Negative Cooperativity between Folinic Acid and Coenzyme in Their Binding to *Lactobacillus casei* Dihydrofolate Reductase†

B. Birdsall, A. S. V. Burgen, E. I. Hyde,‡ G. C. K. Roberts,* and J. Feeney

ABSTRACT: The binding of folinic acid (5-formyl-5,6,7,8-tetrahydrofolate) to *Lactobacillus casei* dihydrofolate reductase has been measured. The natural 6*S*,α*S* diastereoisomer has a binding constant of 1.3 (±0.6) × 10^8 M^{-1} at pH 6.0, 25 °C; the 6*R*,α*S* diastereoisomer binds approximately 10^4-fold more weakly. The natural diastereoisomer of folinic acid binds negatively cooperatively with the coenzymes $NADP^+$ and NADPH, binding 3 times more weakly in the presence of $NADP^+$ and 600 times more weakly in the presence of NADPH than to the enzyme alone. Negative cooperativity has been unequivocally distinguished from competition by measurements of coenzyme binding as a function of folinic acid concentration, of the effects of folinic acid on the ^1H and ^{31}P chemical shifts of the bound coenzyme, and of the effects of folinic acid on the coenzyme dissociation rate constant. The latter experiments also give evidence for the coexistence of two slowly interconverting conformational forms of the ternary enzyme–coenzyme–folinic acid complex. Small changes in structure of the oxidized coenzymes have substantial effects on the cooperativity with folinic acid, with the thionicotinamide analogue showing positive rather than negative cooperativity. The changes in environment of the bound coenzyme produced by folinic acid, as revealed by ^1H and ^{31}P NMR, demonstrate clearly that the negative cooperativity shown by $NADP^+$ and NADPH, respectively, arises by two structurally distinct mechanisms.

It has commonly been observed with NAD(P)H-linked dehydrogenases that substrate analogues bind to the enzyme more tightly in the presence of the coenzyme. For example, the binding of fatty acids and their amides to liver alcohol dehydrogenase (Winer & Theorell, 1959) and that of oxalate and oxamate to lactate dehydrogenase (Winer & Schwert, 1959) have been known for a number of years to be dramatically increased by the coenzyme [for more recent work, see Brändén et al. (1975) and Holbrook et al. (1975)]. We have recently been studying this cooperativity between substrate analogues and coenzyme in *Lactobacillus casei* dihydrofolate reductase (Birdsall et al., 1980a,b). Substantial cooperativity—up to 2000-fold—was observed, with the magnitude of the effect depending markedly on the structure of both the coenzyme and the substrate analogue (inhibitor). A number of inhibitors of dihydrofolate reductase, such as methotrexate and trimethoprim, are clinically useful drugs, and this cooperativity with coenzyme may be of importance in their therapeutic action. For the coenzyme analogues examined, the ratio of binding constants $K_{ternary}/K_{binary}$ was greater than 1 for complexes with methotrexate, trimethoprim, and folate, that is, positive cooperativity (Birdsall et al., 1980a). We now report experiments which demonstrate that folinic acid (5-formyl-5,6,7,8-tetrahydrofolate; leucovorin) shows marked *negative* cooperativity with coenzyme in binding to the reductase.

Experimental Procedures

Materials

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described by Dann et al. (1976). Its concentration was determined from its absorbance at 280 nm, by assaying its catalytic activity and by fluorometric titration with methotrexate (Dann et al., 1976). $NADP^+$, NADPH, the coenzyme analogues[1] ε$NADP^+$, $TNADP^+$, $APADP^+$, and $NHDP^+$, and (±)-folinic acid were obtained from Sigma (London) Chemical Co. Ltd. Samples of (−)-(6*S*,α*S*)-folinic acid and (+)-(6*R*,α*S*)-folinic acid were kindly provided by Drs. P. Charlton and D. W. Young, University of Sussex, Sussex, United Kingdom. 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 DE-52). *N*-[*p*-(Hexylamino)benzoyl]-L-glutamate was synthesized as described by Birdsall et al. (1978).

Methods

Determination of Equilibrium Constants. The equilibrium constants for the binding of folinic acid and for the binding of coenzymes in the presence of folinic acid were measured fluorometrically. All measurements were made at 25 °C in a buffer of 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol] hydrochloride, pH 6.0, containing 0.5 M KCl. Depending on the magnitude of the binding constant to be determined, enzyme concentrations between 20 nM and 25 μM were used; no dependence of binding constant on enzyme concentration was observed. The binding of folinic acid was followed by its quenching of the tryptophan fluorescence of the enzyme (excitation 295 nm, emission 340 nm). The binding of ε$NADP^+$ was followed by the increase in its fluorescence (excitation 340 nm, emission 400 nm), and the binding constants of the other oxidized coenzymes were determined by competition with ε$NADP^+$. The binding of NADPH in the presence of folinic acid was determined by using energy-transfer fluorescence (excitation 280 nm, emission 440 nm). The procedures for data analysis were those described by Birdsall et al. (1978, 1980a).

† From the Division of Molecular Pharmacology, National Institute for Medical Research, London NW7 1AA, United Kingdom. *Received June 26, 1981.*

‡ Supported by an MRC Research Studentship. Present address: Department of Chemistry, University of Washington, Seattle, WA 98195.

[1] Abbreviations used: ε$NADP^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate; $TNADP^+$, thionicotinamide adenine dinucleotide phosphate; $APADP^+$, acetylpyridine adenine dinucleotide phosphate; $NHDP^+$, nicotinamide hypoxanthine dinucleotide phosphate; PADPR-OMe, the methyl β-riboside of 2′-phosphoadenosine 5′-diphosphoribose; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-7186$01.25/0

NMR Spectroscopy. Samples for ^1H NMR contained ~1 mM enzyme, 1–10 mM ligand, 50 mM potassium phosphate, pH* 6.8 (the notation pH* indicates a meter reading uncorrected for the isotope effect on the glass electrode), 500 mM KCl, 1 mM dioxane, and 1 mM EDTA in a volume of 0.35 mL of ^2H_2O. Samples for ^{31}P NMR were made up in ^2H_2O containing 50 mM Bistris, pH* 6.8, 500 mM KCl, and 1 mM EDTA; either 1 mM enzyme in 1.5 mL or 0.5 mM enzyme in 3.0 mL was used.

For some of the ^{31}P NMR experiments, samples of the enzyme–(6*S*,α*S*)-folinic acid complex were prepared by using a method which makes use of the much greater affinity of the enzyme for this diastereoisomer. Acid-washed charcoal was thoroughly washed with Bistris buffer (as above but in H_2O), equilibrated with 1% Dextran T40 in the same buffer, and air-dried at room temperature. A 30-mg sample of treated charcoal was added to 0.5 mL of a solution, on ice, containing 0.96 mM enzyme and 2.02 mM (±)-folinic acid in Bistris buffer. After 20 s, the charcoal was centrifuged down at 14 000 rpm (~8700*g*) in a microcentrifuge. Analysis of the UV spectrum and of the formyl proton signal in the ^1H NMR spectrum [see Feeney et al. (1981)] of the supernatant showed that it contained only the enzyme–(6*S*,α*S*)-folinic acid complex, with undetectable amounts of (6*R*,α*S*)-folinic acid or of free (6*S*,α*S*)-folinic acid. This sample was lyophilized and redissolved in ^2H_2O for the NMR experiment. Sample temperatures (5–35 °C) were controlled to ±1 °C.

^1H NMR spectra (270 MHz) were obtained by the Fourier-transform technique with a Bruker WH270 spectrometer. Between 200 and 2000 transients were averaged for each spectrum, using a spectral width of 4.2 kHz and an acquisition time of 0.5–1 s (4096 or 8192 data points). Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz, and if 4096 data points had been collected, the data table was filled to 8192 points with zeros. Transfer of saturation experiments were performed as described by Hyde et al. (1980a). Chemical shifts are expressed with respect to internal dioxane (3.71 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate), with downfield shifts positive.

