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Hydrogen-1, Carbon-13, and Phosphorus-31 Nuclear Magnetic Resonance Studies of the Dihydrofolate Reductase-Nicotinamide Adenine Dinucleotide Phosphate-Folate Complex: Characterization of Three Coexisting Conformational States[†]

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ABSTRACT: The *Lactobacillus casei* dihydrofolate reductase-folate-NADP⁺ complex is shown by ¹H and ¹³C NMR to exist in three interconverting conformational states, I, IIa, and IIb. The proportions of the three states, as estimated from the intensities of the three separate ¹³C resonances observed in the complex containing [3-carboxamido-¹³C]NADP⁺, are pH dependent. State I predominates at low pH and states IIa and IIb predominate at high pH; the ratio IIa:IIb is pH independent. The pH dependence of the interconversion of states I and IIa + IIb can be explained by a model in which a group on the enzyme has a pK of <5 in state IIa + IIb and >7 in state I. ¹H, ¹³C, and ³¹P NMR has been used to characterize the structural differences between the three states of the complex. As judged by the ¹H and ¹³C chemical shifts of the

bound coenzyme, states I and IIa are similar to one another but quite different from state IIb. This difference appears to be a localized one, since only the nicotinamide 2 and 4 protons, nicotinamide 3-carboxamide ¹³C, and pteridine 7 proton show differences in chemical shift between these states. These differences are, however, large—up to 1.4 ppm for ¹H and 2 ppm for ¹³C. The remaining coenzyme protons, as well as the three ³¹P nuclei, are unaffected. Studies of the C2 proton resonances of the seven histidine residues show that the ionizable group responsible for the interconversion of states I and IIa + IIb is not a histidine (although two histidines show slight differences in environment between states IIa and IIb); the possible identity of this ionizable group and the nature of the conformational differences between the states are discussed.

Dihydrofolate reductase, which is responsible for maintaining the cellular pools of tetrahydrofolate derivatives, is the target of the "antifolate" drugs trimethoprim and methotrexate, the latter being a close structural analogue of the substrate folate. There is considerable, though often indirect, evidence that ligand binding to the enzyme is accompanied by changes in protein conformation, which appear to be different for inhibitors and for substrates [see, e.g., Roberts et al. (1977), Blakely et al. (1978), and Feeney et al. (1980)]. These conformational changes have a considerable influence on the specificity of the enzyme (Birdsall et al., 1978, 1980a; Hyde et al., 1980a; Roberts, 1978) and most probably also underlie the cooperativity—both positive and negative—between coenzymes and substrates or inhibitors in their binding to the enzyme (Birdsall et al., 1980a,b, 1981a).

In addition to, and perhaps related to, these ligand-induced conformational changes, there is evidence that under some

conditions the enzyme coexists as a mixture of two or more conformational states which interconvert only slowly. This appears to be the case in the absence of ligands for the enzyme from *Escherichia coli*, *Lactobacillus casei*, and *Streptococcus faecium* (Pattishall et al., 1976; Dunn et al., 1978; London et al., 1979; Cayley et al., 1981). Recently we have shown that the ternary complex of the *L. casei* enzyme with trimethoprim and NADP⁺ exists as a mixture of comparable amounts of two conformational forms which bind the coenzyme differently and which interconvert at a rate of about 6 s⁻¹ at 31 °C (Gronenborn et al., 1981a,b). Less direct evidence for two (or more) coexisting conformations has also been obtained for the ternary complex of the enzyme with coenzyme and the product analogue folinic acid (Birdsall et al., 1981a). We now report that the ternary complex containing enzyme, NADP⁺, and the substrate folate exists in three states whose relative proportions are pH dependent. A preliminary report of part of this work has appeared (Birdsall et al., 1981b).

Materials and Methods

Dihydrofolate reductase was isolated from *L. casei* MTX/R as described by Dann et al. (1976). Its concentration was

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determined by assaying its catalytic activity and by fluorometric titration with methotrexate (Dann et al., 1976). Folate, ϵ NADP⁺,¹ and NADP⁺ were obtained from Sigma Chemical Co.

Synthesis of Isotopically Labeled NADP⁺. [3-carboxamido-¹³C]NADP⁺ was synthesized as described by Way et al. (1975) and [nicotinamide-4-²H]NADP⁺ by the method of San Pietro (1955). [6-²H]Nicotinic acid was prepared from pyridine-2,5-dicarboxylic acid by the method of Mauzerall & Westheimer (1955). The exchangeable protons of the dicarboxylic acid were exchanged for deuterium by dissolving it in ²H₂O, allowing to stand overnight at 4 °C, and evaporating off the ²H₂O under reduced pressure. The compound was then decarboxylated by refluxing for 25 min in nitrobenzene, and the deuterated nicotinic acid was precipitated by addition of petroleum ether and recrystallized from ethanol. The acid was converted to the amide by treatment with thionyl chloride followed by bubbling ammonia gas through a solution of the acid chloride in dry tetrahydrofuran. The [6-²H]-nicotinamide was characterized by ¹H NMR; overall yield from the dicarboxylic acid averaged 60%. The deuterated nicotinamide was converted to [nicotinamide-6-²H]NADP⁺ by means of the exchange reaction catalyzed by calf spleen NAD⁺ glycohydrolase, as described by Way et al. (1975).

