0.2
NHDP$^+$	+0.05	−0.45	−0.95	−0.5	*c*
TNADP$^+$	*c*	−0.8	0	−0.3	*c*
APADP$^+$	*c*	−0.85	*c*	−0.25	*c*
PADPR-OMe	−0.1	−0.85	+0.1	−0.15	+0.05
NADPH	+0.2	−0.85	0	0	0
NHDPH	+0.2	−0.45	−0.8	0	0
TNADPH	+0.15	−0.85	0	−0.1	−0.05
APADPH	+0.2	−0.85	−0.1	+0.1	+0.05

[a] Only those histidines for which there is a significant change are included. Changes are quoted to the nearest 0.05 ppm for $\Delta\delta_{HA}$ (downfield positive) and to the nearest 0.05 unit for ΔpK; changes > 0.1 in either quantity are probably significant. [b] Quoted relative to the higher of the two pKs characterizing the titration of this resonance in the free enzyme. [c] Not measurable.

Table IV: Changes in Chemical Shift or pK of Histidine Residues of Dihydrofolate Reductase on Coenzyme Binding to Enzyme-Inhibitor Complexes[a]

		resonance			
		A	C		E
inhibitor	coenzyme	ΔpK	$\Delta\delta_{HA}$	ΔpK	ΔpK
methotrexate	NADP$^+$	−0.1	−0.8	+0.05	−0.2
	NADPH	+0.05	−0.75	+0.15	−0.1
trimethoprim	NADP$^+$	−0.15	−0.75	+0.3	+0.1
	NHDP$^+$	+0.15	−0.35	−0.6	+0.2
	TNADP$^+$	−0.1	−0.75	+0.25	+0.1
	NADPH	+0.35	−0.7	+0.25	+0.15

[a] Only those histidines for which there is a significant change are included. Changes are quoted to the nearest 0.05 ppm for $\Delta\delta_{HA}$ and to the nearest 0.05 unit for ΔpK; changes > 0.1 in either quantity are probably significant.

NHDP$^+$ by the dashed curve in Figure 3, these analogues produce a much smaller upfield shift (0.4 vs. 0.8 ppm) of resonance C, together with a marked (~0.8 unit) decrease in the histidine pK value. Since resonance C arises from a histidine in close proximity to the 2′-phosphate group of the bound coenzyme (see below), we have also compared the behavior of the ^{31}P resonance of the 2′-phosphate in the enzyme–NADP$^+$ and –NHDP$^+$ complexes. In the NADP$^+$ complex, this resonance appears at 2.72 ppm (1.7 ppm downfield from the resonance position of the dianionic 2′-phosphate of free NADP$^+$) and is pH independent over the range 5–8 (Feeney et al., 1975). In contrast, the corresponding signal of bound NHDP$^+$ shifts from 2.47 ppm at pH* 5.9 to 2.82 ppm at pH* 8.8, the pH dependence being characterized by a "pK" of ~7.

The effects of coenzyme binding to the enzyme–methotrexate and enzyme–trimethoprim complexes (Table IV) are in general closely similar to those observed in the binary complexes. Resonances B, D, and F are again unaffected, while the 0.8-ppm upfield shift of resonance C is still observed. Neither NADP$^+$ nor NADPH produce any appreciable change in the pK of H_C. However, in the presence of trimethoprim, as for the free enzyme, NHDP$^+$ produces a smaller upfield shift and a decrease in pK of this histidine.

There are two clear differences in the coenzyme effects between the binary and ternary complexes. First, coenzyme binding to the enzyme–inhibitor complexes produces no additional downfield shift of resonance A beyond that already produced by the inhibitors. Second, the decrease in pK of H_E produced by oxidized coenzymes in the binary complexes is either much reduced or absent in the ternary complexes. In the enzyme–trimethoprim–NADP$^+$ complex, both resonances E and F appear as "doublets" in the pH range 6–7 with a "splitting" of 0.04 ppm. We have shown that this "splitting" arises from the coexistence of two slowly interconverting conformational forms of this complex; the characterization of these two conformations is described elsewhere (Gronenborn et al., 1981).

Discussion

The assignment of the seven histidine C2-H resonances of dihydrofolate reductase to individual residues in the sequence is as yet incomplete (Matthews, 1979; Wyeth et al., 1980). Resonance C can be assigned with some confidence to His-64, as can resonance F to His-28, while resonance A has been assigned with rather less certainty to His-22 (Feeney et al., 1980; Wyeth et al., 1980). Resonances B and G probably arise from His-89 and His-153 and resonances D and E from His-18 and His-77, but no firm assignments of these signals have yet been made.