^{31}P NMR spectra were obtained at 40.5 and 81 MHz by the Fourier-transform technique using, respectively, Varian XL-100-15 and Bruker WM-200 spectrometers. Noise-modulated ^1H decoupling was employed for all spectra. For the 40.5-MHz spectra, 1.5-mL samples were used; spectra were collected in the block-averaging mode, typically averaging 300 blocks each of 200 spectra, with a spectral width of 2 kHz, an acquisition time of 0.5 s, and a line broadening of 1.6 Hz. For the 81-MHz spectra, 3-mL samples of half the enzyme concentration were used; up to 28 000 transients were averaged, with a spectral width of 4 kHz, an acquisition time of 1.0 s, and a line broadening of 3 Hz. Chemical shifts are expressed with respect to inorganic phosphate, pH* 8.0 (2.94 ppm downfield from H_3PO_4), with downfield shifts positive.

Coenzyme dissociation rates were measured by ^1H transfer of saturation and ^{31}P line-shape analysis as described by Hyde et al. (1980a,b). In the transfer of saturation experiment (Forsén & Hoffman, 1963), the intensity of a resonance of the free coenzyme, I_F, as a function of the time for which the corresponding resonance of the bound coenzyme is saturated is given by

$$I_F(t) = I_{F_0}k_F \exp[-t(\rho_F + k_F)]/(\rho_F + k_F) + I_{F_0}\rho_F/(\rho_F + k_F) \quad (1)$$

In this expression, I_{F_0} is the intensity of the free signal in the absence of saturation, ρ_F is the spin–lattice relaxation rate of the proton in the free state, and k_F is the apparent pseudo-first-order rate constant for exchange to the bound state. When both the time constant of this exponential decrease in intensity, $1/(\rho_F + k_F)$, and the equilibrium fractional intensity as $t \rightarrow \infty$, $I_F/I_{F_0} = \rho_F/(\rho_F + k_F)$ are measured, both ρ_F and k_F can be calculated. The apparent dissociation rate constant, k_{app}, is then given by

$$k_{app} = P_F k_F / P_B \quad (2)$$

where P_F and P_B are the fractional populations of coenzyme in the free and bound states, respectively. In the ^{31}P spectra, the 2′-phosphate resonance shows behavior characteristic of slow exchange between the free and bound states. The exchange process contributes to the line width of both signals, with the width at half-height, W, of that from the free coenzyme being given by

$$\pi W = 1/T^*_2 + k_{app}P_B/P_F \quad (3)$$

where $1/T^*_2$, the apparent spin–spin relaxation rate, includes contributions from field inhomogeneity and the exponential weighting used to improve the signal-to-noise ratio. The value of k_{app} was estimated from the dependence of W on coenzyme concentration (i.e., on P_B/P_F, calculated from the measured binding constant).

Results

Binding Constants. The tryptophan fluorescence of *L. casei* dihydrofolate reductase is substantially quenched on addition of folinic acid, providing a convenient method for measuring its binding constant. At enzyme concentrations of 1–10 μM, the enzyme fluorescence decreases linearly with the concentration of (±)-folinic acid until 2 mol/mol of enzyme has been added, after which there is no further change. This stoichiometry strongly suggests that only one of the two diastereoisomers of folinic acid binds strongly ($K > 10^7$ M^{-1}) to the enzyme.

This has been confirmed by studies with the separated diastereoisomers. The binding constant of the natural diastereoisomer, (6*S*,α*S*)-folinic acid, was determined by measurements of fluorescence quenching at an enzyme concentration of 20 nM and was found to be 1.3 (±0.6) × 10^8 M^{-1}. Within experimental error, this is the same as the value of 1.2 (±0.5) × 10^8 M^{-1} determined by using (±)-folinic acid and assuming that only one diastereoisomer binds. It follows that the unnatural 6*R*,α*S* diastereoisomer must bind much more weakly than the natural compound. This is also apparent from our earlier ^1H NMR experiments (Feeney et al., 1981) and from the stoichiometry experiment described above; the latter sets an upper limit of about 10^5 M^{-1} to the binding constant of (6*R*,α*S*)-folinic acid. Direct measurements of the binding constant of this diastereoisomer were hampered by the presence of small amounts of a highly fluorescent impurity, but an estimate of ~1.0 × 10^4 M^{-1} was obtained by competition with *N*-[*p*-(hexylamino)benzoyl]-L-glutamate. We conclude that the 6*R*,α*S* diastereoisomer of folinic acid binds at least 5000 times more weakly than the natural compound to *L. casei* dihydrofolate reductase, so that in a solution containing more than 2 mol of (±)-folinic acid per mol of enzyme, only the enzyme–(6*S*,α*S*)-folinic acid complex will be present in significant amounts.

We have also measured the binding constants of tetrahydrofolate and 5-methyltetrahydrofolate, for comparison with that of folinic acid. Under the same conditions, the binding constants of tetrahydrofolate and 5-methyltetrahydrofolate are 2.0 (±0.4) × 10^6 M^{-1} and 3.0 (±0.4) × 10^6 M^{-1}, respectively

Table I: Equilibrium Constants[a] for the Binding of Oxidized Coenzymes to Dihydrofolate Reductase in the Presence and Absence of Folinic Acid

coenzyme	K_C (M^{-1})[b]	K_{FC} (M^{-1})	K_{coop}	ΔG°_{coop} (kcal/mol)
$NADP^+$	6.1×10^4	$2.0 (\pm 0.3) \times 10^4$	0.33	+0.66
$NHDP^+$	9.4×10^3	$4.4 (\pm 0.5) \times 10^3$	0.47	+0.45
$\epsilon NADP^+$	4.9×10^4	$2.0 (\pm 0.2) \times 10^4$	0.41	+0.53
$TNADP^+$	1.4×10^4	$5.3 (\pm 0.6) \times 10^4$	3.8	−0.79
$APADP^+$	8.9×10^3	$6.6 (\pm 0.8) \times 10^3$	0.74	+0.18
PADPR-OMe	5.2×10^4	$4.4 (\pm 0.5) \times 10^4$	0.85	+0.10

[a] K_C is the equilibrium constant for formation of the binary coenzyme–enzyme complex, and K_{FC} is the equilibrium constant for the binding of coenzyme to the enzyme–folinic acid complex. $K_{coop} = K_{FC}/K_C$, and ΔG°_{coop} is the corresponding Gibbs energy change. [b] From Birdsall et al. (1980a).

(measured by using the 6*RS*,α*S* compounds and assuming that only the natural diastereoisomer binds). These values are very similar to one another but a factor of 40–60 lower than that for (6*S*,α*S*)-folinic acid, showing that a formyl group in the 5 position has a substantial effect on binding.

The effects of folinic acid on coenzyme binding (which must, of course, be the same as those of coenzyme on folinic acid binding) have been determined by comparing the coenzyme binding constants measured in the presence of folinic acid with those measured earlier for the binary coenzyme complexes (Birdsall et al., 1980a). The results for the oxidized coenzyme $NADP^+$ and a number of structural analogues are given in Table I. (Many of these experiments, and those in the following sections, were performed with (±)-folinic acid under conditions where only the natural 6*S*,α*S* diastereoisomer bound to the enzyme. Henceforth "folinic acid" will be used to mean the natural diastereoisomer unless otherwise indicated.)

It can be seen from Table I that the oxidized coenzymes examined fall into three classes as far as the effect of folinic acid on their binding is concerned. The three compounds having a normal nicotinamide ring ($NADP^+$, $NHDP^+$, and $\epsilon NADP^+$) bind a factor of 2–3 more weakly to the enzyme–folinic acid complex than to the enzyme alone. The binding of $APADP^+$ and PADPR-OMe is only slightly, and probably not significantly, affected by folinic acid, while $TNADP^+$ binds almost 4 times more tightly in the presence of folinic acid.