Measurement of Binding Constants. The binding of ϵ NADP⁺ to the enzyme was measured fluorometrically and that of NADP⁺ by competition with ϵ NADP⁺ (Birdsall et al., 1980a). Measurements were made at 25 °C in a solution containing 0.5–5.0 μ M enzyme, 20–25 μ M folate, 500 mM KCl, and either 50 mM citrate (pH 4.8–6.0) or 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol] (pH 6.0–7.4).

NMR Spectroscopy. ¹H NMR (270 MHz) spectra were obtained by using a Bruker WH270 spectrometer. Samples (0.35 mL) contained approximately 1 mM enzyme, 1–5 mM folate and/or NADP⁺, 500 mM KCl, and 50 mM phosphate, pH* 5.5–7.3, in ²H₂O. The notation pH* indicates a meter reading uncorrected for the deuterium isotope effect on the glass electrode. Spectra were obtained by using quadrature detection, with a spectral width of 4.2 kHz and a pulse interval of 0.5–1.0 s. Up to 10 000 transients were averaged, using a floating point acquisition routine for the longer runs to overcome the dynamic range problem. Before Fourier transformation, the free induction decay was multiplied by an exponential function, giving a line broadening of 2 Hz, and the data table filled to 8192 points with zeros if necessary. ¹H chemical shifts are expressed relative to internal dioxane (3.71 ppm from 4,4-dimethyl-4-silapentanesulfonate).

¹³C NMR spectra were obtained at 50.3 MHz on a Bruker WM200 spectrometer. Samples (3.0 mL) contained 0.5 mM enzyme, 0.45 mM [3-carboxamido-¹³C]NADP⁺, and 1 mM folate in the same buffer as used for the ¹H experiments. Spectra were obtained by using quadrature detection with a spectral width of 10 kHz, a pulse interval of 0.82 s, and a flip angle of 25°. Typically 70 000–80 000 transients were averaged, and a line broadening of 5 Hz was applied before transformation. ¹³C chemical shifts are expressed relative to external dioxane (50% in ²H₂O).

³¹P NMR spectra were obtained by using a Varian XL-100-15 spectrometer. Samples (1.5 mL) contained approximately 1 mM enzyme, 0.9 mM NADP⁺, 2 mM folate, 500 mM KCl, and 50 mM Bistris, pH* 5.3–7.0, in ²H₂O. Spectra

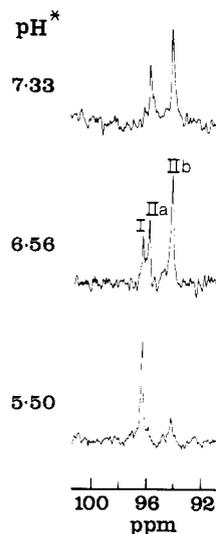


FIGURE 1: 50.3-MHz ¹³C NMR spectra of the nicotinamide carboxamide carbon in the dihydrofolate reductase-folate-[³carboxamido-¹³C]NADP⁺ complex at various pH* values. The resonances from states I, IIa, and IIb are indicated.

were obtained in the block-averaging mode, typically averaging 300 blocks each of 200 transients, with a spectral width of 2 kHz and a pulse interval of 0.5 s, and a line broadening of 1.6 Hz was applied before transformation. ³¹P chemical shifts are expressed relative to inorganic phosphate, pH* 8.0 (2.94 ppm downfield from H₃PO₄).

All ¹³C and ³¹P spectra were obtained with noise-modulated ¹H decoupling. Sample temperature was regulated to ± 1 °C by a stream of heated or cooled dry air; ¹³C and ³¹P spectra were obtained at 11 °C and ¹H spectra between 5 and 40 °C.

Results

¹³C NMR Spectra of the Ternary Complex. Figure 1 shows the carboxamide carbon region of the ¹³C NMR spectrum of a sample containing 0.5 mM *L. casei* dihydrofolate reductase, 1 mM folate, and 0.45 mM [3-carboxamido-¹³C]NADP⁺. From the measured binding constants (Birdsall et al., 1980a; see also below), it is clear that all the observed signals arise from the ¹³C-enriched carbon of NADP⁺ bound to the enzyme as the ternary enzyme-folate-NADP⁺ complex; the resonance of free NADP⁺ would appear at 98.58 ppm and that of the binary enzyme-NADP⁺ complex at 96.98 ppm (Way et al., 1975).

At pH* 5.5, a single major resonance is observed at 96.32 ppm, labeled I in Figure 1. As the pH* is increased, the intensity of resonance I decreases and that of the two other resonances, labeled IIa and IIb, increases. By pH* 7.3 signals IIa and IIb, at 95.88 and 94.26 ppm, respectively, predominate, and resonance I is barely visible. In our earlier studies of [3-carboxamido-¹³C]NADP⁺ binding to the enzyme (Way et al., 1975), two resonances were observed for the enzyme-NADP⁺-folate complex at 25.2 MHz and pH* 6.5, but resonances I and IIa were not fully resolved at the lower field. The chemical shifts of these three resonances are compared to those observed for the same carbon in other complexes (Way et al., 1975) in Table I.

The observation of these three separate resonances from a single ¹³C nucleus in the enzyme-folate-NADP⁺ complex shows clearly that this complex exists as a mixture of (at least) three states whose relative proportions are pH dependent. Although the relative intensities of the observed resonances are pH dependent, their chemical shifts are not (over the range pH* 5.5–7.3), indicating that exchange between the three

¹ Abbreviation: ϵ NADP⁺, nicotinamide 1,*N*⁶-ethenoadenine dinucleotide phosphate.

