Proton Nuclear Magnetic Resonance Saturation Transfer Studies of Coenzyme Binding to *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The chemical shifts of all the aromatic proton and anomeric proton resonances of $NADP^+$, NADPH, and several structural analogues have been determined in their complexes with *Lactobacillus casei* dihydrofolate reductase by double-resonance (saturation transfer) experiments. The binding of $NADP^+$ to the enzyme leads to large (0.9–1.6 ppm) downfield shifts of all the nicotinamide proton resonances and somewhat smaller upfield shifts of the adenine proton resonances. The latter signals show very similar chemical shifts in the binary and ternary complexes of $NADP^+$ and the binary complexes of several other coenzymes, suggesting that the environment of the adenine ring is similar in all cases. In contrast, the nicotinamide proton resonances show much greater variability in position from one complex to another. The data show that the environments of the nicotinamide rings of $NADP^+$, NADPH, and the thionicotinamide and acetylpyridine analogues of $NADP^+$ in their binary complexes with the enzyme are quite markedly different from one another. Addition of folate or methotrexate to the binary complex has only modest effects on the nicotinamide ring of $NADP^+$, but trimethoprim produces a substantial change in its environment. The dissociation rate constant of $NADP^+$ from a number of complexes was also determined by saturation transfer.

The preceding papers (Birdsall et al., 1980a,b) have demonstrated the marked effects of coenzyme binding to dihydrofolate reductase on its affinity for substrates and inhibitors and illustrated some of the effects of structural variation in the coenzyme molecule. NADPH binds to the enzyme some 2000 times more strongly than $NADP^+$ and has larger effects on the binding of other ligands; a number of structural analogues of $NADP^+$, on the other hand, show little variation in binding constant but significantly different effects on methotrexate binding (Birdsall et al., 1980a,b).

In order to arrive at a detailed explanation of these observations in terms of the structure of the various complexes, we must establish whether or not the various coenzyme analogues bind to the enzyme in the same way. One approach to this goal would be to monitor the changes in chemical shift of the proton resonances of the coenzymes on binding, since these should be sensitive to the precise environment of the bound coenzyme. [The complementary approach, using ^{31}P NMR, is described in the following paper (Hyde et al., 1980).] However, the majority of the coenzymes bind relatively tightly to the enzyme so that exchange between the bound and free states is slow on the NMR time scale and two separate sets of spectra are observed. In the presence of the very large number of ^1H resonances from the protein, it is often extremely difficult to resolve and assign the resonances of the bound coenzyme.

To circumvent this problem, we have identified the position of the resonance of the bound coenzyme by saturation transfer experiments (Forsén & Hoffman, 1963; Redfield & Gupta, 1972; Campbell et al., 1976, 1977; Brown & Campbell, 1976; Bendall et al., 1977; Chen et al., 1979; Cayley et al., 1979). In this way we have been able to determine the chemical shifts of all the aromatic protons of several coenzymes in their complexes with the enzyme and thus to make detailed comparisons of their mode of binding.

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‡ Supported by an MRC Research Studentship.

Experimental Section

Materials

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as described by Dann et al. (1976). Its concentration was determined by assaying its activity, by measuring its absorbance at 280 nm, and by fluorometric titration with methotrexate, using precedures described by Dann et al. (1976). Sources of and abbreviations for coenzyme analogues are given by Birdsall et al. (1980a).

Methods

Samples for NMR spectroscopy contained ~1 mM enzyme in 0.35 mL of ^2H_2O containing 1 mM dioxane, 1 mM EDTA, 500 mM KCl, 50 mM potassium phosphate, pH* 6.5 (pH* indicates a pH meter reading uncorrected for the isotope effect on the glass electrode), and 4–6 mM coenzyme. Because of the relative instability of NADPH at this pH, experiments involving this coenzyme were performed at pH* 7.0 (in the above buffer).

To determine the mononucleotide chemical shifts, we incubated either $NADP^+$ or its hypoxanthine, acetylpyridine, or thionicotinamide analogues ($NHDP^+$, $APADP^+$, or $TNADP^+$) in ^2H_2O containing 50 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol], 1 mM EDTA, and 500 mM KCl, pH* 6.5, at 37 °C for 3 days with *Crotalus* nucleotide pyrophosphatase [Sigma (London) Chemical Co. Ltd.]. After the completion of the hydrolysis had been checked by ^{31}P NMR, the mononucleotide chemical shifts were determined directly on an aliquot of the incubation mixture diluted into ^2H_2O containing 1 mM EDTA, 50 mM potassium phosphate, and 500 mM KCl, ph* 6.5, to give a nucleotide concentration of 5×10^{-4} M.