When a decrease in the binding constant is observed on addition of a second ligand, it is obviously important to distinguish between negative cooperativity (in which a ternary complex is formed) and simple competition. This can be done by measuring the apparent binding constant, K_{app}, of the coenzyme at different concentrations of folinic acid. For simple competition

$$K_{app} = \frac{K_C}{1 + K_F[F]} \quad (4)$$

where K_C and K_F are the binding constants for coenzyme and folinic acid, respectively, to the enzyme alone and [F] is the concentration of free folinic acid. From this equation, K_{app} will of course decrease monotonically as [F] is increased. For negative cooperativity, on the other hand

$$K_{app} = \frac{K_C + K_{FC}K_F[F]}{1 + K_F[F]} \quad (5)$$

and K_{app} reaches a plateau value of K_{FC}, the equilibrium constant for the binding of coenzyme to form the ternary complex,[2] as [F] is increased. The apparent binding constant

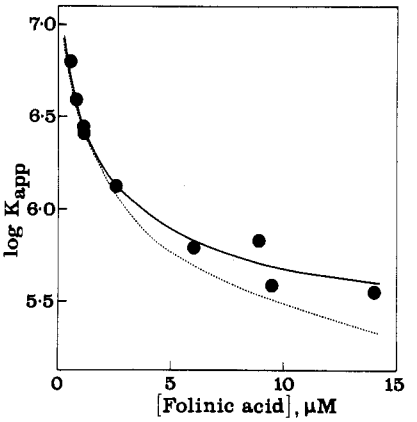


FIGURE 1: Apparent binding constant of NADPH as a function of the free concentration of (6*S*,α*S*)-folinic acid. The broken line was calculated by using eq 4 and the solid line by using eq 5, with $K_{FC} = 1.7 \times 10^5$ M^{-1}.

of $\epsilon NADP^+$ was determined at free folinic acid concentrations of 2.3 and 28 μM, and values of 1.85×10^4 M^{-1} and 2.0×10^4 M^{-1} were obtained. These values are identical within experimental error, as predicted by eq 5, and quite inconsistent with the values of 169 M^{-1} and 14 M^{-1} predicted for the competitive case using eq 4. It is therefore clear that there is negative cooperativity between $\epsilon NADP^+$ and folinic acid in their binding to dihydrofolate reductase. We can reasonably assume that this is also the case for $NADP^+$ and $NHDP^+$, and in fact, negative cooperativity is demonstrated for $NADP^+$ by the NMR experiments described in the following sections. It is particularly notable that a relatively minor structural change, from $NADP^+$ to $TNADP^+$, converts negative cooperativity with folinic acid into positive cooperativity.

The apparent binding constant of NADPH has also been determined in the presence of various concentrations of folinic acid, and the results are shown in Figure 1. The curves in Figure 1 are those described by eq 4 and 5; for eq 5, the "best-fit" curve obtained by varying the value of K_{FC} is shown. The data are better described by the eq 5 than by eq 4. However, the binding of NADPH to the enzyme–folinic acid complex is so much weaker than that to the enzyme alone that the distinction between negative cooperativity and competition is not nearly as clear-cut as in the case of the oxidized coenzymes. Negative cooperativity, rather than competition, between NADPH and folinic acid is unambiguously demonstrated by the NMR experiments described in the following sections which show that (a) in the presence of folinic acid the chemical shift of, for example, the 8 proton of the adenine ring of bound NADPH is different both from that in the binary complex and from that in free NADPH and (b) addition of folinic acid increases the rate constant for dissociation of NADPH from the enzyme.

The value for the binding constant of NADPH to the enzyme–folinic acid complex, K_{FC}, which best fits the data in Figure 1, is 1.7 (±0.5) $\times 10^5$ M^{-1}, compared to 1.0×10^8 M^{-1} for the formation of the binary complex (Dunn et al., 1978). NADPH therefore binds about 600 times less strongly to the enzyme–folinic acid complex than to the enzyme alone (K_{coop}

[2] The degree of cooperativity is defined by the ratio of binding constants $K_{FC}/K_C = K_{coop}$. The corresponding Gibbs energy change is ΔG°_{coop}. K_{coop} and ΔG°_{coop} are also the equilibrium constant and Gibbs energy change of the "disproportionation reaction": EF + EC ⇌ EFC + E. For "positive cooperativity", $K_{coop} > 1$ and $\Delta G^\circ_{coop} < 0$, while for "negative cooperativity", $K_{coop} < 1$ and $\Delta G^\circ_{coop} > 0$; if $K_{coop} = 1$ and $\Delta G^\circ_{coop} = 0$, the binding of the two ligands is totally independent.

Table II: ^{1}H Chemical Shift Changes on Coenzyme Binding to Dihydrofolate Reductase, Measured by Transfer of Saturation

	chemical shift[a] (ppm)							
proton[b]	E-NADP$^+$[c]	E-NADP$^+$-folinic acid	E-NADP$^+$-folate[d]	E-TNADP$^+$[c]	E-TNADP$^+$-folinic acid	E-TNADP$^+$-folate	E-NADPH[c]	E-NADPH-folinic acid
A2	−0.86	−0.95	−0.83	−0.82	−0.94	−0.89	−0.93	−0.90
A8	−0.39	−0.70	−0.47	−0.54	−0.73	−0.53	−0.28	−0.54
N2	0.61	*f*	0.56	−0.03	−0.37	0.32	0.15	0.04
N4	1.36	0.08	0.14	0.11	0.12	0.51	0.64	*e*
N5	0.97	*f*	1.41	*e*	*e*	*e*	*e*	*e*
N6	0.73	0.41	0.69	0.09	0.09	0.40	0.47	0.43
A1′	0.56	0.57	0.53	0.54	0.61	0.48	0.47	0.50
N1′	−0.42	*e*	−0.43	*e*	*e*	*e*	*e*	

[a] Shifts (positive downfield) in the complex relative to the appropriate mononucleotide. [b] A2 and A8 are the protons on the adenine ring, N2, N4, N5, and N6 are those on the nicotinamide ring; A1′ and N1′ are the two ribose anomeric protons. [c] From Hyde et al. (1980a). [d] Data for the "high pH" conformer of this complex; B. Birdsall, A. Gronenborn, E. I. Hyde, G. C. K. Roberts, J. Feeney, G. M. Clore, and A. S. V. Burgen, unpublished results. [e] Not detected. [f] In rapid exchange with free coenzyme; see text.

= 1.7 (±0.5) × 10^{-3}; $\Delta G°_{coop}$ = +3.8 (±0.2) kcal/mol). This value of K_{coop} can be compared with the ratio of coenzyme dissociation rate constants in the absence and presence of folinic acid (k_C/k_{FC}) measured by NMR. These measurements are described below; anticipating that discussion, we can use the weighted average of the rate constants for coenzyme dissociation from the two forms of the ternary complex for this comparison. This gives k_C/k_{FC} = 1.2 × 10^{-3}. For NADP$^+$, K_{coop} = 0.33 and k_C/k_{FC} = 0.2 (see Table V below). In each case, the values of K_{coop} and k_C/k_{FC} are clearly very similar, showing that the effects of folinic acid on the coenzyme binding constants can be substantially accounted for by an effect on the dissociation rate constants and providing further evidence for negative cooperativity.

^{1}H and ^{31}P NMR Chemical Shifts. The existence of this marked negative cooperativity between folinic acid and coenzyme indicates that each ligand alters the environment of the other, probably by producing a conformational change in the protein. To characterize these changes in ligand environment, we have determined the ^{1}H and ^{31}P chemical shifts of oxidized and reduced coenzymes in their ternary complexes with enzyme and folinic acid.

Transfer of saturation experiments were used, as described by Hyde et al. (1980a), to locate the resonances of the aromatic protons of NADP$^+$, TNADP$^+$, and NADPH in these ternary complexes. The chemical shifts of protons of the bound coenzymes measured in this way are given in Table II, together with those measured in the binary complexes and (for NADP$^+$ and TNADP$^+$) in the ternary complex with folate (Hyde et al., 1980a). In Table II, these shifts are quoted relative to those of the mononucleotides rather than those of the free coenzymes themselves. The latter are influenced by the variable degree of intramolecular stacking of the free coenzymes so that, as discussed by Hyde et al. (1980a), the environment of the coenzyme when bound is more accurately reflected by using the mononucleotides as reference compounds.