Table I: Chemical Shifts of the Carboxamide Carbon Resonance of NADP⁺ Bound to *L. casei* Dihydrofolate Reductase

complex	chemical shift (ppm from dioxane) ^a
E-folate-NADP ⁺ resonance I	96.32
resonance IIa	95.88
resonance IIb	94.26
E-NADP ⁺ ^b	96.98
E-NADP ⁺ -aminopterin ^{b,c}	95.68

^a Downfield positive. ^b From Way et al. (1975). ^c Aminopterin is 4-deoxy-4-aminofolate.

states of the complex is slow on the NMR time scale. The line widths of the three resonances (after correction for the effect of the exponential weighting applied to the free-induction decay) are 5.0–6.5 Hz at 11 °C, setting an upper limit to the interconversion rates of 19 s⁻¹ at this temperature.

Provided that the spin-lattice relaxation rate of the carboxamide carbon is similar in the three states of the complex, the relative intensities of the three resonances I, IIa, and IIb can be used as estimates of the relative proportions of the corresponding states. The ratio of intensities of signals IIb and IIa is, within experimental error, constant at 1.67 (±0.02) over the pH* range 5.5–7.3. The pH dependence of the intensities thus involves interconversion of I and IIa + IIb. The fraction of the total intensity in resonance I as a function of pH* is shown as the points in Figure 2 (the lines are calculated from two alternative models of the pH dependence described under Discussion). Resonance I is seen to contain 50% of the total intensity at approximately pH* 6.0.

Histidine Residues. The pH dependence of the ¹³C NMR spectrum suggested that the ionizable group involved might be the imidazole ring of a histidine residue.

We have examined the C2 proton resonances of the seven histidine residues of the enzyme over the range pH* 5.1–7.5. The chemical shifts and pK values are very similar to those reported earlier for the enzyme-methotrexate-NADP⁺ complex (see Table II). All of the histidine resonances showed continuous titration curves over the most of the range. However, as shown in Figure 3, the C2 proton resonances A and E (most probably from histidine residues 22 and 18, respectively; Wyeth et al., 1980) each split into two signals of unequal intensity as the pH* is increased above 6.5. It appears that the environment of these two residues must differ somewhat between state IIa and state IIb, the two states which predominate at high pH.

¹H and ³¹P NMR of the Bound Ligands. To characterize the differences between the three conformational states of the complex, we have measured the chemical shifts of the ¹H and ³¹P resonance of the bound ligands. The ¹H resonances of the

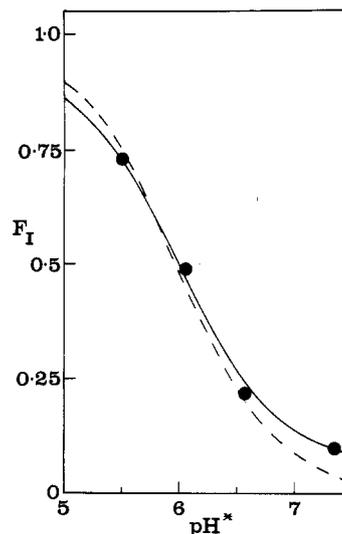


FIGURE 2: Fraction of the total intensity of the carboxamide carbon resonances in resonance I as a function of pH*. The points are the experimental data and the lines theoretical relationships derived from the two models described under Discussion: (---) model i, pK = 5.97; (—) model ii, eq 1, pK_I = 7.2, pK_{II} = 4.8, and K_E = 15.8.

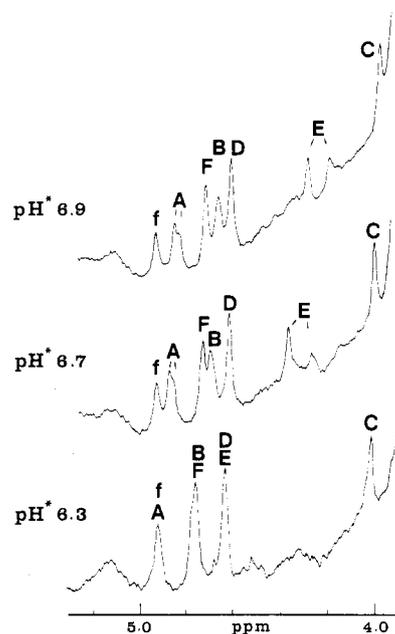


FIGURE 3: Histidine C2 proton resonance region of the 270-MHz ¹H NMR spectrum of the enzyme-folate-NADP⁺ complex at several pH* values. The histidine resonances, A–F, and the 7-proton signal of free folate, f, are indicated. Note that resonances A and E split into two at high pH*.

bound coenzyme have been located and assigned by two methods, transfer of saturation (Forsén & Hoffman, 1963;

Table II: Chemical Shifts^a and pK Values of Histidine Residues of Dihydrofolate Reductase in the Enzyme-Folate-NADP⁺ and Enzyme-Methotrexate-NADP⁺ Complexes

complex	resonance														
	A		B		C		D		E		F		G		
	δ _{HA}	pK ^b	δ _{HA}	pK ^b	δ _{HA}	pK ^b	δ _{HA}	pK ^b	δ _{HA}	Δδ ^c	pK ^c	δ _{HA}	pK ^b	δ _{HA}	pK
E-folate-NADP ⁺	4.95	8.1 ^d	4.79	8.0	4.03	8.15	4.65	8.4	4.90	0.95 ^d	6.60 ^d	4.79	8.3	5.86	
E-methotrexate-NADP ⁺ ^e	4.92	7.8	4.79	7.8	4.03	8.1	4.65	8.4	5.00	1.0	6.50	4.79	8.3	5.86	