In the crystal structure of the enzyme–methotrexate–NADPH complex (Matthews et al., 1978, 1979), only one histidine residue, His-64, has its imidazole ring in direct contact with the coenzyme. The relationship of His-64 to the adenine ring and 2′-phosphate of the coenzyme in the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979) is shown in Figure 4. The imidazole ring partially overlaps

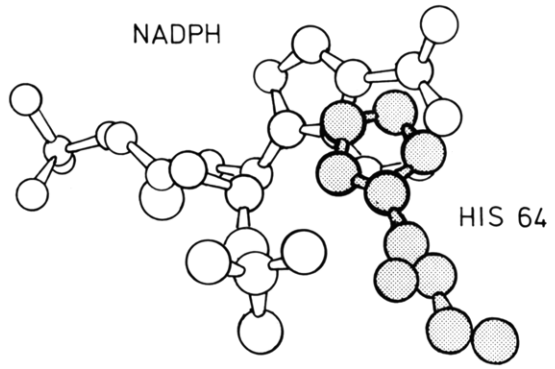

FIGURE 4: Relationship of His-64 to the adenosine 2′-phosphate moiety of NADPH in the enzyme–methotrexate–NADPH complex. Based on the crystallographic work of Matthews et al. (1978, 1979).

and is in van der Waals contact with the six-membered ring moiety of the adenine, although the imidazole and adenine rings are not coplanar. At the same time, $N_{\delta 1}$ of the imidazole is within hydrogen-bonding distance of one of the oxygen atoms of the 2′-phosphate. The proximity of the C2 proton of His-64 to the adenine suggests that the large upfield shift of its resonance (resonance C) on coenzyme binding might arise from the ring current shielding contribution from the adenine ring. We have calculated the ring current shift expected on the basis of the crystal structure by using the Johnson-Bovey (Johnson & Bovey, 1958) equation with the parameters proposed for adenine and hypoxanthine by Giessner-Prettre & Pullman (1970) [see also Giessner-Prettre et al. (1976)]. The calculated upfield shift for the C2 proton resonance is 0.79 ppm from the adenine ring and 0.33 ppm from the hypoxanthine ring. These are close to the observed chemical shift changes of 0.85 and 0.45 ppm, respectively, and are thus consistent with the idea that the relationship between His-64 and the adenine (or hypoxanthine) ring is similar, in all the coenzyme complexes, to that observed in the crystal. In addition, the resonance of the adenine 2-proton shifts substantially upfield when the coenzyme binds (Hyde et al., 1980a), as would be expected from the geometry shown in Figure 4, since it will be affected by the imidazole ring current.

The proximity of His-64 to the 2′-phosphate group would also be expected to produce a substantial decrease in the pK of the 2′-phosphate and an increase in the histidine pK. The pK of the 2′-phosphate is decreased by at least 3 units on binding (Feeney et al., 1975), but there is no indication of an increase of more than 0.3 unit in the pK of His-64 on the binding of any of the coenzymes. Indeed, the hypoxanthine analogues produce a *decrease* of 0.6–0.95 unit in the pK of His-64.

To reconcile these observations with the interaction between His-64 and the 2′-phosphate revealed in the crystal structure, it is necessary to postulate that in the free enzyme His-64 is close to an anionic (carboxylate) group of the protein. Coenzyme binding would then involve replacing one imidazole–anion interaction with another, having little net effect on the imidazole pK. This proposal would also be consistent with the observation that, in the free enzyme and the enzyme–methotrexate complex, the chemical shift of the C2 proton of His-64 is influenced by *two* ionizable groups in the pH range 5–8.

The markedly different effect of $NADP^+$ and $NHDP^+$ on the pK of His-64 and the different behavior of the 2′-phosphate resonance in these two cases show clearly that the relative positions of His-64 and the 2′-phosphate are *not* the same in these two complexes. Since the chemical differences between $NADP^+$ and $NHDP^+$ are in the heterocyclic ring, the difference in the His-64–2′-phosphate relationship must presumably originate from a difference in the orientation of this ring or in the relationship of His-64 to it. This is in contrast to the impression given by the ring current shift calculations. However, we have been able to measure the shift of only one resonance from His-64, and it is clear that there will be a number of possible orientations of the imidazole relative to the hypoxanthine ring which would give very similar shifts of this resonance and yet very different imidazole–phosphate interactions. Thus (in contrast to earlier conclusions from more limited data; Birdsall et al., 1980), modification of the adenine ring of the coenzyme, like modification of the nicotinamide ring (Hyde et al., 1980a,b), does lead to a detectable difference in the mode of binding of the coenzyme.