In the case of the NADP$^+$ complex, transfer of saturation effects were readily observed for the A2, A8, A1′, N4, and N6 protons but not for the N2 and N5 protons. The line widths of the N4 and N6 proton resonances of the free coenzyme increased appreciably with increasing temperature in the range 5–35 °C, as would be expected if exchange between bound and free states is slow (cf. eq 3; this exchange broadening is discussed in more detail below). By contrast, the N2 and N5 proton resonances of the free coenzyme sharpened significantly with increasing temperature, showing that for these protons exchange between bound and free states is fast on the NMR time scale. Since the rate of exchange of all the nicotinamide protons must be the same, it follows that the chemical shift difference between bound and free states must be substantially smaller for the N2 and N5 protons than for N4 and N6. Since no progressive change in chemical shift of the N2 and N5 resonances with increasing NADP$^+$ concentration was observed, and from the estimated rates of exchange (see following section), we estimate that the resonances of the N2 and N5 protons of the bound coenzyme must be within 15 Hz (0.06 ppm) of the corresponding resonances of the free coenzyme. The N2 and N5 resonances of NADP$^+$ are 0.290 and 0.154 ppm, respectively, upfield of those of NMN$^+$ (at 11 °C; Hyde et al., 1980a), so that relative to the mononucleotide (the scale used for the other protons in Table II) the shifts of N2 and N5 in the complex will be roughly −0.2 ppm.

The shifts of the adenine and nicotinamide ring protons of NADP$^+$ in the enzyme–NADP$^+$–folinic acid complex are clearly different from those in either the enzyme–NADP$^+$ or the enzyme–NADP$^+$–folate[3] complexes. In the latter complexes, all the nicotinamide proton signals (with the exception of N4 in the ternary folate complex) are shifted substantially downfield, whereas in the ternary complex with folinic acid only N6 shows a large downfield shift. The adenine proton resonances are also affected by the presence of folinic acid, with both, but particularly A8, being shifted further upfield. No other substrate analogue or inhibitor which we have examined has affected the adenine proton chemical shifts on forming the ternary complex [see, e.g., Hyde et al. (1980a)]. The C2 proton resonance of His-64, whose imidazole ring is in contact with the adenine ring in the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979), has the same chemical shift, 3.95 ppm, in the enzyme–folinic acid–NADP$^+$ or –NADPH complexes as in other binary and ternary coenzyme complexes (Gronenborn et al., 1981c).

Only two of the nicotinamide protons of NADPH could be located in the spectrum of the ternary complex with folinic acid, so that little can be said of the environment of this ring. However, the A8 proton resonance of NADPH is farther upfield in the ternary than in the binary complex by a similar amount to that observed for NADP$^+$ (0.26 vs. 0.31 ppm).

The environment of the bound coenzyme can also be investigated by examining its ^{31}P spectrum (Feeney et al., 1975; Hyde et al., 1980b). The 81-MHz ^{31}P spectra of the enzyme–NADPH and enzyme–NADPH–folinic acid complexes

[3] We have recently found (Birdsall et al., 1981; B. Birdsall, A. Gronenborn, E. I. Hyde, G. C. K. Roberts, J. Feeney, G. M. Clore, and A. S. V. Burgen, unpublished results) that this complex exists in a pH-dependent equilibrium between three conformers; the data in Table II are for the form which predominates at pH* 6.8.

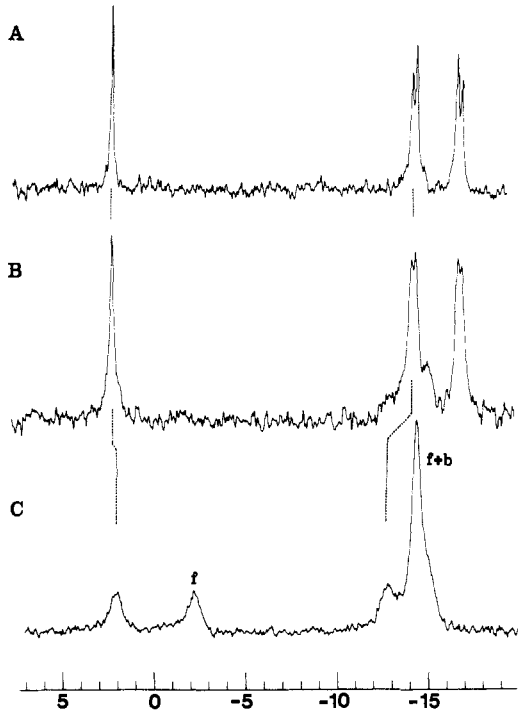


FIGURE 2: 81-MHz ^{31}P spectra of coenzyme bound to dihydrofolate reductase. (A) Enzyme–NADPH binary complex, [NADPH]/[enzyme] = 1. (B) Enzyme–NADPH–folinic acid ternary complex, [NADPH]/[enzyme] = 1. (C) Enzyme–$NADP^+$–folinic acid ternary complex, [$NADP^+$]/[enzyme] = 1.5. The 2′-phosphate resonance of free $NADP^+$ (at −0.22 ppm) is labeled f, and the peak comprising both pyrophosphate resonances from free $NADP^+$ and one pyrophosphate resonance from bound $NADP^+$ is labeled f + b; the second pyrophosphate signal from the bound $NADP^+$ can be seen just to low field of the latter peak.

Table III: ^{31}P Chemical Shifts of Coenzymes, Free and Bound to Dihydrofolate Reductase

coenzyme or complex	chemical shift[a] (ppm) 2′-phosphate	pyrophosphate
$NADP^+$	−1.88	−14.14, −14.47
E–$NADP^+$[b]	2.7	−14.3, −16.2
E–$NADP^+$–folinic acid	2.15	−12.7, −15.1
E–$NADP^+$–folate[b]	2.7	−14.4, −16.3
$TNADP^+$	−1.90	−14.15, −14.47
E–$TNADP^+$	2.4[c]	−14.0, −15.2
E–$TNADP^+$–folinic acid	2.5[c]	−14.5, −14.5
E–$TNADP^+$–folate[b]	2.5[c]	−14.4, −15.5
PADPR-OMe	−1.88	−13.7
E-PADPR-OMe[b]	2.7	−14.2, −16.1
E-PADPR-OMe–folinic acid	2.15	−14.9, −16.7
E-PADPR-OMe–folate[b]	2.5[c]	−14.2, −16.1
NADPH	−1.19	−13.75, −13.78
E-NADPH	2.7	−14.3, −16.2
E-NADPH–folinic acid	2.7	−14.3, −16.2

[a] Relative to inorganic phosphate, pH* 8 (2.94 ppm downfield from H_3PO_4). [b] From Hyde et al. (1980b). [c] ±0.25 ppm.

are shown in Figure 2A,B. Neither the 2′-phosphate nor the pyrophosphate resonances are affected in chemical shift by the presence of folinic acid, although all three signals are broadened, due to the increased rate of dissociation of the coenzyme from the ternary complex (see below).

By contrast, addition of folinic acid to the enzyme–$NADP^+$ complex does affect the ^{31}P chemical shifts of the bound coenzyme, as shown in Figure 2C [the ^{31}P chemical shifts of $NADP^+$ and NADPH in their respective binary complexes with the enzyme are the same (cf. Table III) so that the line positions in Figure 2A can be compared with those in Figure 2C]. The spectrum shown in Figure 2C was obtained from a sample containing $NADP^+$ in a molar ratio to the enzyme of 1.5, and separate resonances from the 2′ phosphate of free and bound coenzyme can be seen at −0.22 and 2.15 ppm, respectively. Both signals are very broad, due to the relatively rapid exchange of $NADP^+$ between the free and bound states (see below). In the binary complex, the 2′-phosphate resonance appears at 2.7 ppm, so that folinic acid binding has produced an upfield shift of 0.55 ppm. It can also be seen from Figure 2C that one of the two pyrophosphate resonances has been shifted 1.6 ppm downfield by folinic acid. The other pyrophosphate signal is in rapid exchange with the pyrophosphate signal of free coenzyme; its chemical shift, measured by using a sample having an [$NADP^+$]/[enzyme] ratio of 0.5, is given in Table III, where it can be seen that it too is shifted downfield on folinic acid binding.

