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Phosphorus-31 Nuclear Magnetic Resonance Studies of the Binding of Oxidized Coenzymes to *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The ^{31}P NMR spectra of $NADP^+$ and a number of its structural analogues have been obtained from their binary and ternary complexes with *Lactobacillus casei* dihydrofolate reductase. The 2′-phosphate resonance is shifted downfield 2.7–2.9 ppm in all cases. Line-shape analysis of this resonance as a function of coenzyme concentration gave values for the dissociation rate constant of the coenzyme from many of the complexes. The values obtained are discussed in terms of the kinetic mechanism of coenzyme binding. The chemical shifts of the pyrophosphate resonances vary from one complex to another over a range of 3.8 ppm. The assignment of these signals to the individual pyrophosphate ^{31}P nuclei and the structural origins of the chemical shift changes are discussed. From these data, and the ^1H NMR experiments described in the preceding paper [Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980) *Biochemistry* (third paper of four in this issue)], it is concluded that the "nicotinamide" end of the thionicotinamide and acetylpyridine coenzyme analogue binds to the enzyme quite differently from that of the natural coenzyme $NADP^+$.

Over the last few years, ^{31}P NMR spectroscopy has proven to be of considerable value in the study of the binding of phosphorylated compounds to proteins; its uses in this area have recently been reviewed by Cohn & Nageswara Rao (1979). In our studies of dihydrofolate reductase, we have earlier used this technique to demonstrate that the 2′-phosphate group of the coenzyme NADPH or $NADP^+$ is in the dianionic form when bound to the enzyme (Feeney et al., 1975, 1977; Birdsall et al., 1977) and that the conformation about one of the C5′–O bonds of NADPH changes by ~60° on binding (Feeney et al., 1975), an observation subsequently confirmed by X-ray crystallography (Matthews et al., 1978, 1979).

We have now shown that the presence of inhibitors such as methotrexate affects the binding constants of $NADP^+$ and structurally related compounds (Birdsall et al., 1980a,b) and that these effects are accompanied by significant changes in the environment of the nicotinamide ring of the bound coenzyme (Hyde et al., 1980). In this paper we use ^{31}P NMR to explore the environment of the phosphate groups of the coenzyme in these various complexes and to estimate, from a line-shape analysis, the lifetime of the coenzyme in the complexes.

Experimental Section

Materials

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described by Dann et al.

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(1976). Sources of coenzymes and inhibitors are given by Birdsall et al. (1980a).

Methods

The enzyme was dissolved to a concentration of ~1 mM in ^2H_2O containing 500 mM KCl, 1 mM EDTA, 50 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol], pH* 6.5 (the notation pH* indicates a pH meter reading uncorrected for the isotope effect on the glass electrode).

The equilibrium constants for formation of the various complexes were determined in this buffer at 11 °C by using the fluorometric methods described in detail by Birdsall et al. (1980a).

NMR Spectroscopy. ^{31}P NMR spectra were obtained at 40.5 MHz by using a Varian XL-100-15 spectrometer equipped with Fourier transform facilities controlled by a VDM-620i computer. A sample volume of 1.4 mL was used, in a 12-mm tube fitted with a Teflon plug to prevent vortexing. The sample temperature was maintained at 11 ± 1 °C. Noise-modulated proton decoupling was employed for all spectra unless otherwise noted. Spectra were acquired in the block averaging mode; typically 300 blocks each of 200 spectra were averaged. An acquisition time of 0.5 s was used for a spectral width of 2 kHz. Before Fourier transformation, the free induction decay was multiplied by an exponential with a time constant of 0.2 s to improve the signal-to-noise ratio; this leads to a line broadening of 1.6 Hz.

Chemical shifts are expressed relative to the signal of inorganic phosphate at pH* 8.0 (this is 2.94 ppm downfield from H_3PO_4); negative numbers denote upfield shifts.

Exchange Theory and Data Analysis. It is well-known that the exchange of a nucleus between magnetically nonequivalent sites can have substantial effects on the NMR spectrum (Pople et al., 1959). Here we are concerned with nuclei of the coenzyme which is exchanging between two states, free in solution and bound to the enzyme, according to the equilibrium

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \tag{1}$$

with an equilibrium constant

$$K' = k_1/k_{-1} = [EL]/[E][L] \tag{2}$$

(where E and L represent enzyme and ligand, respectively). In each of these states, the nucleus has a characteristic chemical shift, ν_F or ν_B, spin–spin relaxation rate, $1/T_{2F}$ or $1/T_{2B}$, and lifetime, τ_F or τ_B (where the subscripts F and B indicate quantities associated with the free and bound states, respectively, and $1/\tau_B = k_{-1}$).

If the exchange of the nucleus between the two states is *slow*, defined by

$$2\pi|\nu_B - \nu_F| \gg 1/\tau_F + 1/\tau_B \tag{3}$$

separate signals will be observed, at ν_F and ν_B, for the free and bound ligand. For the bound ligand, the line width, $\Delta\nu_{1/2}$, is given by

$$\pi\Delta\nu_{1/2B} = 1/T_{2B} + k_{-1} \tag{4}$$

and similarly for the free ligand

$$\pi\Delta\nu_{1/2F} = 1/T_{2F} + 1/\tau_F = 1/T_{2F} + P_B k_{-1}/P_F \tag{5}$$

where P_F and P_B are the mole fractions of ligand in the free and bound states and

$$P_B = [EL]/[EL] + [L] = 1 - P_F \tag{6}$$

The line width of the signal from nuclei in the bound state will thus be independent of ligand concentration, while that of the "free" signal will decrease with increasing ligand concentration. A plot of $\pi\Delta\nu_{1/2F}$ vs. P_B/P_F will be a straight line with a slope of k_{-1}. The ratio P_B/P_F is equal to [EL]/[L], which can be calculated from the total concentrations of enzyme and ligand if K' is known. For the complexes studied here, the total enzyme concentration $[E]_T > 10/K'$, so that

$$P_B/P_F \simeq [E]_T/([L]_T - [E]_T)$$

At the other extreme, if the exchange is *fast*, i.e.