^a δ_{HA}, chemical shift of C2 proton resonances of protonated imidazole (ppm from dioxane; ±0.03 ppm); Δδ = δ_{HA} - δ_A. ^b pK value estimated from incomplete titration curve by constraining Δδ to 0.9–1.0 ppm; ±0.1 unit. ^c pK and Δδ from complete titration curve; ±0.05 unit and ±0.05 ppm, respectively. ^d Titration curve fitted by using the mean chemical shift of the two components of the resonance at high pH (Figure 3). ^e From Gronenborn et al. (1981c); pK values adjusted to correspond to the calibration used in the present work.

Table III: Chemical Shifts of Protons of NADP⁺ Bound to *L. casei* Dihydrofolate Reductase Determined by Transfer of Saturation Experiments

proton ^a	chemical shift (ppm from dioxane) ^b	
	E-folate-NADP ⁺	E-NADP ⁺ ^c
N2	6.13, 6.37	6.42
N4	5.38, 6.70	6.60
N5	5.96	5.52
N6	6.27	6.31
N1'	1.99	2.04
A2	3.67	3.64
A8	4.34	4.42
A1'	3.00	3.03

^a Protons in the adenosine and nicotinamide moieties are denoted A and N, respectively; A1' and N1' are the two ribose anomeric protons. ^b Downfield positive. ^c From Hyde et al. (1980a).

Hyde et al., 1980a) and the use of specifically deuterated NADP⁺. We have found that the nicotinamide 2 and 4 protons of the bound NADP⁺ (denoted N2 and N4, respectively) give rise to three separate resonances, corresponding to the three conformational states of the complex.

In the transfer of saturation experiment, irradiation at the position of a resonance of the bound ligand leads to a decrease in intensity of the corresponding resonance of the free ligand, provided that the rate of exchange of the ligand between the two states is fast enough. The resonances of bound ligands can thus be located by systematic irradiation through the relevant region of the spectrum. Experiments of this sort have been carried out on the enzyme-folate-NADP⁺ complex at pH* values between 5.4 and 7.3 and at temperatures between 10 and 40 °C. The chemical shifts of the proton resonances of bound NADP⁺ located in this way are summarized in Table III, where they are compared with those in the enzyme-NADP⁺ complex (Hyde et al., 1980a). For most of the coenzyme protons, transfer of saturation effects were observed at one specific irradiation frequency for each proton, and the chemical shifts of the bound coenzyme identified in this way were in good agreement with those reported earlier on the basis of experiments at a single pH and temperature (Hyde et al., 1980a). However, for the 2 and 4 protons of the nicotinamide ring, we have now observed apparent transfer of saturation effects at two (but not three) distinct frequencies (see Table III). The frequencies at which these effects were observed were independent of pH.

The transfer of saturation experiments indicate, therefore, the existence of two different chemical shifts for both N2 and N4 of the bound coenzyme in this complex, corresponding to two of the three states identified from the spectra of [¹³C]-NADP⁺. However, as discussed elsewhere (Clare et al., 1981), when two (or more) forms of the complex are present, the rate of exchange of the ligand from the complex is a much more important determinant of the observed transfer of saturation effects than is the ratio of the two forms of the complex. We cannot, therefore, use the magnitude of the transfer of saturation effect or its pH dependence to identify the N2 and N4 resonances we have located as coming from form I, IIa, or IIb of the complex.

This can only be done on the basis of the intensities of directly observed resonances. The bottom spectrum in Figure 4 shows the low-field region of the ¹H spectrum of the complex at pH* 5.3 in the presence of ~1 molar equivalent of excess NADP⁺. The resonance at 5.83 ppm is histidine C2 proton resonance G (Wyeth et al., 1980), while the three resonances

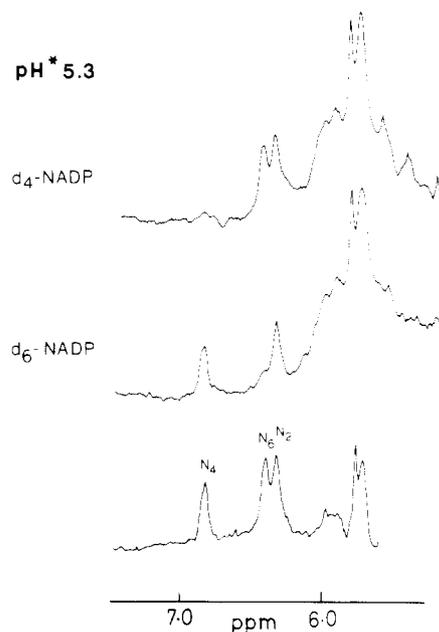


FIGURE 4: Low-field region of the 270-MHz ¹H NMR spectrum of the enzyme-folate-NADP⁺ complex at pH* 5.3 (bottom), together with those of the corresponding complexes formed with [*nicotinamide-4-²H]*NADP⁺ and [*nicotinamide-6-²H]*NADP⁺. The bottom spectrum was obtained from a sample which had been treated as described by Gronenborn et al. (1981c) to exchange virtually all the peptide NH protons for deuterium, so that contributions from NH resonances are minimal. In the other two samples, exchange was less complete, so that NH resonances appear between 5.5 and 6.2 ppm.