Table IV: Chemical Shifts of Protons of Folinic Acid, Free and Bound to Dihydrofolate Reductase

complex	chemical shift[a] (ppm) formyl	benzoyl 3,5 protons	benzoyl 2,6 protons
folinic acid	4.91, 4.18	2.96	3.88
E-folinic acid[b]	5.26	1.93	3.45
E-folinic acid–$NADP^+$	5.10	1.83	3.48

[a] Relative to dioxane, downfield positive. [b] From Feeney et al. (1981).

This table summarizes the ^{31}P chemical shifts of $NADP^+$, $TNADP^+$, PADPR-OMe, and NADPH in their binary complex and a number of ternary complexes. It is notable that in $TNADP^+$ neither the 2′-phosphate nor the pyrophosphate resonances are affected by folinic acid binding, whereas in PADPR-OMe the 2′-phosphate signal is shifted upfield just as in $NADP^+$, but the pyrophosphate resonances are also shifted upfield rather than downfield. The effects of folinic acid on the 2′-phosphate resonance of $NADP^+$ together with those on the A2 and A8 proton resonances discussed above show clearly that the adenine end of the bound coenzyme is affected by folinic acid binding. This is in marked contrast to the lack of effect of folate, methotrexate, or trimethoprim (Hyde et al., 1980a,b).

The formyl and benzoyl proton resonances of folinic acid in the enzyme–$NADP^+$–folinic acid complex have also been located by transfer of saturation experiments. Their chemical shifts are given in Table IV with, for comparison, those of folinic acid free and in its binary complex with the enzyme. While the benzoyl protons of folinic acid undergo a large upfield shift on binding to form the binary complex (Feeney et al., 1981), addition of $NADP^+$ to give the ternary complex has very little further effect. The two chemical shifts for the formyl proton of free folinic acid quoted in Table IV correspond to the two rotameric states of the formyl group (Feeney et al., 1980). Only one of these, that with the formyl proton oriented toward the 4-oxo substituent on the pteridine ring, which gives the 4.91-ppm signal in the folinic acid, is observed in the binary (Birdsall et al., 1980c; Feeney et al., 1981) or ternary complexes. The 0.35-ppm downfield shift of the formyl proton in the binary complex is reduced to 0.19 ppm in the presence of $NADP^+$.

Kinetics: Evidence for Two Conformations of the Complex. The rate constant for the dissociation of the coenzyme from its complexes with dihydrofolate reductase can be measured both by ^1H transfer of saturation experiments and by line-

Table V: Apparent Rate Constants for Dissociation of Coenzyme from Enzyme–Coenzyme and Enzyme–Coenzyme–Folinic Acid Complexes

	k_{app} (s^{-1})		
complex	^1H transfer of saturation[a]	stopped-flow	^{31}P line-shape analysis[b]
E–NADP$^+$[c]	22 (±5)		25 (±5)
E–NADP$^+$–folinic acid	20 (±5)		125 (±15)
E–NADPH		0.06[d]	
E–NADPH–folinic acid	6.5 (±2)		50 (±10)

[a] At 11 °C, 50 mM phosphate, pH* 6.8, and 500 mM KCl. [b] At 11 °C, 50 mM Bistris, pH* 6.8, and 500 mM KCl. [c] From Hyde et al. (1980a,b). [d] From Dunn et al. (1978) and Dunn (1978).

shape analysis of the 2′-phosphate ^{31}P resonance (Hyde et al., 1980a,b). We have used both methods (outlined under Experimental Procedures) to study the kinetics of dissociation of NADP$^+$ or NADPH from the enzyme–coenzyme–folinic acid complexes. For the transfer of saturation experiments, the A2 proton resonance of NADPH and both A2 and N6 proton resonances of NADP$^+$ were used. The values of the apparent dissociation rate constants estimated by both methods are given in Table V.

For both NADP$^+$ and NADPH, the apparent dissociation rate constant from ternary complex determined from ^{31}P line-shape analysis is about 6 times that measured by ^1H transfer of saturation. The closely similar values obtained for the enzyme–NADP$^+$ complex by the two methods suggest that the marked discrepancy observed for the ternary complex cannot be attributed to systematic error or to the different buffers used.

The simplest explanation for this discrepancy is that the enzyme–coenzyme–folinic acid complexes exist in two states from which the coenzyme dissociates at different rates. The single 2′-phosphate signal observed for the bound coenzyme would be an average of the resonances from these two states, so that the dissociation rate constant estimated by line-shape analysis would be a weighted average of those from the two states. However, if the chemical shifts of the N6 proton, for example, were sufficiently different in the two states, separate resonances would be observed. The signal located by transfer of saturation would then correspond to only *one* of the two forms,[4] and the dissociation rate constant measured in this experiment would differ from the weighted average measured by ^{31}P line-shape analysis.

In a thorough search of the relevant region of the spectrum at several temperatures, only one frequency position giving transfer of saturation was found for the N6 proton (and similarly for the A2, N8, N4, and A1′ positions). This could be explained if the N6 resonance from the second form of the complex were sufficiently close to that of the free coenzyme for there to be rapid exchange between these two states. On this hypothesis, both the chemical shift and line width of this "free" signal should depend upon coenzyme concentration in a characteristic way. A small concentration dependence of the chemical shift of the "free" N6 proton signal was in fact observed; when 25% of the coenzyme was bound, the N6 proton signal was 0.045 ppm (12.1 Hz) downfield of the position for coenzyme alone at the same concentration. The concentration dependence of the line width of this signal is shown in Figure 3. If the only form of the complex present was that whose N6 proton was located by transfer of saturation, then the line width of the "free" signal would be given by eq 3, with k_f measured by the saturation transfer experiment. The line widths predicted on this basis are shown as the dotted line in Figure 3; they are obviously much smaller than those observed. On the other hand, a model involving two forms of the complex *is* able to describe the data adequately (with a single adjustable parameter), as indicated by the shaded curve in Figure 3 (calculated as described under Appendix).

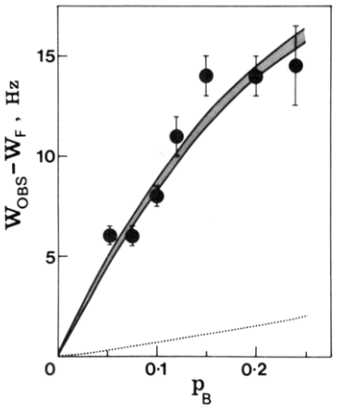


FIGURE 3: Line width of the "free" N6 proton resonance of NADP$^+$ in the presence of the enzyme–NADP$^+$–folinic acid complex as a function of the fraction of the coenzyme bound to the protein (calculated by using the binding constant in Table I). Data could not be reliably obtained for $P_B > 0.25$ ([NADP$^+$]/[enzyme] < 4) because the broad N6 resonance overlapped a number of peptide NH resonances from the protein. The line width is plotted as observed line width (W_{obsd}) minus the line width of coenzyme alone (W_F) under the same conditions. The dashed line was calculated by using eq 3, with k_{app} from the transfer of saturation experiments. The shaded area represents a family of curves calculated on the basis of the three-site model described under Appendix.

By proposing that two conformational states of the complex exist, we are thus able to account in a self-consistent way for the ^1H transfer of saturation, chemical shift, and line width and ^{31}P line width data. These data are not sufficient for a quantitative analysis of the general three site exchange model, but, as described under Appendix, it can be shown that a simplified model is quantitatively consistent with the data and allows limits to be placed on the values of the kinetic parameters.