$$2\pi|\nu_B - \nu_F| \ll 1/\tau_F + 1/\tau_B \tag{7}$$

a single resonance line will be observed at a frequency

$$\nu = \nu_F P_F + \nu_B P_B \tag{8}$$

which will depend on the ligand concentration. The fast exchange case is commonly recognized by the observation of such a single, concentration-dependent resonance. However, we have recently shown (Feeney et al., 1979) that this is not a sufficient criterion for the applicability of eq 8 and that substantial errors can result from the use of this equation under inappropriate circumstances. We have therefore analyzed all spectra, other than those in which two clearly resolved signals were observed, by means of the full line-shape equation for exchange between two uncoupled sites (Binsch, 1969, and references cited therein). The amplitude of the absorption mode signal as a function of frequency (the line shape) is given by the imaginary part of the complex quantity

$$G(\nu) = -iC\left[\frac{2P_F P_B \tau - \tau^2(P_F\alpha_B + P_B\alpha_F)}{P_F P_B - \tau^2\alpha_F\alpha_B}\right] \tag{9}$$

where

$$\tau = (1/\tau_F + 1/\tau_B)^{-1} = P_F/k_{-1}$$

$$\alpha_F = -[2\pi i(\nu_F - \nu) + 1/T_{2F} + P_B k_{-1}/P_F]$$

and

$$\alpha_B = -[2\pi i(\nu_B - \nu) + 1/T_{2B} + k_{-1}]$$

For spectra in which a single "averaged" peak was observed, the three unknown parameters ν_B, T_{2B}, and k_{-1} were estimated by nonlinear least-squares analysis of the dependence of chemical shift and line width on ligand concentration. For each set of parameter values and each ligand concentration, the chemical shift and line width were estimated from eq 9 [for details, see Feeney et al. (1979)]. These estimates were compared with the experimental values, and the parameters were adjusted so as to obtain the best fit (in a least-squares sense) to the data as a function of ligand concentration by using a program written by Dr. J. G. Batchelor. For the coenzyme analogue PADPR-OMe, the spectra showed two broad and incompletely resolved signals, characteristic of intermediate exchange, which could not be treated either as a single peak, for this least-squares analysis in terms of shift and line width, or as two well-separated peaks, for eq 5. The values of ν_B, T_{2B} and k_{-1} were estimated by visual comparison of spectra at a series of ligand concentrations with spectra simulated by using eq 9.

Results

The ^{31}P spectra of $NADP^+$ free in solution and bound to dihydrofolate reductase are shown in Figure 1. The 2′-phosphate group gives rise to a signal at –0.22 ppm which shifts downfield by 2.9 ppm on binding to the enzyme. The two pyrophosphate ^{31}P resonances have closely similar chemical shifts in free $NADP^+$, so that the outer lines of the quartet

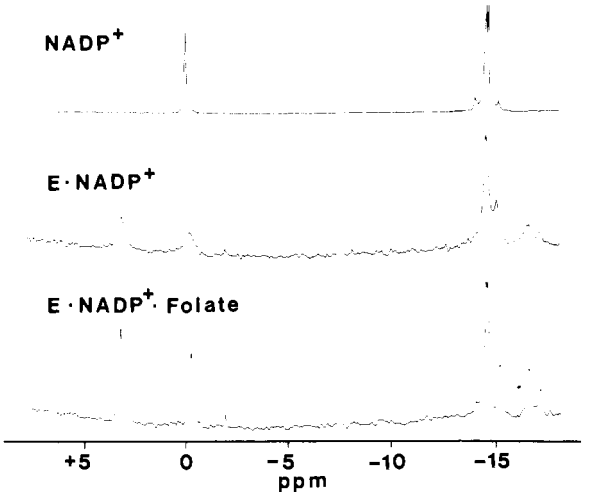

FIGURE 1: 40.5-MHz ^{31}P NMR spectra of $NADP^+$, free in solution (top) and in the presence of dihydrofolate reductase (center) and the dihydrofolate reductase–folate complex (bottom). The center and bottom spectra were obtained from samples having a coenzyme/enzyme ratio of approximately 2:1, and separate resonances for free and bound coenzyme are observed. [The small resonance at approximately −2 ppm in the bottom spectrum arises from a small (~5%) amount of decomposition of the coenzyme.]

arising from this AB spin system have low intensity; the chemical shifts are −14.2 and −14.5 ppm, and $^2J_{PP}$ = 20 Hz [see also Sarma & Mynott (1972)]. On binding to the enzyme one pyrophosphate resonance shifts substantially upfield, so that their separation is substantially greater than in the free coenzyme and the quartet structure is thus much more apparent. In the complex with the enzyme, all the ^{31}P resonances of $NADP^+$ are relatively broad; the line widths are substantially greater than those observed with NADPH (Feeney et al., 1975). Addition of folate to the enzyme–$NADP^+$ complex leads to a marked sharpening of the ^{31}P signals, as well as a further upfield shift of the pyrophosphate resonances (Figure 1). Since folate increases the binding constant of $NADP^+$ (Birdsall et al., 1980a) and decreases its dissociation rate constant (Hyde et al., 1980), it is reasonable to attribute the broad lines in the binary complex to an exchange contribution to the line width (cf. eq 4) which is decreased in the ternary complex. These exchange effects have been further investigated by examining the 2′-phosphate resonance.

Exchange Effects on the 2′-Phosphate Resonance. Figure 2A shows the 2′-phosphate signal of $NADP^+$ in the presence of dihydrofolate reductase. The observation of two distinct, well-resolved resonances for coenzyme/enzyme ratios greater than 1 clearly indicates that exchange of $NADP^+$ between the free and bound states is slow on the NMR time scale. The line width of the lower field (bound) signal is not affected by increasing the $NADP^+$ concentration, whereas the higher field (free) signal not only increases in intensity but also sharpens with increasing concentration. Figure 2B shows the line width plotted against P_B/P_F according to eq 5. A good straight line is obtained, and from its slope we obtain k_{-1} = 25 (±3) s^{-1}. This value is also consistent with the line width of the bound signal; if we assume that the line width in the absence of exchange is the same as that in the enzyme–$NADP^+$–methotrexate complex (5 Hz), the linewidth in the binary complex gives $k_{-1} \sim 30$ (±5) s^{-1}.