to low field of 6.2 ppm arise, as indicated, from three of the nicotinamide protons of the bound coenzyme. This assignment was made by comparison with the spectra of the same complex formed with [*nicotinamide-4-²H]*NADP⁺ or [*nicotinamide-6-²H]*NADP⁺, shown in Figure 4. Comparison of these spectra shows clearly that the resonance at 6.82 ppm in the bottom spectrum arises from the N4 proton of the bound coenzyme, and that at 6.43 ppm from the N6 proton. Since [*nicotinamide-5-²H]*NADP⁺ was not available to us, the N5 resonance of the bound NADP⁺ could not be identified; it may be the signal at 5.80 ppm, close to histidine resonance G.

Each of the three unambiguously assigned resonances has an intensity equivalent to one proton at pH* 5.3. However, as the pH is increased, their intensity decreases until they become undetectable at pH* >6.7. This strongly suggests that they are from the coenzyme in conformation I of the complex.

None of these three nicotinamide proton resonances corresponds in chemical shift to the positions identified in the transfer of saturation experiments. This can be explained by postulating that the resonances located by transfer of saturation are those of states IIa and IIb, the rate of dissociation of the coenzyme from state I being too slow for any saturation transfer to be observed, even when conformation I predominates at low pH (Clare et al., 1981, present calculations illustrating the range of exchange rates and relaxation rates which would give this result).

This assignment is strongly supported by the results of experiments with the deuterated coenzyme at high pH. Figure 5 compares the low-field region of the ¹H spectrum of the complex (formed with [*nicotinamide-6-²H]*NADP⁺) at pH* 5.3 and pH* 7.2. The resonance of the N4 proton in conformation I is indicated in the spectrum obtained at low pH. The two resonances labeled N4(IIa) and N4(IIb) in the spectrum obtained at high pH are at precisely those chemical shifts at which transfer of saturation effects were observed for

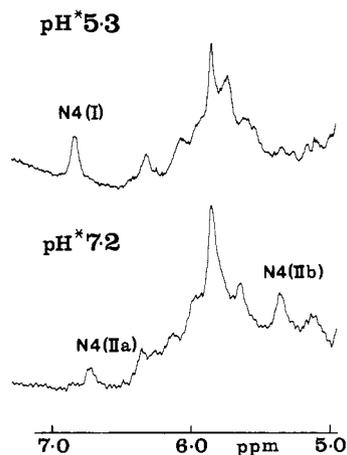


FIGURE 5: Low-field region of the 270-MHz ¹H NMR spectrum of the enzyme-folate-NADP⁺ complex at pH* 5.3 and pH* 7.2, showing the resonances of the N4 proton in states I, IIa, and IIb. The complex was formed with [*nicotinamide-6-²H]NADP⁺, since the N6 resonance of any free NADP⁺ present partially overlaps the N4(IIb) signal.*

Table IV: ¹H Chemical Shift Changes on NADP⁺ Binding to *L. casei* Dihydrofolate Reductase

proton	chemical shift ^a (ppm)				
	E-folate-NADP ⁺			E-metho- trexate- NADP ⁺ ^b	E- NADP ⁺ ^b
	I	IIa	IIb		
N2	0.51	0.56	0.32	0.53	0.61
N4	1.58	1.46	0.14	0.92	1.36
N5	1.25 ^d		1.41	0.79	0.97
N6	0.85		0.69	0.73	0.73
N1'	<i>c</i>	-0.45		-0.45	-0.42
A2	<i>c</i>	-0.83		-0.86	-0.86
A8	<i>c</i>	-0.47		-0.50	-0.39
A1'	<i>c</i>	0.53		0.53	0.56

^a Shifts (positive downfield) in the indicated complex relative to the appropriate mononucleotide [see Hyde et al. (1980a)].

^b From Hyde et al. (1980a). ^c Not determined. ^d Tentative assignment.

the N4 proton. These two resonances can be unambiguously assigned to the N4 proton of the bound coenzyme, since they are absent from the spectrum of the complex formed with [*nicotinamide-4-²H]NADP⁺. They must arise from states IIa and IIb since they are only observable at high pH and the signal at 6.82 ppm has already been assigned to N4 in conformation I. The resonance at 6.70 ppm has an intensity of approximately one-third of a proton at pH* 7.2, indicating that it arises from conformation IIa, the less populated of the two high pH forms. The second resonance position located by transfer of saturation, labeled N4(IIb) in Figure 5, must thus arise from state IIb. A similar assignment of the two N2 resonances located by transfer of saturation to states IIa and IIb is less clear-cut, since comparison of their intensities is more difficult, due to overlap with peptide NH signals, but the most probable assignment is IIa, 6.37 ppm, and IIb, 6.13 ppm. The chemical shifts of the coenzyme proton resonances in each of the three conformations of the complex are summarized in Table IV, where they are expressed relative to those of the corresponding mononucleotides so as to correct for the contribution of intramolecular stacking in the free coenzyme [see Hyde et al. (1980a)].*

Two separate positions for the resonance of the pteridine 7 proton of bound folate have also been identified. Transfer of saturation experiments readily located one 7-proton resonance at 5.62 ppm, 0.67 ppm downfield from its position in free folate. At low pH, a resonance was observed at 4.50 ppm