Discussion

Whenever two different ligands bind simultaneously to a protein, some degree of mutual effect on their binding constants or "free energy coupling" (Weber, 1975) is to be expected. For example, both positive and negative free energy couplings between the binding of protons and that of other ligands is commonly observed. As noted in the introduction, positive cooperativity ($K_{coop} > 1$, $\Delta G°_{coop}$ negative) between coenzyme and substrate binding is often seen even in simple dehydrogenases. Negative cooperativity between different ligands ($K_{coop} < 1$, $\Delta G°_{coop}$ positive) is of course also possible, and many examples are known among oligomeric "allosteric" proteins, with perhaps the most familiar being the interaction between oxygen and diphosphoglycerate binding to hemoglobin (Benesch et al., 1968; Benesch & Benesch, 1974). In simple proteins, however, negative cooperativity of the sort described here has more rarely been reported. (It should be noted that we are using the term "negative cooperativity" in a purely

[4] When transfer of saturation experiments are used to locate "hidden" resonances, they give no indication of the population of the species whose resonance is located; efficient transfer of saturation can be observed from species with very low populations (Clore et al., 1981).

operational sense, as outlined in footnote 2. It is not intended to imply anything about the molecular mechanism of the effect.) Among the dehydrogenases, negative cooperativity has been observed between chloride ion and NADH binding to lactate dehydrogenase (Winer, 1963; Anderson, 1981) while in bovine serum albumin negative cooperativity between anilinonaphthalenesulfonate and 3,5-dihydroxybenzoate has been reported (Kolb & Weber, 1975).

In *L. casei* dihydrofolate reductase, one of the smallest known dehydrogenases, we have observed both large positive and large negative free-energy coupling between coenzyme and substrate analogue binding. For NADPH and methotrexate, $\Delta G°_{coop}$ is −3.84 kcal/mol (Birdsall et al., 1980a), while for NADPH and folinic acid, $\Delta G°_{coop}$ is +3.76 kcal/mol. Although these Gibbs energy changes are not large compared to those accompanying formation of the binary complexes of these ligands (−11 to −13 kcal/mol: Hood & Roberts (1978), Birdsall et al. (1980a), and present work), they are larger than any of the free-energy couplings tabulated by Weber (1975). [For example, the interaction between oxygen and diphosphoglycerate binding to hemoglobin is characterized by a free-energy coupling of only +1.3 kcal/mol (Tyuma et al., 1971).] Free-energy couplings of the magnitude seen in dihydrofolate reductase lead to very substantial effects on the distribution of the ligands between binary and ternary complexes. Consider solutions containing concentrations of coenzyme and substrate analogue such that in each case the enzyme is half-saturated with each ligand ($\bar{y}_1 = \bar{y}_2 = 0.5$). If the binding of the two ligands is independent ($\Delta G°_{coop} = 0$), then the fraction of the enzyme in the form of the ternary complex, with both ligands bound, $\bar{y}_{12}$, will of course be 0.25. For NADPH and methotrexate, however, with $\Delta G°_{coop}$ = −3.84 kcal/mol, when $\bar{y}_1 = \bar{y}_2 = 0.5$, then $\bar{y}_{12} = 0.48$, so that 96% of the enzyme molecules having ligand bound will be in the form of the ternary complex. In contrast, for NADPH and folinic acid, with $\Delta G°_{coop}$ = +3.7 kcal/mol, when $\bar{y}_1 = \bar{y}_2 = 0.5$, then $\bar{y}_{12} = 0.02$, and most of the enzyme will be in the form of binary complexes, with only 2% existing as the ternary complex.

The observed negative cooperativity between NADPH and folinic acid may be important for the pharmacological use of folinic acid. This compound is widely used in conjunction with high-dose methotrexate therapy (Shapiro et al., 1977). It is employed as a "rescue" compound, being administered 2–6 h after methotrexate to prevent some of the toxic effects of the latter compound by increasing the levels of tetrahydrofolate derivatives in the cell and perhaps by displacing methotrexate from dihydrofolate reductase, thus speeding up its "washout" from the cell. In the absence of coenzyme, folinic acid binds very tightly to the reductase. If there were similarly tight binding to the mammalian enzyme, the "rescue" effects of folinic acid would tend to be offset by the fact that it would itself be a powerful inhibitor of the enzyme. However, since it is likely that in the cell the enzyme is usually saturated with NADPH, it is the 600-fold weaker binding to the enzyme–NADPH complex which is important in this context, and folinic acid binding to the reductase will be substantially reduced if not abolished. Folinic acid is thus able to reverse methotrexate toxicity without itself producing significant additional inhibition of the reductase.

The results obtained in the present work for the different coenzyme analogues and the comparisons of these with our earlier studies of cooperativity with folate, trimethoprim, and methotrexate (Birdsall et al., 1980a) show that this cooperativity depends markedly on ligand structure and, hence, has a substantial influence on the specificity of the enzyme. For example, the ratio of the binding constant of methotrexate to that of folinic acid is about 16 in the binary complexes but 6.4×10^6 in their ternary complexes with NADPH (Hood & Roberts, 1978; Birdsall et al., 1980a; present work). This dramatic effect of the binding of a second ligand on the relative affinities for structurally related compounds extends to compounds of much more similar structure. Thus the ratio of binding constants of NADPH and $NADP^+$ is 1640 in the binary complex, 89 500 in the ternary complex with methotrexate, and 8.4 in the ternary complex with folinic acid—a range of Gibbs energy differences of 1.3–6.7 kcal/mol. Similarly, the ratio of binding constants of $NADP^+$ and $TNADP^+$ ranges from 0.4 in the folinic acid ternary complex to 29 in that containing folate. The nicotinamide ring obviously plays a major role in determining the nature of the interaction between folinic acid and coenzyme. In the absence of a nicotinamide ring (PADPR-OMe), there is no significant interaction, while an oxidized nicotinamide ring gives modest negative cooperativity and a reduced nicotinamide ring large negative cooperativity. It is particularly striking that the single atom difference between $NADP^+$ and $TNADP^+$ converts negative to positive cooperativity.

The relationship between structure and binding constant among, for example, coenzyme analogues is clearly *not* a unique one. The effect of the ligand occupying the substrate binding site is at least comparable with the effects of structural modification of the coenzyme itself, so that one must consider a series of structure-binding relationships, each applicable to a particular enzyme–substrate or –substrate analogue complex.

Such relationships can only be understood in molecular terms if the mode of binding of the structurally related ligands can be compared in detail. For the enzyme–coenzyme–folinic acid complexes, these comparisons are complicated by the finding that these complexes exist in two or more slowly interconverting conformational states. A number of other instances of slow interconversion between conformational states have been observed with dihydrofolate reductase. For the enzyme in the absence of ligands, evidence for the coexistence of two or more conformations has been obtained from equilibrium binding studies of the *Escherichia coli* reductase (Pattishall et al., 1976), from stopped-flow kinetic studies of the *L. casei* enzyme (Dunn et al., 1978), and from ^{13}C NMR studies of the *Streptococcus faecium* enzyme (London et al., 1979). More recently, we have demonstrated the existence of slowly interconverting forms of the enzyme–$NADP^+$–trimethoprim (Gronenborn et al., 1981a,b) and enzyme–$NADP^+$–folate (Birdsall et al., 1981; B. Birdsall, A. Gronenborn, E. I. Hyde, G. C. K. Roberts, J. Feeney, G. M. Clore, and A. S. V. Burgen, unpublished results) complexes of the *L. casei* enzyme.

At present we do not have sufficient information to compare the coenzyme binding and chemical shifts for the two forms of the enzyme–coenzyme–folinic acid complexes individually (as could be done for the enzyme–$NADP^+$–trimethoprim complex; Gronenborn et al., 1981b). However, even a general comparison of chemical shifts and binding constants gives some useful indications of the origins of negative cooperativity in this system.