The coenzyme analogue $TNADP^+$, in which the carboxamide group on the nicotinamide ring has been replaced by a thioamide, shows different behavior. As shown in Figure 3, a single 2′-phosphate resonance is observed which shifts progressively upfield as the $TNADP^+$ concentration is increased. This is characteristic of rapid exchange between the bound and free states. However, Figure 3 shows that the line width of the averaged signal first increases and then decreases as the ligand concentration is increased, a maximum line width beind observed at a coenzyme/enzyme ratio of ~1.5. Simulation experiments (Feeney et al., 1979) demonstrated that the observation of a maximum in the line width at these concentrations is a clear indication that the exchange is not rapid enough to allow eq 8 to be used. We have therefore analyzed both the chemical shift and the line width as a function of coenzyme concentration using the full line-shape equation, as outlined under Experimental Section. The data are presented in Figure 4, along with the theoretical curves calculated with the parameter values which give the best fit

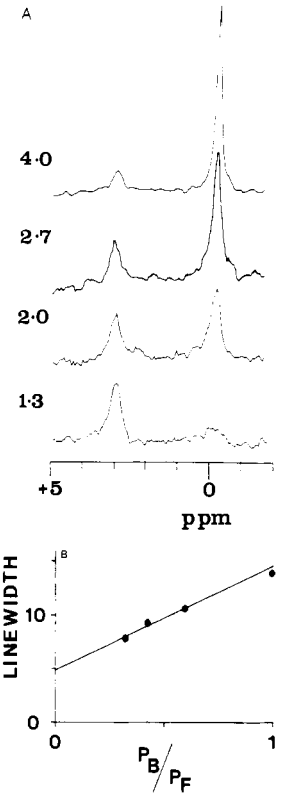

FIGURE 2: (A) 2′-Phosphate ^{31}P resonance of $NADP^+$ in the presence of dihydrofolate reductase as a function of $NADP^+$ concentration, expressed as moles of $NADP^+$ per mole of enzyme. (B) Line width of the 2′-phosphate resonance of free $NADP^+$ as a function of P_B/P_F, according to eq 5.

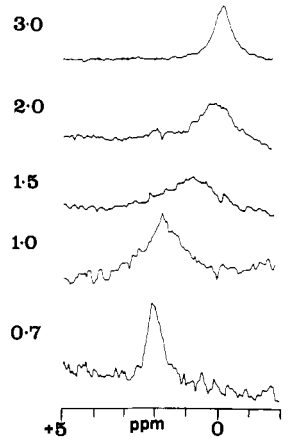

FIGURE 3: 2′-Phosphate ^{31}P resonance of $TNADP^+$ in the presence of dihydrofolate reductase as a function of $TNADP^+$ concentration, expressed as moles of $TNADP^+$ per mole of enzyme.

Table I: Dissociation Rate Constants of Coenzymes from Their Binary and Ternary Complexes with Dihydrofolate Reductase

	dissociation rate constant[a] (s^{-1})					
	NADP+					
complex	11 °C	25 °C	NHDP+	APADP+	TNADP+	PADPR-OMe
enzyme-coenzyme	25 (±3)[b]	33 (±4)[c]	58 (±9)[b]	250 (±50)[d]	230 (±50)[d]	180 (±40)[d]
enzyme-folate-coenzyme	1.4 (±0.5)[c]			13 (±2)[b]	160 (±60)[d]	200 (±40)[d]
enzyme-methotrexate-coenzyme		3.8 (±1)[c]		90 (±14)[b]	65 (±10)[b]	55 (±10)[b]

[a] Unless otherwise indicated, in 50 mM Bistris and 500 mM KCl, pH* 6.5, at 11 °C. [b] Determined by using eq 5. [c] Determined by ^{1}H saturation transfer measurements (Hyde et al., 1980) in 50 mM phosphate and 500 mM KCl, pH* 6.5, at the indicated temperature. [d] Determined by using eq 9.

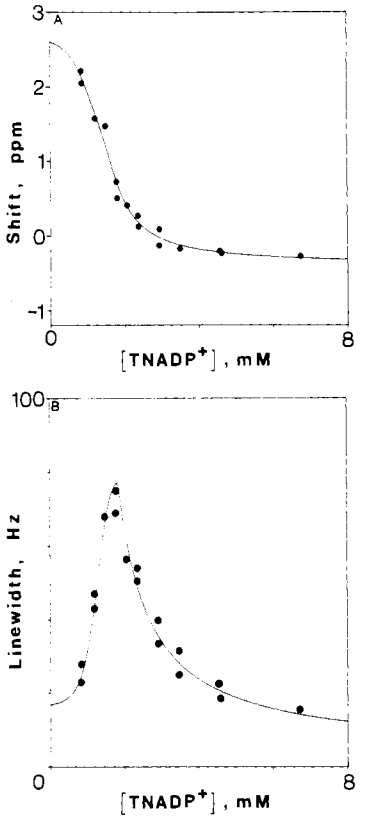

FIGURE 4: Chemical shift (A) and line width (B) of the 2′-phosphate resonance of TNADP+ as a function of coenzyme concentration. In each case, the line is the best-fit curve obtained from the line-shape analysis (eq 9) described in the text, using the parameters given in the text and Tables I–III.

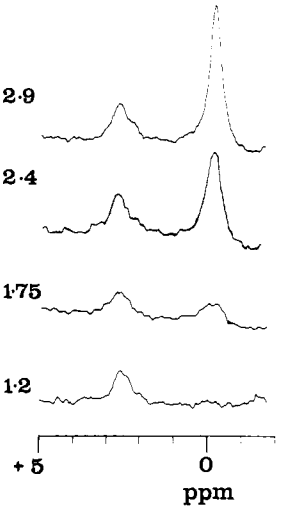

FIGURE 5: 2′-Phosphate ^{31}P resonance of TNADP+ in the presence of the dihydrofolate reductase–methotrexate complex as a function of TNADP+ concentration, expressed as moles of TNADP+ per mole of enzyme.