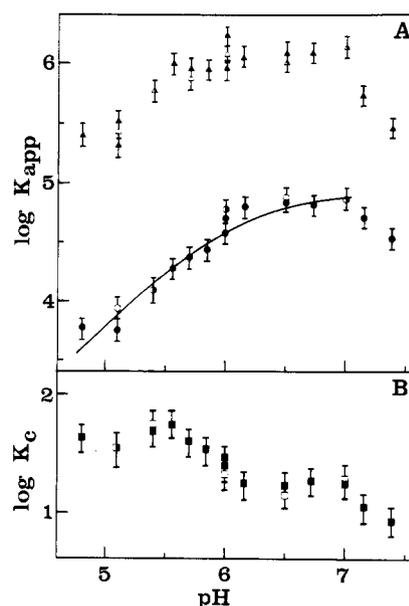


FIGURE 6: (A) pH dependence of the binding constant of ϵ NADP⁺ (solid symbols) and NADP⁺ (open symbols) to dihydrofolate reductase (circles) and to the dihydrofolate reductase-folate complex (triangles). The line is calculated for a pK of 6.1 in the free coenzyme decreasing to <3.5 in the complex. (B) pH dependence of the cooperativity between folate and ϵ NADP⁺ (solid symbols) or NADP⁺ (open symbols) binding to dihydrofolate reductase. K_c is the ratio of coenzyme binding constants in the presence and absence of folate.

which, at 45 °C, showed a weak transfer of saturation to the 7-proton resonance of free folate. By analogy with the coenzyme resonances, the resonance showing a large transfer of saturation effect (i.e., that at 5.62 ppm) most probably arises from state IIa and/or state IIb, while that at 4.50 ppm, which shows only weak transfer of saturation, comes from state I. In the enzyme-methotrexate-NADP⁺ complex, the resonance of the 7 proton of methotrexate also appears at 4.50 ppm (B. Birdsall, A. Gronenborn, G. C. K. Roberts, and J. Feeney, unpublished work).

The ³¹P NMR spectrum of NADP⁺ in the enzyme-folate-NADP⁺ complex at pH* 6.5 has been reported earlier (Hyde et al., 1980b). We have now repeated this experiment at pH* 5.3 and 7.0 and find that all three resonances (from the 2'-phosphate and the two pyrophosphate ³¹P nuclei) have the same chemical shift at all three pH* values. The environments of the 2'-phosphate and the pyrophosphate are thus identical in all three states of the complex.

pH Dependence of NADP⁺ Binding. If the affinity of the enzyme for NADP⁺ differs between state I and states IIa and IIb, then the pH dependence of the relative populations of these states will be reflected in the pH dependence of NADP⁺ binding. The pH dependence of the logarithms of the association constants for the binding of NADP⁺ and ϵ NADP⁺ to the enzyme alone and to the enzyme-folate complex is shown in Figure 6A. Since the binding of ϵ NADP⁺ can be readily followed, using the increase in ethenoadenine fluorescence on binding (Birdsall et al., 1980a), most of the measurements were made with this analogue. As noted earlier (Birdsall et al., 1980a) and confirmed by the data in Figure 6, the binding constant of ϵ NADP⁺ is indistinguishable, within experimental error, from that of NADP⁺ in both binary and ternary complexes. In the presence or absence of folate, the coenzyme binding constant increases as the pH is increased from 5 to 7, followed by a rather abrupt decrease above this pH. Qualitatively similar behavior is seen for NADPH binding to the enzyme (Dunn et al., 1978).

We have shown by ^{31}P NMR that the pK of the 2'-phosphate group of NADP^+ decreases substantially (to <3.5) when it binds to the enzyme (Feeney et al., 1975) or the enzyme-folate complex (Hyde et al., 1980b). As shown by the good agreement between the data points and the theoretical line in Figure 6A, the pH dependence of ϵNADP and NADP^+ binding to the enzyme alone can be quantitatively accounted for (in the range pH 5–7) by this change in pK . The form of the pH dependence of coenzyme binding to the enzyme-folate complex is not exactly the same; this can be appreciated by noting (Figure 6B) that the ratio of binding constants for the binary and ternary complexes, $K_c = K_{\text{ternary}}/K_{\text{binary}}$, varies from approximately 18 at pH 6.5–7.0 to approximately 43 at pH 5–5.5. This additional pH dependence could arise from a difference in affinity for the coenzyme between state I and states IIa and IIb; any such difference can thus be no more than a factor of about 2.5.

Discussion

The observation of three distinct resonance positions for the carboxamide carbon and the N2 and N4 protons of the bound coenzyme shows clearly that the dihydrofolate reductase- NADP^+ -folate complex exists in three distinct, slowly interconverting states.

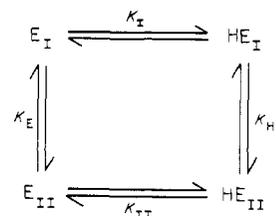
It is also evident that the equilibrium between state I and states IIa and IIb is pH dependent. Two simple models can be considered as explanations of this pH^* dependence: (i) It could arise directly, as a chemical shift effect, from the ionization of a single group near the nicotinamide ring of the bound coenzyme. This model would require the existence of two forms of the complex, whose population ratio is pH independent (at 1.67). At low pH^* , the carboxamide carbon of the NADP^+ (as well as the nicotinamide protons) would have an identical chemical shift in both forms, but as the neighboring group ionizes, it would have a large effect on the chemical shift in only one of the two forms, so that they would give clearly separated resonances at high pH^* . In this model resonance I corresponds to the protonated state of both conformations, and resonances IIa and IIb correspond to the two conformations in the unprotonated state. This model predicts that the pH^* dependence of the intensity of resonance I will be described by the Henderson-Hasselbalch equation, and the dashed line in Figure 2 is calculated in this way, with a pK of 5.97.