NMR Spectroscopy. The 270-MHz ^1H NMR spectra were obtained with a Bruker WH-270 spectrometer using the Fourier transform technique. Between 50 and 200 transients were averaged for each spectrum using the pulse sequence $(t-\tau-\pi/2-AT-T)_n$. During time t (0.1–3.0 s) selective irradiation was applied at a chosen frequency; τ was a short (2-ms) delay to allow for electronic recovery. After the $\pi/2$ observation pulse, data was acquired for a time AT (0.5–1 s; 4096

0006-2960/80/0419-3738$01.00/0

or 8192 data points, using a 4200-Hz spectral width), and this was followed by an additional delay T (1–5 s) to allow reasonably complete recovery of magnetization before initiating the next sequence. Before Fourier transformation, the free induction decay was multiplied by an exponential function to improve the signal-to-noise ratio, leading to a line broadening of 2 Hz, and when 4096 data points were used, the data table was filled to 8192 points with zeros before Fourier transformation.

To identify the resonance positions of the aromatic protons of the bound coenzyme, we used a sample containing 5 mol of coenzyme/mol of enzyme and monitored the intensity of the resonances of free coenzyme as the frequency of the selective irradiation was moved in steps of 10–20 Hz from approximately 2.0 to 7.0 ppm (from dioxane; all chemical shifts are quoted relative to an internal dioxane reference, downfield shifts being positive). In these experiments a long pulse (3 s) of irradiation was used to maximize selectivity. In order to find the optimum conditions for observation of saturation transfer, we varied the sample temperature in the range 2–45 (±1) °C.

If care is taken to ensure that the signal of the bound proton is completely saturated in a time short compared to t and that the delay T is long enough for complete recovery of magnetization, then the new equilibrium intensity of the signal from the corresponding proton of the *free* coenzyme is (Solomon, 1955; Forsén & Hoffman, 1963)

$$I_{F_\infty} = I_{F_0}\rho_F/(\rho_F + k_F) \quad (1)$$

where ρ_F is the spin–lattice relaxation rate of the proton in the free state and k_F is the apparent first-order rate constant for exchange to the bound state. The approach to this new equilibrium is a simple exponential function of the irradiation time t:

$$I_F(t) = I_{F_0}k_F \exp[-t(\rho_F + k_F)]/(\rho_F + k_F) + I_{F_0}\rho_F/(\rho_F + k_F) \quad (2)$$

By combining measurements of the equilibrium intensity, giving $\rho_F/(\rho_F + k_F)$, and of the time constant of the exponential decrease in intensity as a function of t, $1/(\rho_F + k_F)$, both ρ_F and k_F can be calculated. The dissociation rate constant is then given by

$$k_{off} = P_F k_F/P_B \quad (3)$$

where P_B and P_F represent the fraction of the coenzyme in the bound and free states, respectively. The data were fitted to eq 2 by unweighted nonlinear regression.

With TNADP$^+$ at high temperature (≥30 °C), exchange between the bound and free states was rapid on the NMR time scale. The coenzyme proton chemical shifts were measured as a function of coenzyme concentration and fitted, again by nonlinear regression, to the "weighted-average" equation appropriate to fast exchange:

$$\partial_{obsd} = [EL](\partial_B - \partial_F)/[L]_t + \partial_F \quad (4)$$

where ∂_{obsd} is the observed chemical shift and ∂_B and ∂_F are those characteristic of the bound and free states, respectively, $[L]_t$ is the total ligand concentration and [EL] is the concentration of the complex, an expression for which can be obtained from the mass action equation.

Spin–lattice relaxation times were measured by the standard "inversion–recovery" method (Vold et al., 1968).

Results

Binding of NADP$^+$. Addition of 1 molar equiv of NADP$^+$ to *L. casei* dihydrofolate reductase leads to substantial changes throughout the ^{1}H NMR spectrum of the protein. Increasing concentrations of NADP$^+$ have no further effect, and the broad NADP$^+$ resonances which become visible are at exactly the same frequencies as those of NADP$^+$ alone. This behavior is characteristic of a ligand in slow exchange between the bound and free states. Separate spectra will thus be observed for the two states, and the resonances of the bound coenzyme will be just as difficult to resolve and assign as those of the protein itself.