Table II: Equilibrium Constants for the Binding of Coenzyme Analogues to Dihydrofolate Reductase

	K'[a] (M^{-1})	
coenzyme	enzyme alone	+folate[c]
NADP+	1.5 (±0.2) × 10^5	*b*
NHDP+	2.6 (±0.4) × 10^4	*b*
APADP+	1.4 (±0.2) × 10^4	1.4 (±0.5) × 10^5
TNADP+	3.3 (±0.5) × 10^4	1.6 (±0.2) × 10^5
PADPR-OMe	1.4 (±0.2) × 10^5	1.3 (±0.3) × 10^5

[a] Determined fluorometrically, in 50 mM Bistris and 500 mM KCl, pH* 6.5, at 11 °C. [b] Not determined. [c] Extrapolated to saturating folate concentrations from experiments conducted at 10–22 μM folate (0.1 μM enzyme); see Birdsall et al. (1980a).

to the data. When ν_B, T_{2B}, k_{-1}, and the binding constant, K', were treated as unknowns, the value of K' obtained, 3.5 × 10^4 M^{-1}, was in good agreement with that obtained by fluorescence methods, 3.3 × 10^4 M^{-1}. In order to improve the precision with which the remaining parameters could be determined, the curve fitting was repeated by taking K' from the fluorescence experiments as a known parameter. The dissociation rate constant obtained in this way is 230 (±50) s^{-1}.

We have shown (Birdsall et al., 1980a) that the binding of TNADP+ is increased approximately fivefold (at 25 °C) in the presence of methotrexate. The ^{31}P spectra obtained in the presence of methotrexate (Figure 5) demonstrate that this increase in binding is associated with a decrease in dissociation rate constant, since the appearance of the spectra is now characteristic of *slow* exchange of the coenzyme between bound and free states. Analysis of the concentration dependence of the line width of the signal from the free coenzyme according to eq 5 gives a value of 65 (±10) s^{-1} for k_{-1}.

Using either the full line-shape equation (eq 9) or that for slow exchange (eq 5), we have determined the dissociation rate constants of a number of coenzyme analogues from their complexes with the enzyme and from their ternary complexes with methotrexate or folate. The values obtained are given in Table I. The dissociation rate constants of NADP+ from its ternary complexes were too low to be measured accurately from ^{31}P line widths; the values in Table I are those measured by ^{1}H saturation transfer (Hyde et al., 1980). As noted above, when the full line-shape equation was used, a more precise value of k_{-1} was obtained if an independent measurement of K' could be used in the analysis. Accordingly, we measured K' for all the binary and some of the folate ternary complexes by fluorescence methods under the same temperature, pH, and buffer conditions used for the NMR experiments. The values obtained are given in Table II; the trends are essentially identical with those seen at 25 °C and pH 6.0 (Birdsall et al., 1980a), the values being ~2.5 times greater.

Tables I and II show, as expected from eq 1 and 2, a general parallel between the values of the equilibrium constant K' and

Table III: ^{31}P Chemical Shifts of Coenzyme Analogues Bound to Dihydrofolate Reductase Alone and in Ternary Complexes with Folate or Methotrexate

	chemical shift[a] (ppm)							
	free		binary complex		+folate		+methotrexate	
coenzyme	δ(2'P)	δ(PP)	δ(2'P)	δ(PP)	δ(2'P)	δ(PP)	δ(2'P)	δ(PP)
$NADP^+$	−0.22	−14.14 −14.47	2.7	−14.3 −16.2	2.7	−14.4 −16.3	2.7	−14.9 −16.5
$NHDP^+$	−0.20	−14.13	2.5	−14.5 −16.3	2.5	−14.6 −16.3	2.5	−14.9 −16.6
$APADP^+$	−0.24	−14.15 −14.47	2.5[b]	−13.8 −15.3	2.7[b]	−14.5 −16.1	2.7	−12.9[b] −14.9
$TNADP^+$	−0.24	−14.15 −14.47	2.4[b]	−14.0 −15.2	2.5[b]	−14.4 −15.5	2.7	−14.3[b]
PADPR-OMe	−0.22	−13.7	2.7[b]	−14.2 −16.1	2.5[b]	−14.2 −16.1	2.7	−13.9 −16.2

[a] Chemical shifts of the 2'-phosphate [δ(2'P)] and of the two pyrophosphate nuclei [δ(PP)] given in ppm from inorganic phosphate, pH* 8.0, downfield positive. Where only a single figure is quoted for δ(PP), the resonances of the two pyrophosphate nuclei were too close together to be resolved. Estimated precision (for bound coenzymes) ±0.1 ppm, unless otherwise noted. [b] Estimated precision is ±0.25 ppm (broad resonances).

the dissociation rate constant k_{-1}. The increase in K' produced by folate or methotrexate (Birdsall et al., 1980a) is associated with a decrease in k_{-1}. The stability of these various coenzyme complexes, as indicated by K' and k_{-1}, covers a 200-fold range. The chemical shift of the 2'-phosphate resonance (given in Table III) is, however, remarkably constant in all the complexes, suggesting that the variations in binding constant do not arise from variations in the interaction of this part of the coenzyme molecule with the enzyme. The only exception to this is $NHDP^+$, whose 2'-phosphate signal is 0.2 ppm to higher field of that of the other coenzymes in both binary and ternary complexes.

Pyrophosphate Resonances. The changes in chemical shift of the pyrophosphate resonances on binding to the enzyme are of the same order as, or less than, those of the 2'-phosphate signal; in particular, as shown for $NADP^+$ in Figure 1, one of the pyrophosphate signals is close to that of the free coenzyme. As a consequence, in samples containing both bound coenzyme and free coenzyme, complex exchange effects were often observed on the pyrophosphate resonances. In order to minimize these effects and obtain accurate values for the chemical shifts of the bound coenzyme, it was necessary to obtain spectra under conditions where essentially all the coenzyme is bound to the enzyme (i.e., at low coenzyme/enzyme ratios). As a compromise between this and signal-to-noise requirements, we used a molar ratio of 0.5 (i.e., 5×10^{-4} M coenzyme and 1.0×10^{-3} M enzyme). Typical spectra are shown in Figure 6; under these conditions the fraction of the coenzyme bound to the enzyme varied from a minimum of 0.88 in the binary complexes to >0.99 in the ternary complexes. The measured pyrophosphate chemical shifts, summarized in Table III, are thus essentially those of the complexes.