To explore the relationship between changes in coenzyme environment and negative cooperativity, we can compare the changes in $NADP^+$ chemical shifts produced by folate with those produced by folinic acid and the effects on $TNADP^+$ and PADPR-OMe with those on $NADP^+$. The most obvious difference between folate and folinic acid is that the latter,

Table AI: Estimated Populations, Dissociation Rate Constants, and Chemical Shifts of the Two Conformations of the Enzyme-$NADP^+$-Folinic Acid Complex

	populations		dissociation rate constants (s^{-1})		N6 proton chemical shifts (ppm)		
case[a]	f_1	f_2	k_{-1}	k_{-2}	$(\delta_{B_2}-\delta_F)$[b]	δ_{B_2}[c]	δ_{B_1}[c,d]
I	0.12 (±0.03)	0.88 (±0.03)	170	119 (±25)	0.16	−0.11 (±0.02)	0.41
II	0.38 (±0.11)	0.62 (±0.11)	53 (±25)	170	0.24	−0.03 (±0.02)	0.41

[a] The two cases correspond to the extreme values consistent with the data; for case I, $k_{-1} = 170\ s^{-1}$, and for case II, $k_{-2} = 170\ s^{-1}$. Calculations based on the simplified model, eq A1. [b] Magnitude estimated from line-width calculations; sign from observed shift. [c] Relative to mononucleotide. [d] Measured by transfer of saturation.

but not the former, alters the environment of the adenine end of the coenzyme. The resonances of the A2 and A8 protons and of the 2′-phosphate are all at higher field in the folinic acid complex (in at least one form of the ternary complex). In the crystal structure of the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979), the imidazole ring of His-64 is in contact with both the adenine ring and the 2′-phosphate. However, this particular feature of the environment of the adenine end of the coenzyme does not appear to be affected by folinic acid binding, since the chemical shift of the C2-proton resonance of His-64 is the same in the enzyme–coenzyme–folinic acid complexes as in the other coenzyme complexes, in which it seems to be determined largely by the adenine ring current (Gronenborn et al., 1981c).

Although these changes at the adenine end of the coenzyme, together with the downfield shifts of the pyrophosphate ^{31}P resonances, clearly distinguish folinic acid binding from folate binding, it is more difficult to relate them in a simple way to the occurrence of negative cooperativity. Thus in the enzyme–TNADP$^+$–folinic acid complex, whose formation is characterized by positive cooperativity, the A2 and A8 protons have the same chemical shift as in the $NADP^+$ complex, but the 2′-phosphate and pyrophosphate resonances do not, having instead the same shifts as in the enzyme–TNADP$^+$–folate complex. The coenzyme analogue lacking the nicotinamide ring, PADPR-OMe, shows no cooperativity with folinic acid; in its ternary complex, the 2′-phosphate resonance behaves like that of $NADP^+$, but the pyrophosphate signals do not (shifting, in fact, in the opposite direction).

It appears, then, that no one feature of the coenzyme environment alone correlates in a simple way with the existence of negative cooperativity. Only when *all* the characteristic changes occur is negative cooperativity seen. Depending on the interactions of the coenzyme with the nicotinamide subsite, partial changes in environment of the rest of the coenzyme can result, and these can be accompanied by no cooperativity or by positive cooperativity.

The changes in the pyrophosphate and nicotinamide chemical shifts of $NADP^+$ produced by folinic acid binding bear some resemblance to those seen in conformation II of the enzyme–$NADP^+$–trimethoprim complex (Gronenborn et al., 1981b). The chemical shifts of the nicotinamide protons (relative to the mononucleotide) seem to be as small in form 2 of the folinic acid complex as they are in conformation II of the trimethoprim complex, and the pyrophosphate ^{31}P shifts are also very similar (−12.7 and −15.1 ppm for the folinic acid complex and −12.9 and −14.9 for the trimethoprim complex). Although these changes in ^{31}P chemical shift probably indicate that the binding of folinic acid does change the conformation of the pyrophosphate "backbone" of the coenzyme, this change must differ from that produced by trimethoprim, since the ^{31}P–^{31}P coupling constant has its normal value of 20 Hz in the folinic acid complex, compared to 11 Hz in conformation II of the trimethoprim complex (Gronenborn et al., 1981b).

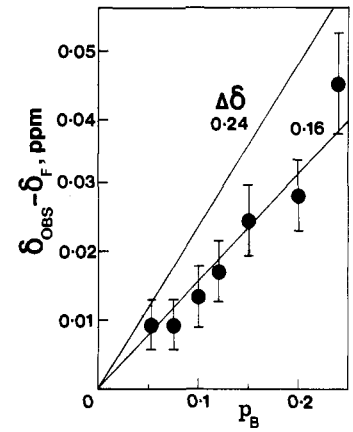
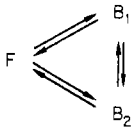


FIGURE 4: Chemical shift of the "free" N6 proton resonance of $NADP^+$ (relative to that of $NADP^+$ alone) in the presence of the enzyme–$NADP^+$–folinic acid complex as a function of the fraction of the coenzyme bound to the enzyme (calculated from the binding constant in Table I). The two lines were calculated by using eq A6 and the values of $\Delta\delta$ $(=\delta_{B_2} - \delta_F)$ in Table AI.

In contrast to the widespread effects on the environment of $NADP^+$, the reduced coenzyme NADPH is much less affected by folinic acid, in spite of the very large negative cooperativity between these two ligands. The effects of folinic acid on the reduced nicotinamide ring cannot be properly assessed, since we have only been able to measure the chemical shifts of the N2 and N6 protons in the ternary complex; these do not, however, differ greatly from those of the corresponding protons in the binary complex. The A8 proton of NADPH is affected by folinic acid and in a very similar way to that of $NADP^+$. Thus, folinic acid, in contrast to other substrate analogues, does affect the environment of the adenine end of NADPH. This change in environment is not, however, the same as the change produced in that of the corresponding part of $NADP^+$, since neither the A2 proton nor the 2′-phosphate resonances of NADPH are shifted by addition of folinic acid. Similarly, the pyrophosphate signals of $NADP^+$ are affected by folinic acid, but those of NADPH are not.

The relatively modest negative cooperativity between folinic acid and $NADP^+$ ($\Delta G°_{coop} = +0.66$ kcal/mol) is thus associated with much more extensive changes in the coenzyme environment than is the very large negative cooperativity between folinic acid and NADPH ($\Delta G°_{coop} = +3.76$ kcal/mol). It appears to be generally true in *L. casei* dihydrofolate reductase that the environment of the reduced coenzyme is less affected by the binding of other ligands or by structural changes in the coenzyme itself than that of the oxidized coenzyme (E. Hyde, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished results). In the present instance, the different effects on the oxidized and reduced coenzymes show clearly that the negative cooperativity between folinic acid and coenzyme is produced by two distinct mechanisms.

Scheme I



Acknowledgments

We are most grateful to Drs. P. Charlton and D. W. Young (University of Sussex) for generously providing samples of the separated diastereoisomers of folinic acid and to Gill Ostler and John McCormick for skillful technical assistance.

Appendix

Analysis of the Kinetic Data in Terms of a Simplified Three-Site Exchange Model. The exchange of coenzyme between the free state (F) and two forms of the complex (B_1 and B_2) can be described by Scheme I. In the case of the enzyme–$NADP^+$–folinic acid complex, we have not been able to observe directly separate resonances from B_1 and B_2, and we cannot determine all the parameters in this general scheme. In order to establish that the measured chemical shifts and line widths are quantitatively consistent with a model of this type, we have analyzed them using a simplified model in which it is assumed that the rate of interconversion between the two forms of the complex is slow compared to the dissociation of the coenzyme from each, giving

$$B_1 \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} F \underset{k_{+2}}{\overset{k_{-2}}{\rightleftharpoons}} B_2 \qquad \text{(A1)}$$

No direct measurement of the rate of interconversion of B_1 and B_2 is available for the enzyme–$NADP^+$–folinic acid complex. However, if this rate is similar to the rates of interconversion of different conformational forms measured for the enzyme–$NADP^+$–trimethoprim complex (2 s^{-1} at 26 °C; Gronenborn et al., 1981a,b) and the enzyme–$NADP^+$–folate complex ($\ll$19 s^{-1} at 11 °C; Birdsall et al., 1981; B. Birdsall, A. Gronenborn, E. I. Hyde, G. C. K. Roberts, J. Feeney, G. M. Clore, and A. S. V. Burgen, unpublished results), then this assumption would be justified.