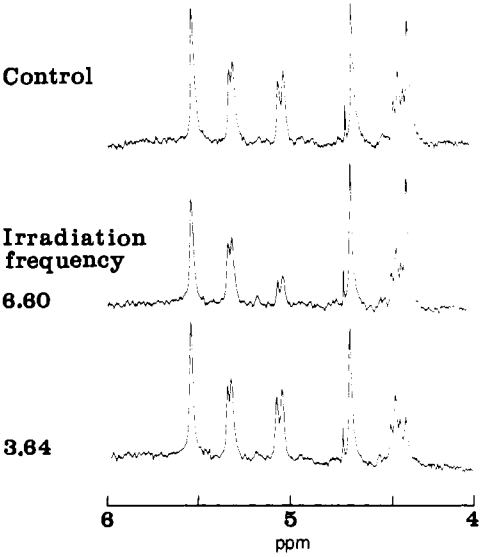

FIGURE 1: Aromatic ^{1}H resonances of 5 mM NADP$^+$ in the presence of 1 mM dihydrofolate reductase, showing the effects of selective irradiation before the observation pulse at 6.60 or 3.64 ppm. From the left, the resonances are those of the N2, N6, N4, A8, N5, and A2 protons.

This problem can, however, be overcome by double-resonance experiments. When 5 mol of NADP$^+$/mol of enzyme is added to a solution of dihydrofolate reductase at 11 °C, the relatively sharp signals from the aromatic protons of the free coenzyme can readily be observed (Figure 1). Irradiation at a series of frequencies spaced systematically throughout the aromatic region of the spectrum revealed that at certain specific irradiation frequencies each of the different proton resonances of free NADP$^+$ showed a marked and selective decrease in intenisty. Figure 1 shows examples of this for the nicotinamide 4-proton (N4) and the adenine 2-proton (A2) resonances. The simplest explanation of these effects is the phenomenon of saturation transfer. If we irradiate at the resonance frequency of a proton of bound NADP$^+$, perturbing the relative populations of its two energy levels, then, provided that the exchange rate is at least of the same order as the relaxation rate, this perturbation will persist as the NADP$^+$ molecule exchanges from the bound to the free state and will be manifested in the spectrum as a change in the intensity of the resonance of the *same* proton in *free* NADP$^+$. This double-resonance experiment thus allows us to identify the resonance positions of the protons of NADP$^+$ bound to the enzyme and at the same time to assign them (since the assignments of the signals of free NADP$^+$ are known).

Although this explanation of the results of the double-resonance experiments in terms of a direct transfer of saturation is by far the simplest, there is one other possibility which must be considered. Irradiation at the resonance frequency of a proton of the protein which contributes to the dipolar relaxation of a proton of the bound coenzyme would perturb the energy level populations of the latter by means of the nuclear Overhauser effect, and this perturbation could then be transferred to the free state by chemical exchange, as before.

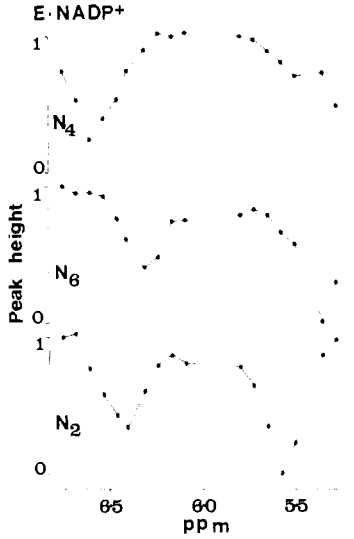

FIGURE 2: Intensities of the N2, N4, and N6 proton resonances of free $NADP^+$ (4 mM, in the presence of 1 mM enzyme–$NADP^+$ complex) as a function of the frequency of irradiation. The low-field (left-hand) minima are from saturation transfer, and those at high field are from direct saturation of the free $NADP^+$ resonances.

Nuclear Overhauser effects between protons of the protein and those of a rapidly exchanging ligand have been described in a number of systems [e.g., Balaram et al. (1972) and James & Cohn (1974)], and we have in fact observed a transferred nuclear Overhauser effect of this sort [see below and Albrand et al. (1979)], although between two protons of a bound ligand rather than between ligand and protein. In the limiting case of rapid spin diffusion (Kalk & Berendsen, 1976), irradiation at any point in the ^1H spectrum of the protein could lead to a decrease in the intensity of the resonances of the free ligand.