In comparing the pyrophosphate chemical shifts of free and bound coenzymes, it should be noted that we do not know which pyrophosphate signal of the free coenzyme corresponds to which in the bound coenzyme. For simplicity, we shall express chemical shift changes on binding relative to the mean of the two chemical shifts in the free state. These are sufficiently similar that this approximation does not affect the interpretation, and the true chemical shifts are given in Table III.

When $NADP^+$ binds to the enzyme, as noted above, one of the pyrophosphate resonances is shifted 1.9 ppm upfield, while the other is hardly affected (<0.2 ppm). Addition of folate has very little effect on either pyrophosphate signal, but

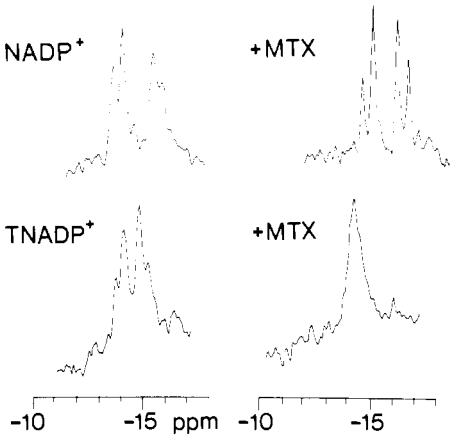

FIGURE 6: Pyrophosphate ^{31}P resonances of $NADP^+$ and $TNADP^+$ in their complexes with dihydrofolate reductase (left) and the dihydrofolate reductase–methotrexate complex (right). In each case the coenzyme/enzyme ratio is 1:2, so that essentially all the coenzyme is bound to the enzyme.

methotrexate produces significant additional upfield shifts, particularly of the lower field signal (Figure 6), so that in this ternary complex the two pyrophosphate signals are 2.2 and 0.6 ppm upfield of those of the free coenzyme. The binding of $TNADP^+$ has distinctly different effects on its pyrophosphate chemical shifts (Figure 6). On forming the binary complex, one of the pyrophosphate signals shifts upfield by 0.9 ppm, only about half as much as the shift observed with $NADP^+$, while the other shifts slightly downfield. Addition of folate produces small but significant upfield shifts of both signals, but in the ternary complex with methotrexate only a single broad resonance centered at −14.3 ppm is observed (Figure 6). This must mean that the two pyrophosphate nuclei have very similar chemical shifts in this complex and, hence, that one of them has been shifted substantially downfield on the binding of methotrexate. This effect is also seen with $APADP^+$; in the ternary complex with methotrexate, two broad signals are seen for the pyrophosphate ^{31}P nuclei, one of which, at −12.9 ppm, is 1.4 ppm *downfield* from the mean resonance position of the free coenzyme (Table III).

With the exception of the ternary enzyme–methotrexate–$TNADP^+$ or –$APADP^+$ complexes, the resonances of the bound coenzyme (at a molar ratio of 0.5 coenzyme/enzyme) were sharp enough to allow the ^{31}P–^{31}P spin coupling constant

to be measured. Values of 18–20 Hz were measured; allowing for the fact that the relatively broad lines will tend to make the measured splitting rather less than the true coupling constant, these values are probably not significantly different from the value of 20 Hz in the free coenzymes.

Discussion

These ^{31}P NMR experiments provide two kinds of information which are potentially useful in attempts to understand the binding of coenzymes to dihydrofolate reductase and their cooperative interactions with inhibitors. Information about the rate of coenzyme dissociation from the various complexes is available from the line-shape analysis, while the environment of the phosphoryl groups in these complexes is reflected in their ^{31}P chemical shifts.

Dissociation Rate Constants. For the simple single-step binding process indicated by eq 1, one can calculate the association rate constant as $K'k_{-1}$ (eq 2). In the simplest case, the association rate constant will be diffusion limited and thus the same for all the coenzyme analogues. From the data for the binary complexes in Tables I and II, values of k_1 calculated for NADP$^+$, NHDP$^+$, TNADP$^+$, and APADP$^+$ fall in the range (1.5–7.7) $\times 10^6$ M^{-1} s^{-1}. These compare reasonably well with the values of 8×10^6 M^{-1} s^{-1} for NADP$^+$ and 7×10^6 to 1.6×10^7 M^{-1} s^{-1} for a series of reduced coenzymes measured by Dunn et al. (1978) at pH 6.0, 25 °C. Similarly, the increase in binding constant produced by folate is clearly largely due to a decrease in k_{-1}. When the different coenzyme analogues were compared, the ratio of the binding constants of analogues A and B, K'_A/K'_B, is generally within less than a factor of 3 of the ratio k_{-1B}/k_{-1A}. Taking into account the twofold range in k_1 observed by Dunn et al. (1978), the data for these compounds are entirely consistent with the simple model of eq 1.

However, while the binding constant of PADPR-OMe is essentially identical with that of NADP$^+$ [Table II and Birdsall et al. (1980a)], its dissociation rate constant is more than 7 times greater. The simple relationship between K' and k_{-1} (eq 2) thus does not hold in all cases, and the binding process may be more complex than that indicated by eq 1. Independent evidence for this was obtained by Dunn et al. (1978). They observed that for NADP$^+$ and TNADPH the dissociation rate constant estimated under pseudo-first-order conditions was about a factor of 3 greater than that estimated by displacement of the coenzyme from a preformed binary complex. These anomalies could be explained if the binding of the coenzymes to the enzyme was a two-step process:

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL^* \qquad (10)$$

The dissociation rate constant measured by displacement would be the slower of k_{-2} and k_{-1}, whereas that obtained from the pseudo-first-order experiment would be a function of k_{-1}, k_2, and k_{-2}. However, the relatively small discrepancy between the two estimates of the dissociation rate constant suggests that the equilibrium constant for the second step is not much, if at all, greater than 1.

The dissociation rate constant of NADP$^+$ from its binary complex measured by NMR (either from ^{31}P line widths or from ^1H saturation transfer measurements; Hyde et al., 1980) clearly corresponds to the slower of the two values measured by stopped flow; the NMR value at pH* 6.5, 25 °C, of 33 (±5) s^{-1} is in agreement with the stopped-flow value of 43 (±3) s^{-1} at pH 6.0, 25 °C (Dunn et al., 1978). This agreement is to be expected whatever the detailed mechanism of binding, since in both experiments one is measuring the rate-limiting step in the complete dissociation of the coenzyme from the enzyme.