(ii) In the second model, it is postulated that the ionization of a group in the complex is coupled to a change in conformation of the protein and that it is this which produces the change in chemical shift. The three resonances observed would thus correspond to three conformational states of the protein. The ionizable group in question would have the same pK in the conformations giving resonances IIa and IIb (since their ratio is pH independent) but a higher pK in the state corresponding to signal I. The pH dependence of the intensity of resonance I predicted by this model can be analyzed in terms of the simple two-state model of Scheme I, since the conformations giving signals IIa and IIb can, for this purpose, be combined into state II. The proportion in state I is then given by

$$F_I = \frac{1 + [\text{H}^+]/K_I}{(1 + [\text{H}^+]/K_I) + K_E(1 + [\text{H}^+]/K_{II})} \quad (1)$$

where K_I and K_{II} are the dissociation constants of the ionizable group in states I and II, respectively, and $K_E = [\text{E}_{II}]/[\text{E}_I]$. Provided that K_I and K_{II} are sufficiently different, this equation predicts a pH dependence very similar to that given by the

Scheme I: Model for the Interconversion of States I and II (i.e., IIa + IIb)^a



^a E_I and E_{II} represent the unprotonated forms of the two states of the complex and HE_I and HE_{II} the corresponding protonated forms. $K_I = [\text{H}^+][\text{E}_I]/[\text{HE}_I]$ and $K_E = [\text{E}_{II}]/[\text{E}_I]$, with analogous definitions for K_{II} and K_{HE} .

Henderson-Hasselbalch equation. This is shown as the solid line in Figure 2, calculated with $\text{pK}_I = 7.2$, $\text{pK}_{II} = 4.8$, and $K_E = 15.8$.

It is apparent from Figure 2 that these two models cannot be distinguished by the data available on the pH dependence of resonance intensities. Model ii gives a slightly better fit to the data than does model i, but this is offset by the fact that eq 1 contains three adjustable parameters, compared to one in the Henderson-Hasselbalch equation. A clear-cut distinction between the two models would require measurement of the asymptotic values of F_I at low and high pH. However, at the signal-to-noise ratio attainable in these experiments, accurate measurements of the intensity of signals representing less than about 10% of the total cannot be made.

Although model i is clearly the simpler of the two, since it postulates two rather than three states of the complex, there are two points which suggest that model ii is in fact the more plausible. First, model i requires that at low pH both the carboxamide carbon resonance and all the nicotinamide proton resonances of NADP^+ have precisely the same chemical shift in the two conformations. Since, as argued below, the differences between the conformational states of this complex are largely localized to the immediate environment of the nicotinamide ring, such a coincidence is improbable. Second, in model i the rate of interconversion between the protonated (signal I) and unprotonated (signals IIa and IIb) states will simply be determined by the rate of proton exchange on and off the ionizable group. For a diffusion-limited proton transfer ($k_{\text{on}} \approx 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) and a pK of 6, $k_{\text{off}} \approx 10^4 \text{ s}^{-1}$. Although the rate of the forward reaction could of course be decreased below the diffusion limit by steric hindrance, the measured upper limit of 19 s^{-1} would nonetheless seem to be improbably slow for such a proton transfer.

If we therefore adopt the second model, involving a slow conformational change coupled to the ionization process, then we can conclude that the pK of the ionizable group involved must be substantially higher in state I than in states IIa and IIb. Although the pK values used to calculate the solid curve in Figure 2 give a good fit to the data, the limitations to the data noted above mean that one can only reliably obtain limits to the parameter values: $\text{pK}_I > 7.0$, $\text{pK}_{II} < 5.0$, $(\text{pK}_I + \text{pK}_{II})/2 = 6.0$, and $K_E > 9$.

The measurements of the pH dependence of NADP^+ binding suggest that there is only a small difference, about a factor of 2.5, between conformation I and conformations IIa and IIb in their affinity for the coenzyme. The transfer of saturation experiments suggest that the dissociation rate constant of the coenzyme from state I of the ternary complex is lower than that from states IIa and IIb by a rather larger factor. It is thus likely that the coenzyme binding to at least one of the states is kinetically complex.

Characteristics of the Three Conformations of the Complex. The carboxamide ¹³C and N2 and N4 ¹H resonances of the nicotinamide ring of the bound coenzyme have similar chemical shifts in conformations I and IIa, but quite different shifts in conformation IIb. The remaining proton signals of the bound coenzyme could not be identified in state I, since appropriately deuterated NADP⁺ was not available, but from the transfer of saturation experiments, they have at least very similar and probably identical chemical shifts in states IIa and IIb. The ³¹P spectra show that the pyrophosphate "backbone" and the 2'-phosphate at the adenine end of the coenzyme have the same environment in all three states. It appears, then, that the structural differences between the three states of the complex involve primarily the region around the nicotinamide ring of the bound coenzyme. The observations on the bound folate molecule are consistent with this picture, since there is a substantial difference in chemical shift between the different states for the pteridine 7 proton, but no detectable difference for the benzoyl carbonyl carbon (Pastore et al., 1976); one would expect the folate 7 proton to be very close to the coenzyme N4 if this complex resembles the catalytically competent enzyme-folate-NADPH complex. In addition, only a single resonance is seen for the 2 carbon of folate in the *S. faecium* enzyme-folate-tetrahydro-NADP complex (Cocco et al., 1981). The small differences between states IIa and IIb in the environment of histidines-18 and -22 also fit in with this picture, since both residues are part of a loop of polypeptide chain which runs close to the nicotinamide ring (Matthews et al., 1978, 1979).