The high degree of selectivity in the effects seen here obviously rules out a nonspecific mechanism such as spin diffusion. As well as being selective, these effects are also general, in the sense that they are observed (with the appropriate irradiation frequencies) for each of the $NADP^+$ protons examined. It is obviously unlikely that each of the $NADP^+$ protons is sufficiently close to an aromatic proton of the protein for a nuclear Overhauser effect to be observed. The involvement of protein protons in these double-resonance effects can in fact be ruled out, since precisely the same effects are seen with a sample of selectively deuterated enzyme [prepared as described by Feeney et al. (1977)] which contains only 10 aromatic protons (of tyrosine residues) rather than the 97 of the normal protein. None of the optimum irradiation positions for producing intensity changes in the free $NADP^+$ resonances coincides with the resonance positions of these tyrosine protons; only one, that of A1′, is within 0.5 ppm. The resonance of the bound A1′ proton has in fact been observed directly in the spectrum of a different selectively deuterated analogue (Feeney et al., 1980) at precisely the frequency indicated by the double-resonance experiments. Similarly, the A2 proton resonance of bound $NADP^+$ can be seen in the spectrum of the tyrosine analogue (Feeney et al., 1977) and has been assigned by observing transfer of saturation in both directions.

We can be confident, therefore, that this method does allow us to determine the chemical shifts of the protons of the bound coenzyme. Figure 2 shows the intensity of several of the resonances of free $NADP^+$ as a function of the irradiation frequency, indicating the precision, approximately ±0.06 ppm, with which these shifts can be measured. In this way we have been able to determine the chemical shifts of all the protons on the nicotinamide and adenine rings of the bound coenzyme

Table I: ^1H Chemical Shifts of $NADP^+$, Free and in Its Binary and Ternary Complexes with Dihydrofolate Reductase

	chemical shift[a] (ppm from dioxane)				
proton	$NADP^+$	E–$NADP^+$[b]	E–$NADP^+$–methotrexate[c]	E–$NADP^+$–folate[d]	E–$NADP^+$–trimethoprim[b]
A2	4.365	3.64	3.64	3.67	3.66
A8	4.656	4.42	4.31	4.34	4.20
N2	5.522	6.42	6.34	6.37	5.71
N4	5.042	6.60	6.16	5.38	5.33
N5	4.398	5.52	5.34	5.96	4.56
N6	5.312	6.31	6.31	6.27	5.51
A1′	2.337	3.03	2.95	3.00	3.03
N1′	2.270	2.04	2.01	2.03	*e*

[a] Free coenzyme, ±0.002 ppm; bound coenzyme, ±0.06 ppm. [b] 11 °C. [c] 40 °C. [d] 25 °C. [e] Not detected.

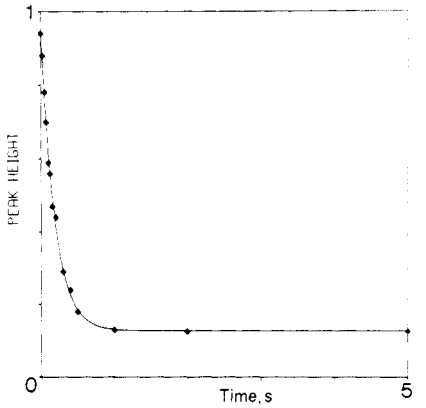

FIGURE 3: Intensity of the A2 proton resonance of free $NADP^+$ as a function of the time of irradiation at 3.64 ppm, the resonance position of the A2 proton in the enzyme–$NADP^+$ complex. The line is the best fit to eq 2 using the parameters given in the text.

and also those of the two ribose anomeric (1′) protons. These chemical shifts are given in Table I, along with those of free $NADP^+$ for comparison. The four nicotinamide ring proton resonances all show strinkingly large downfield shifts on binding to the enzyme, ranging from 0.88 ppm for N2 to 1.54 ppm for N4. The adenine protons show somewhat smaller shifts in the opposite direction (upfield).