As indicated under Results, we assume, in addition to model A1, the following:

(i) The chemical shifts of the ^{31}P resonances are such that B_1 and B_2 are in rapid exchange with each other and in slow exchange with F. The apparent dissociation rate constant measured from the ^{31}P line width will then be

$$k_{app,P} = f_1 k_{-1} + f_2 k_{-2} \qquad \text{(A2)}$$

where f_1 and f_2 (with $f_1 + f_2 = 1$) represent the relative populations of B_1 and B_2.

(ii) The chemical shifts of the N6 proton resonances are such that B_2 and F are in rapid exchange with each other and in slow exchange with B_1. The apparent dissociation rate constant measured from the transfer of saturation experiments will then be (for small P_B, i.e., large [$NADP^+$]/[enzyme] ratios, as used in these experiments)

$$k_{app,H} \simeq f_1 k_{-1} \qquad \text{(A3)}$$

From eq A2 and A3 and taking the experimental values for the enzyme–$NADP^+$–folinic acid complex from Table V, we have

$$f_1 k_{-1} = 20\ s^{-1} \qquad f_2 k_{-2} = 105\ s^{-1} \qquad \text{(A4)}$$

In the ^{31}P spectrum, separate, though broad, 2′-phosphate signals are seen for F and (B_1 + B_2) at 40.5 MHz, with a chemical shift difference of 96 Hz. Clearly if either k_{-1} or k_{-2} were much greater than 96 s^{-1}, these separate signals would not be observed. Line-shape simulations showed that, on this basis, 170 s^{-1} is a rather generous upper limit to the values of k_{-1} and k_{-2}. Combining the two limiting conditions, k_{-1} = 170 s^{-1} and k_{-2} = 170 s^{-1}, with eq A4, we can estimate the ranges of f_1, f_2, k_{-1}, and k_{-2}, which are consistent with the measured k_{app} values; these are given in Table AI. The line width of the "free" N6 proton resonance (i.e., that of F + B_2) can, on this model, be approximated as

$$\pi(W_{obsd} - W_F) = \frac{f_1 P_B}{1 - f_1 P_B} k_{-1} + f_2 P_B \left(\frac{1}{T_{2B}} - \frac{1}{T_{2F}} \right) + f_2 P_B (1 - f_2 P_B)^2 (\Delta\omega^2 / k_{-2}) \qquad \text{(A5)}$$

where W_{obsd} is the measured line width and W_F that of $NADP^+$ alone under the same conditions. T_{2B} and T_{2F} are the spin–spin relaxation times of the N6 proton in the bound and free states, respectively, and $\Delta\omega = 2\pi(\delta_{B_2} - \delta_F)$, where δ_{B_2} and δ_F are the N6 proton chemical shifts in the B_2 and F states, respectively. The first term on the right-hand side is the contribution from the slow exchange with B_1, the second represents the contribution from the line width of the bound species (B_2), and the third is again an exchange contribution, from the moderately rapid exchange between B_2 and F.

Of the parameters in eq A5, P_B can be calculated from the concentration of enzyme and $NADP^+$ and the binding constant (Table I), while ranges for f_1, f_2, k_1, and k_2 are given in Table IV. The value of $1/T_{2B}$ was estimated from the line width of the bound N6 proton signal of $NADP^+$ in complexes where it can be observed directly (Gronenborn et al., 1981a). Thus only $\Delta\omega$ remains unknown. The shaded area in Figure 3 represents a family of curves calculated from eq A5 by using a "best-fit" value of $\Delta\omega^2$ and the range of f_1, f_2, k_1, and k_2 values indicated in Table AI. It is clear that this equation provides an adequate fit to the data.

The values of $\Delta\omega^2$ required to fit the line-width data provide estimates of the magnitude (but not the sign) of $\delta_{B_2} - \delta_F$. The chemical shift of the "free" N6 proton resonance will be given by

$$\delta_{obsd} = P_B \delta_{B_2} + P_F \delta_F \qquad \text{(A6)}$$

The direction of the observed shift (downfield) gives the sign of $\delta_{B_2} - \delta_F$ as positive, and the limits obtained from the line-width analysis are included in Table AI. The concentration dependence of the chemical shift predicted by these values is compared with the data in Figure 4. The lower value of $\delta_{B2} - \delta_F$ leads to excellent agreement with the observed chemical shifts, although the errors involved in measuring these rather small changes in chemical shift of a broad line are such that a precise value cannot be obtained.

It is clear that the three-site exchange model (eq A1) is quantitatively consistent with the data, and it follows that the general three-site model would be similarly consistent. Since the available data were only adequate to characterize the simplified model (eq A1), the reliability of the limits on the kinetic parameters given in Table AI depends entirely on the correctness of the assumption that the rate of interconversion of B_1 and B_2 is slow.

References

Anderson, S. R. (1981) *Biochemistry 20*, 464.

Benesch, R. E., & Benesch, R. (1974) *Adv. Protein Chem. 28*, 211.

Benesch, R., Benesch, R. E., & Yu, C. I. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 526.

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. (1980a) *Biochemistry 19*, 3723.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980b) *Biochemistry 19*, 3732.

Birdsall, B., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1980c) *FEBS Lett. 120*, 107.

Birdsall, B., Gronenborn, A., Clore, G. M., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981) *Biochem. Biophys. Res. Commun. 101*, 1139.

Brändén, C.-I., Jörnvall, H., Eklund, H., & Furngren, B. (1975) *Enzymes, 3rd Ed. 11*, 1.

Clore, G. M., Roberts, G. C. K., Gronenborn, A., Birdsall, B., & Feeney, J. (1981) *J. Magn. Reson. 45*, 151.

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. (1978) Ph.D. Thesis, Council for National Academic Awards, United Kingdom.

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

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

Feeney, J., Albrand, J. P., Boicelli, C. A., Charlton, P. A., & Young, D. W. (1980) *J. Chem. Soc., Perkin Trans. 2*, 176.

Feeney, J., Birdsall, B., Albrand, J. P., Roberts, G. C. K., Burgen, A. S. V., Charlton, P. A., & Young, D. W. (1981) *Biochemistry 20*, 1837.

Forsén, S., & Hoffmann, R. A. (1963) *J. Chem. Phys. 39*, 2892.

Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981a) *Nature (London) 290*, 273.

Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981b) *Mol. Pharmacol. 20*, 145.

Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981c) *Biochemistry 20*, 1717.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossman, M. G. (1975) *Enzymes, 3rd Ed. 11*, 191.

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

Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980a) *Biochemistry 19*, 3738.

Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980b) *Biochemistry 19*, 3746.

Kolb, D. A., & Weber, G. (1975) *Biochemistry 14*, 4476.

London, R. E., Groff, J. P., & Blakley, R. L. (1979) *Biochem. Biophys. Res. Commun. 86*, 779.

Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. J. G., 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.

Pascal, M., & Schuber, F. (1976) *FEBS Lett. 66*, 107.

Pattishall, K. H., Burchall, J. J., & Harvey, R. J. (1976) *J. Biol. Chem. 251*, 7011.

Shapiro, W. R., Mehta, B. M., & Hutchison, D. J. (Eds.) (1977) *Cancer Treat. Rep. 61*, 505.

Tyuma, I., Shimizu, K., & Imai, K. (1971) *Biochem. Biophys. Res. Commun. 43*, 423.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1.

Winer, A. D. (1963) *Acta Chem. Scand. 17 (Suppl. 1)*, S203.

Winer, A. D., & Schwert, G. W. (1959) *J. Biol. Chem. 234*, 1155.

Winer, A. D., & Theorell, H. (1959) *Acta Chem. Scand. 13*, 1038.