This similarity of the nicotinamide chemical shifts in states I and IIa probably indicates a similarity in the environment of the nicotinamide ring in these two states. The environment of the coenzyme in states I and IIa is generally similar to that in the enzyme-NADP⁺ or enzyme-NADP⁺-methotrexate complex (Way et al., 1975; Hyde et al., 1980a), although it is not identical with either of these (see Tables I and IV).

The effects of the conformational equilibrium between states IIa and IIb are striking in that they are both large and localized. Three resonances (nicotinamide N4 proton and carboxamide carbon and folate pteridine 7 proton) show differences in chemical shift of more than 1 ppm between these two states. A third resonance, the nicotinamide N2, shows a smaller shift (0.24 ppm), while the remaining coenzyme protons and phosphorus nuclei are unaffected. All the nuclei affected by this equilibrium are thus very close together in space. It is therefore reasonable to suggest that the conformational equilibrium is itself a localized one. This is in contrast to the conformational equilibrium observed in the enzyme-trimethoprim-NADP⁺ complex, for which the equilibrium constant is also close to one, but which involves a substantial change in the mode of binding of the whole nicotinamide ring and in the conformation of the pyrophosphate moiety (Gronenborn et al., 1981a,b; B. Birdsall, A. Gronenborn, A. W. Bevan, G. M. Clore, G. C. K. Roberts, and J. Feeney, unpublished work).

The nature of this localized conformational difference between states IIa and IIb of the enzyme-folate-NADP⁺ complex remains to be established.

Nature of the Ionizable Group Involved. The pH dependence of the ¹³C spectrum allows us to place constraints on the possible pK values of the ionizable group involved in the interconversion of the conformational states of the complex, but it is more difficult to identify the nature of this group.

From the models required to explain the ¹³C data, it is possible to predict the behavior of the resonance(s) of the

ionizable group itself. At pH* 5 it will have the chemical shift characteristic of the protonated form and at pH* 7 that characteristic of the unprotonated form; the transition between the two will have its midpoint at pH* 6 and will be characterized by *slow* exchange behavior, that is, by changes in resonance intensity rather than by progressive changes in chemical shift.

None of the seven histidine C2 proton resonances shows this kind of behavior, and we conclude that the ionizable group involved in the conformational interconversion is *not* an imidazole.

On the basis of the estimated pK values (pK_I > 7, pK_{II} < 5, and (pK_I + pK_{II})/2 = 6), two other possibilities are (a) the 3 nitrogen of bound folate or (b) a carboxylate group. [*L. casei* dihydrofolate reductase contains no thiol groups (Bitar et al., 1977)].

In free folate, the formation of an anion by removal of a proton from N3 is characterized by a pK of 8.0 and is accompanied by the appearance of a strong absorption band at 366 nm (Rabinowitz, 1960; Hood & Roberts, 1978). As reported earlier for the binary complex (Hood & Roberts, 1978), the UV difference spectrum accompanying folate binding to the enzyme-NADP⁺ complex is pH independent in the range pH 5.5-7.5 and does not contain a feature corresponding to the appearance of a band at 366 nm (B. Birdsall, unpublished work). This makes it unlikely that this ionization is the one responsible for the conformational interconversion in the ternary complex, although the possibility of a compensating change in the ultraviolet spectrum arising from some other source cannot be completely ruled out.

Turning to carboxylate groups, it is of course possible that a conformational change linked to the ionization of any carboxylate group in the protein could be propagated to the active site. However, if one is to accept the arguments outlined above that the differences between these states are localized to the immediate surroundings of the nicotinamide ring, then it is reasonable to suggest that the carboxyl group of interest should be close to this ring. In the enzyme-methotrexate-NADPH complex, the only charged amino acid side chain within 15 Å of the nicotinamide carboxamide carbon is Asp-26 (Matthews et al., 1978, 1979; Matthews, 1979), suggesting that this might be the carboxylate responsible for the conformational interconversion seen in the enzyme-folate-NADP⁺ complex. It would have a "normal" pK (<5.0) in states IIa and IIb, but an unusually high pK (>7.0) in state I. Matthews (1979) has noted that the pK of this residue might be high in complexes of folate with the enzyme, due to a markedly reduced solvent accessibility. In the enzyme-methotrexate-NADPH complex (Matthews et al., 1978) Asp-26 is close to the 2 carbon of the pteridine ring, but in the *S. faecium* enzyme-folate-tetrahydro-NADP complex the folate 2-carbon resonance showed no change in chemical shift on binding and no evidence of splitting (Cocco et al., 1981). It is not yet known whether the ternary complex containing tetrahydro-NADP bears a close structural resemblance to that containing NADP⁺. In any case it is clear that the pteridine ring of folate binds to the enzyme in a different orientation to that of methotrexate (Hitchings & Roth, 1980; Charlton et al., 1979), so that the 2 carbon of folate may not be close to Asp-26.

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