When exchange is rapid, it is very difficult to distinguish between the models of eq 1 and 10 by NMR experiments of the kind described here. The general line shape equation for three-site exchange is complex [Patterson & Ettinger, 1960; see also Baldo et al. (1975)] and involves the introduction of a minimum of four additional unknown parameters. With the available signal-to-noise ratio, any attempt to analyze the data for TNADP$^+$, for example (cf. Figures 3 and 4), in terms of three-site rather than two-site exchange would clearly be unprofitable. However, when exchange between the sites is *slow*, it can be much easier to make the distinction, and the number of well-populated sites can be simply obtained from the number of separate signals observed. In this way, we have shown (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) that the ternary complex of NADP$^+$ and trimethoprim with dihydrofolate reductase exists in two conformational forms, since two separate sets of coenzyme ^1H and ^{31}P resonances are observed. This provides strong support for the existence of a two-step binding process, such as eq 10, for this particular complex. As shown in Figure 6, in the binary complex or the ternary complexes with folate or methotrexate only a single set of NADP$^+$ resonances are detectable. This could arise in two ways; either K_2 is substantially different from 1, so that the second form of the complex is present in too low a concentration to be detectable (effectively converting the model of eq 10 to that of eq 1), or the exchange between EL and E*L is rapid on the NMR time scale so that a single averaged resonance is observed. For the NADP$^+$ complexes studied here, there is no NMR evidence which would lead one to reject the simple model of eq 1. However, for APADP$^+$ and TNADP$^+$, both in their binary complexes and in their ternary complexes with methotrexate, the ^{31}P resonances (measured under conditions of >88% binding of the coenzyme) are substantially broader than those of the corresponding NADP$^+$ complexes. The dominant ^{31}P relaxation mechanism in these complexes is probably chemical shift anisotropy relaxation (B. Birdsall, G. C. K. Roberts, and J. Feeney, unpublished work), and there is no obvious reason why this should differ by a factor of 4 between structurally very similar complexes. The most likely alternative explanation is exchange broadening; as noted by Baldo et al. (1975), the observation of exchange broadening under conditions where the ligand is fully bound would imply the existence of an exchange process in the bound state and thus of two interconverting forms of the complex. We have not yet, however, been able to *prove* that the line broadening arises from exchange.

2′-Phosphate Chemical Shifts. The substantial downfield shift of the 2′-phosphate resonance on binding to the enzyme has been shown (Feeney et al., 1975; Birdsall et al., 1977) to arise from two sources. The 2′-phosphate group of the bound coenzyme is in the dianionic form (at least above pH 4.5), leading to a pH-dependent downfield shift, and there is a further downfield shift of 1.7 ppm, reflecting the altered environment of the bound phosphate. In the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979), the 2′-phosphate forms hydrogen bonds with His-64 and Thr-63 and coulombic interactions with Arg-43 and His-64. In dihydrofolate reductase from *Escherichia coli* RT500, His-64 of the *L. casei* enzyme is replaced by a serine residue (Stone et al., 1977), and the "extra" shift of the 2′-phosphate (i.e., the shift difference between bound NADP$^+$ and free dianionic NADP$^+$) is only 0.25 ppm rather than 1.7 ppm (Cayley et al., 1980). This suggests that a substantial contribution to the

large downfield shift of the 2′-phosphate signal on binding to the *L. casei* enzyme arises in some way from the His-64–2′-phosphate interaction. The imidazole ring of this histidine residue is essentially fully protonated in the enzyme–$NADP^+$ complex at pH* 6.5 (B. Birdsall, A. Gronenborn, G. C. K. Roberts, and J. Feeney, unpublished work). However, it seems unlikely that the proximity of this positive charge is responsible for much of the change in ^{31}P chemical shift. Gorenstein et al. (1976) have found that, when cytidine 3′-monophosphate binds to bovine pancreatic ribonuclease, its ^{31}P resonance shifts by less than 0.25 ppm (after correction for the change in ionization state), although the phosphate group is interacting directly with *two* protonated histidine residues. In addition, the His-64–phosphorus distance is too great for there to be an appreciable "ring-current" shift of the ^{31}P resonance.

The ^{31}P chemical shifts of phosphates are very sensitive to small changes in the O–P–O bond angle (Gorenstein, 1975; Gorenstein & Kar, 1975; Ribas Prado et al., 1979), and it seems likely that interaction with a histidine rather than a serine residue produces a slight bond angle distortion and, hence, a large change in the ^{31}P shift. The imidazole ring of His-64 also interacts with the adenine ring of the coenzyme (Matthews et al., 1979; see also Hyde et al., 1980), the two rings being stacked parallel to one another. When the adenine is changed to a hypoxanthine ring (in $NHDP^+$), this interaction might well be sufficiently affected to produce a slight change in the position of the imidazole of His-64 and, hence, the 0.2 ppm change observed in the 2′-phosphate chemical shift.

With this exception, the 2′-phosphate chemical shift is virtually constant in the various complexes. In particular, the increase in binding constant produced by folate or methotrexate is not accompanied by any change in the 2′-phosphate environment; from the ^1H experiments (Hyde et al., 1980), this is true of the adenosine moiety as a whole.

Pyrophosphate Chemical Shifts. There is a good deal more variation in the chemical shifts of the pyrophosphate resonances, and here the coenzymes fall into two distinct groups: $NADP^+$ and $NHDP^+$ on the one hand and $APADP^+$ and $TNADP^+$ on the other. For $NADP^+$ and $NHDP^+$, one pyrophosphate signal is shifted ~2 ppm upfield, while the other is scarcely affected. These shifts are similar to, though slightly smaller than, those seen with NADPH (Feeney et al., 1975). By contrast, with $APADP^+$ and $TNADP^+$ one pyrophosphate resonance shifts ~1 ppm upfield and the other shifts 0.3–0.5 ppm downfield. In the binary complex, PADPR-OMe resembles $NADP^+$ rather than $APADP^+$, though this situation is reversed in the ternary complex with methotrexate.