Since there are no other histidine residues in contact with the coenzyme, the effects of coenzyme binding on resonances A, E, and G (histidine residues 22, 18 or 77, and 89 or 153) must be attributed to ligand-induced conformational changes. The effects on these three residues have a number of features in common. First, oxidized and reduced coenzymes have different effects in each case, while the structural analogues such as TNADP(H) have very similar effects to the natural coenzymes. Second, all three histidines are also affected by inhibitor binding, and the effects of inhibitor and coenzyme are not independent. For example, resonance A is shifted downfield by ~0.1 ppm at pH* 6.5 on binding either coenzyme or inhibitor, but there is no further shift on forming the ternary complex. Trimethoprim, methotrexate, and $NADP^+$ all produce a decrease in the pK of H_E (probably His-18), but when $NADP^+$ binds to the enzyme–trimethoprim complex it leads to an increase in the pK of this residue. This interdependence of the conformational effects of coenzyme and inhibitors may be related to the cooperativity in binding between these two groups of ligands (Birdsall et al., 1980).

Matthews et al. (1978), comparing the crystal structure of the methotrexate–NADPH complex of the *L. casei* enzyme and the methotrexate complex of the *Escherichia coli* enzyme (Matthews et al., 1977), have suggested that a "loop" comprising residues 12–22 changes conformation on NADPH binding. This change in conformation might then account for the observed effects of coenzyme binding on resonance A (His-22) and also for those on resonance E, if this arises from His-18. We have recently shown (Feeney et al., 1980) that the surface accessibility of His-22 (resonance A) *increases* on addition of inhibitors to the enzyme–$NADP^+$ complex, demonstrating that inhibitors as well as coenzymes can change the conformation of this region of the protein. The NMR evidence thus suggests that this loop differs in conformation between complexes containing oxidized and reduced coenzyme and that it is most probably involved in the conformational changes responsible for coenzyme–inhibitor cooperativity.

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Studies on the Microsomal Mixed-Function Oxidase System: Mechanism of Action of Hepatic NADPH–Cytochrome P-450 Reductase†

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ABSTRACT: The mechanism of hepatic NADPH–cytochrome P-450 reductase has been investigated by using a stopped-flow technique. The reduction of the oxidized native enzyme (FAD–FMN) by NADPH proceeds by both one-electron equivalent and two-electron equivalent mechanisms. The air-stable semiquinone form (FAD–FMNH·) of the native enzyme, which is characterized by an absorption shoulder at 635 nm, is also rapidly reduced to another semiquinone form (FADH·–$FMNH_2$) by NADPH with the disappearance of the shoulder at 635 nm, but the absorbance change at 585 nm is relatively constant. The FAD moiety in the FMN-depleted enzyme is rapidly reduced by NADPH, and reduced FAD is oxidized in successive one-electron steps by O_2 or potassium ferricyanide. These results indicate the possibility of intramolecular one-electron transfer between FAD and FMN. The rate of cytochrome P-450 reduction decreases in the presence of FMN-depleted enzyme but is nearly restored to the value of the original enzyme with FMN-reconstituted enzyme. These data suggest that FAD is the low-potential flavin, which serves as an electron acceptor from NADPH. On the other hand, FMN, which is the high-potential flavin, appears to participate as an electron carrier in the process of electron transfer from NADPH to cytochrome P-450 during the mixed-function catalytic cycle.

Hepatic NADPH–cytochrome P-450 reductase (EC 1.6.2.4), the flavoprotein component of a liver microsomal mixed-function oxidase, contains one molecule each of FAD[1] and FMN per molecule of the enzyme, but no other redox-active components (Iyanagi & Mason, 1973). The reductase directly reduces cytochrome P-450 in the presence of phospholipids or detergents without the intermediary of an iron–sulfur protein (Lu & Coon, 1968). The reductase provides the two electrons required for mixed-function oxidation of substrate (Mason, 1958).

We have atempted previously to clarify the redox properties of NADPH–cytochrome P-450 reductase by computer simulating the overall potentiometric and spectrophotometric titration curves (Iyanagi et al., 1974). On the assumption that six components (F_1, F_1H·, and F_1H_2, and F_2, F_2H·, and F_2H_2, where F_1 is the high-potential flavin and F_2 is the low-potential flavin) derived from the two flavins (F_1 and F_2) are in equilibrium during the course of the titration of the enzyme with

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[1] Abbreviations used: For purpose of discussion, FAD, FADH·, $FADH_2$, FMN, FMNH·, and $FMNH_2$ are used to symbolize the flavin species; FlH, oxidized flavin; FlH_2, neutral flavoprotein semiquinone; and FlH_3, neutral fully reduced flavoprotein (Hemmerich et al., 1965); 3-AcPyADP, oxidized 3-acetylpyridine adenine dinucleotide; EPR, electron paramagnetic resonance.

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