Before discussing these chemical shift changes in any more detail, we must attempt to establish which of the pyrophosphate resonances corresponds to the adenosine 5′-phosphate, P_a, and which corresponds to the nicotinamide riboside 5′-phosphate, P_n, in the bound coenzyme. In the case of NADPH, this assignment can be made from a comparison of the observed ^1H–^{31}P spin–spin coupling (Feeney et al., 1975) and the crystallographically determined conformation of bound NADPH (Matthews et al., 1978, 1979; D. A. Matthews and D. J. Filman, personal communication). The higher field of the two pyrophosphate resonances of bound NADPH shows very small ^1H–^{31}P coupling constants, indicating that both the 5′ protons are gauche to the phosphorus, while the much larger ^1H–^{31}P coupling to the phosphorus giving the lower field signal implies a conformation about the C5′–O bond in which the C4′–C5′–O–P dihedral angle differs by ~50° (Feeney et al., 1975). On the basis of the reported conformation of the coenzyme in the crystalline complex (Matthews et al., 1979) the lower field resonance was assigned to P_a (Matthews, 1979). However, higher resolution data have led to a revision of the initial crystallographic conclusions and it now appears that in the NADPH–methotrexate complex, the higher field pyrophosphate resonance (at −16.5 ppm) is that of P_a and the lower field resonance is that of P_n (D. A. Matthews and D. J. Filman, personal communication).

Single-resonance ^{31}P spectra of the enzyme–$NADP^+$–folate and enzyme–$NADP^+$–methotrexate spectra show that the higher field pyrophosphate signal has small ^1H–^{31}P coupling and the lower field signal has much larger coupling. Together with the similarity of the chemical shifts to those in the NADPH complex, this allows us to assign the resonance at −14.4 to −14.9 ppm to P_n and that at −16.3 to −16.5 to P_a. Because of the exchange broadening of the ^{31}P resonances, the effects of ^1H–^{31}P coupling are obscured in the spectra of the other coenzyme complexes, and a straightforward assignment is not possible. For the binary complexes of $NADP^+$ and PADPR-OMe, and ternary enzyme–folate–$APADP^+$ or PADPR-OMe complexes, the pyrophosphate chemical shifts are so similar to those of the ternary $NADP^+$ complexes that it is only reasonable to assume that here too the resonance at −16.1 ppm is that of P_a.

In the remaining complexes (the binary and ternary complexes of $APADP^+$ and $TNADP^+$ and the enzyme–methotrexate–PADPR-OMe complex), there is always a pyrophosphate resonance in the region −13.8 to −14.9 ppm, while in the complexes for which assignments can be made with some confidence, the resonance of P_n is between −14.3 and −14.9 ppm. We therefore tentatively assign the pyrophosphate signal in this range to P_n in all complexes.

The 2-ppm upfield shift of the P_a resonances of $NADP^+$, $NHDP^+$, and PADPR-OMe on binding to the enzyme presumably arises from the same kind of small bond angle distortions invoked above for the 2′-phosphate, although the relationship of such effects to ^{31}P chemical shifts in pyrophosphates as opposed to monophosphates has not yet been studied in any detail. It is interesting that for P_n there is a change in the C5′–O torsion angle on binding, but any bond angle distortions have no net effect on the ^{31}P shift, while for P_a there is no change in C5′–O torsion angle but sufficient bond angle changes to produce a large ^{31}P shift change.

The close similarity between $NADP^+$ and $NHDP^+$ extends to their ternary complexes. Addition of folate or methotrexate leads to a slight further upfield shift of both pyrophosphate signals; in each case the lower field signal, P_n, is shifted the most. Formation of the ternary complexes thus produces only small changes in the environment of the pyrophosphate. In the preceding paper (Hyde et al., 1980) we showed that addition of folate or methotrexate had only localized effects on the nicotinamide ring of $NADP^+$ or $NHDP^+$ so that the ^1H and ^{31}P data taken together indicate only rather modest changes in the environment of these coenzymes on formation of these particular ternary complexes, in spite of the quite substantial increase in binding constants (Birdsall et al., 1980a).

The acetylpyridine and thionicotinamide analogues present a rather different picture. On forming the binary complex, the resonance of P_a shows a much smaller upfield shift than the corresponding resonance of $NADP^+$. The chemical shifts of the protons of the nicotinamide ring (Hyde et al., 1980) indicate that the mode of binding of this ring is markedly different in the analogues in which the amide group has been modified. The ^{31}P results show that this difference extends

to the pyrophosphate group. In the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979) residues 97–99 are close to both the nicotinamide ring and the pyrophosphate [Gly-98 and Gly-99 are conserved in all dihydrofolate reductases of known sequence (Freisheim et al., 1978, 1979) except the plasmid enzyme described by Stone & Smith (1979)]. If the altered interactions of the acetylpyridine or thionicotinamide ring in the nicotinamide binding subsite led to even a slight movement of this part of the chain, then the environment of the pyrophosphate would in turn be altered.

Formation of the ternary complexes of $APADP^+$ or $TNADP^+$ leads, on the whole, to larger chemical shift changes than are seen with $NADP^+$, and these changes are markedly different for the folate and methotrexate complexes. Folate produces upfield shifts of both pyrophosphate resonances; for $TNADP^+$ these are quite modest, but for $APADP^+$ they are large enough for the enzyme–folate–$APADP^+$ complex to have a ^{31}P spectrum virtually identical with that of the enzyme–folate–$NADP^+$ complex. Both $NADP^+$ and $APADP^+$ show a large increase in binding constant on addition of folate (Birdsall et al., 1980a); however, this is accompanied by a change in pyrophosphate chemical shift only in the case of $APADP^+$, and in addition, PADPR-OMe, which shows no cooperativity with folate (Birdsall et al., 1980a), also has very similar ^{31}P shifts in the ternary complex. The situation can be clarified by also considering the nicotinamide proton chemical shifts. Addition of folate to the enzyme–$NADP^+$ complex has little effect on either the nicotinamide ^1H (Hyde et al., 1980) or pyrophosphate ^{31}P chemical shifts, whereas with $APADP^+$ there are substantial changes in both ^1H (E. Hyde, unpublished work) and ^{31}P spectra, in both cases in a direction such as to make them more similar to those of the enzyme–$NADP^+$–folate complex. The similar increases in the binding constants of $NADP^+$ and $APADP^+$ on addition of folate must thus arise by different mechanisms, since they are associated with quite different changes in the environment of the coenzyme. The changes in nicotinamide and pyrophosphate environment tend to go in parallel; this is consistent with the idea that effects can be transmitted from one to the other through residues 97–99. Further support for this comes from the observations with PADPR-OMe, which has no nicotinamide ring and shows no cooperativity and no ^{31}P shift changes.

Addition of methotrexate has profound effects on the pyrophosphate chemical shifts of $APADP^+$ and $TNADP^+$. At least one of the pyrophosphate signals shifts downfield substantially; with the assignments proposed above, the P_a resonance of $APADP^+$ shifts downfield by 2.4 ppm on methotrexate binding, and that of $TNADP^+$ shifts downfield by $\sim$0.9 ppm, while the P_n resonance shifts upfield 1 ppm for $APADP^+$ and $\sim$0.3 ppm for $TNADP^+$. It is evident that the inhibitor methotrexate produces completely different changes in the environment of these coenzymes from those produced by the substrate folate. This is consistent with other evidence that folate and methotrexate bind in a different orientation (Hitchings, 1980; Charlton et al., 1979) and produce different changes in protein conformation (Feeney et al., 1977; Kimber et al., 1977; Roberts et al., 1977). The similarity of the ^{31}P spectrum of the enzyme–$APADP^+$–methotrexate complex to that of the enzyme–$TNADP^+$–trimethoprim complex, for which a more detailed analysis has been possible (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments), suggests that addition of methotrexate may have changed the conformation of the bound coenzyme. In any case, methotrexate obviously produces far more profound changes in the environment of these coenzyme analogues than in that of $NADP^+$ itself. Together with the ^1H experiments reported in the preceding paper, this strongly suggests that the mode of binding of $APADP^+$ and $TNADP^+$ to dihydrofolate reductase is quite distinct from that of the natural coenzyme.

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Interaction of Bovine Carbonic Anhydrase with (Neutral) Aniline, Phenol, and Methanol†

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ABSTRACT: We have investigated the interaction of bovine carbonic anhydrase with neutral aniline, phenol, and methanol molecules. The measurements are of optical spectra and solvent water and methanol proton magnetic relaxation rates of solutions of Co^{2+}-substituted enzyme. We recently proposed a model [Koenig, S. H., Brown, R. D., & Jacob, G. S. (1980) *Proceedings of the Symposium on Biophysics and Physiology of Carbon Dioxide*, Springer-Verlag, West Berlin and Heidelberg], based on the interaction of enzyme with monovalent anions, that accounts for the pH dependences observed for a wide variety of phenomena, including the apparent pK_a for enzymatic activity. We now extend the model to include the observed effects of neutral molecules. Aniline and phenol, though isoelectronic, shift the observed pK_a values in opposite directions, and both appear to bind at the aromatic binding site to which sulfonamide inhibitors and aromatic esters are known to bind. The resulting binary complexes behave as altered enzymes, with different values of the pK_a for activity, but otherwise are similar to the native enzyme. In terms of our model, aniline and phenol alter the relative affinities of water and anions for the same coordination position of the metal ion at the active site. The effect is opposite in sign for the two molecules because of the differing proton affinities of the NH_2 and OH moieties of the phenol ring in each case. By extension, our results indicate that data from experiments using aromatic buffers such as imidazole and lutidine should be analyzed with some care; effects previously attributed to buffer in solution may well be due to binding of neutral buffer molecules to the aromatic binding site in the active region of the enzyme. The interaction of methanol with carbonic anhydrase is quite different, and very weak. Methanol does displace water at the metal, but to first order there is little, if any, preferential binding of methanol compared to water. Observations by others that alcohols inhibit esterase activity with inhibition constants on the order of 1 M are not attributable to binding of alcohol to enzyme but rather, in our view, result from the increased solubility of aromatic ester substrates in the alcohol-modified solvent.

The zinc-containing enzyme carbonic anhydrase (EC 4.2.1.1), which catalyzes the reversible reaction $CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$, has also been found to catalyze the hydrolysis of a variety of esters (Tashian et al., 1964; Malmström et al., 1964; Pocker & Stone, 1965, 1967) and the hydration of aliphatic aldehydes (Pocker & Meany, 1965a,b). A longstanding question has existed regarding the identity of the metal-activated ligand in the enzyme that is responsible for the nucleophilic attack on a bound CO_2 during the hydration reaction. A widely held model for enzymatic activity, originally proposed by Davis (1959), identifies this nucleophile as a zinc-bound hydroxide ion. The observed pH dependence of the catalysis (greater hydration and esterase activity at higher values of pH) is then attributed to ionization of a metal-coordinated water ligand to produce the zinc-bound hydroxide nucleophile. This model, as well as most others proposed for the activity-controlling ionizing group (cf. Pocker & Sarkanen, 1978), cannot explain the observed rate of magnetic relaxation of solvent water protons first reported by Fabry et al. (1970) (cf. Koenig & Brown, 1972). Moreover, Lindskog, a long-time proponent of the "hydroxide" mechanism, concludes a recent review by noting that "... as long as unequivocal evidence for the existence of Zn^{2+}-bound OH^- in the enzyme is lacking, this model must be continually questioned and tested against alternative models" (Lindskog, 1980).

An alternate explanation for the pH dependence of enzymatic activity was recently proposed by us (Koenig et al., 1980). In our model, there is no ionization on the enzyme that determines the observed pH-dependent activity; rather, the pH-dependent phenomenon can be explained by the existence of both active enzyme and anion-inhibited enzyme, with relative concentrations that depend on the type and concentration of monovalent anion present in solution, and on pH. The active enzyme was proposed to have a zinc-bound water ligand, capable of exchanging rapidly with solvent, that can be replaced by a monovalent anion and an associated proton from solution to produce inactive enzyme. Thus, active enzyme predominates at high pH, and anion-inhibited enzyme predominates at low pH, with the pK_a for activity being determined by the composition of the solvent.

The essence of the model is that a constant charge environment is maintained in the active site, both statically and

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