The saturation transfer experiment can also be used to estimate the dissociation rate constant of $NADP^+$ from the complex by determining the dependence of the observed intensity changes on the time for which the selective irradiation is applied (see eq 1 and 2). The amplitude of the resonance of the A2 proton of free $NADP^+$ as a function of irradiation time is shown in Figure 3, together with the best-fit curve obtained by nonlinear regression using eq 2; from this analysis we obtain $k_{off} = 20\ (\pm 5)\ s^{-1}$ and $\rho_F = 0.71\ s^{-1}$. These experiments have been repeated with the N2 proton resonance, giving $k_{off} = 24\ (\pm 5)\ s^{-1}$ and $\rho_F = 2.22\ s^{-1}$. Line-shape analysis of the ^{31}P resonances of $NADP^+$ (at the same temperature but in a different buffer) gives $k_{off} = 25\ (\pm 5)\ s^{-1}$ (Hyde et al., 1980). The good agreement obtained between these independent experiments confirms that the experimental data are correctly described by eq 2. For a dissociation rate constant of this magnitude, an exchange contribution to the line width of the coenzyme ^1H resonances, as observed in the ^{31}P spectrum (Hyde et al., 1980), would be expected. The existence of such a contribution is confirmed by the observation that the line widths of the resonances of free $NADP^+$ in the presence of the enzyme *increase* with increasing temperature, and it no doubt contributes to the difficulty in directly ob-

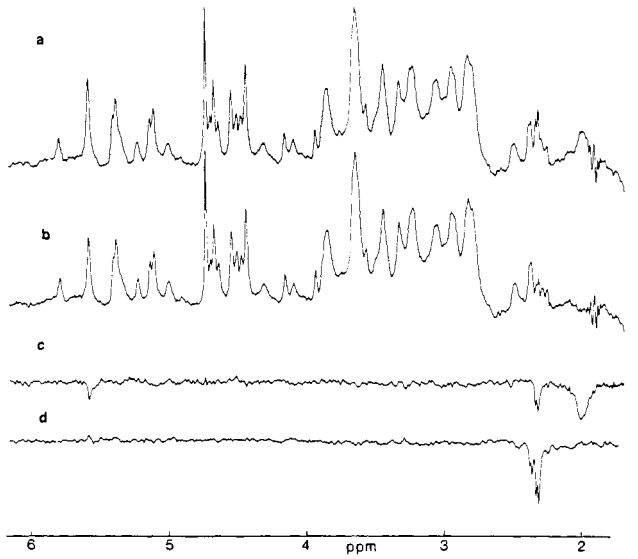

FIGURE 4: Aromatic region of the ^1H NMR spectra of the enzyme–$NADP^+$–methotrexate complex, in the presence of 4 equiv of free $NADP^+$. (a) Control, irradiation at 8.0 ppm; (b) irradiation at 2.01 ppm; (c) difference between (a) and (b); (d) after addition of NADPH to displace the $NADP^+$ from the enzyme, the difference between the control (a) and a spectrum obtained with irradiation at 2.27 ppm.

serving the resonances of the bound coenzyme.

Ternary Complexes Containing $NADP^+$. In the presence of methotrexate, $NADP^+$ binds 12 times more tightly to the enzyme (Birdsall et al., 1980a). This tighter binding would be expected to be accompanied by a decrease in k_{off}, making transfer of saturation experiments more difficult. Indeed, no clear effects could be seen for the ternary complex at 11 °C. Increasing the dissociation rate constant by increasing the temperature made it possible to carry out the experiments on the ternary complex but more difficult to do so on the binary enzyme–$NADP^+$ complex, owing to exchange broadening of the signals. At 25 °C it was possible to do the experiment reasonably well for both complexes. At this temperature, the dissociation rate constants (measured by the method illustrated in Figure 3) were 33 (±5) s^{-1} for the binary complex and 3.8 (±1) s^{-1} for the ternary complex. The decrease in the dissociation rate produced by methotrexate thus essentially accounts for the increase in the binding constant. Although the experiment could be done for both complexes at 25 °C, substantially larger effects on the peak height of the resonances of free $NADP^+$ were seen at 40 °C for the ternary complex, while for the binary complex (see above) the irradiation frequency having the maximum effect could be defined more precisely at 11 °C than at 25 °C since the signals were sharper; these are in the values quoted in Table 1. Comparison with the 25 °C experiment shows that there is no detectable temperature dependence of the chemical shifts in either complex, so that a valid comparison can be made. There are significant differences in the chemical shifts of the A8 and N5 protons, but the largest effect of methotrexate is on the N4 proton, whose resonance is 0.44 ppm to higher field in the ternary than in the binary complex.

One of the saturation transfer experiments on the ternary complex is shown in Figure 4; irradiation at 2.01 ppm leads to a marked decrease in intensity of the resonance, at 2.27 ppm, of the N1′ proton of the free coenzyme, thus identifying the resonance position of this proton in the bound state. However, the difference between the spectra with and without irradiation at 2.01 ppm (Figure 4c) shows that there is also a clear decrease in intensity of the N2 proton resonance of free $NADP^+$

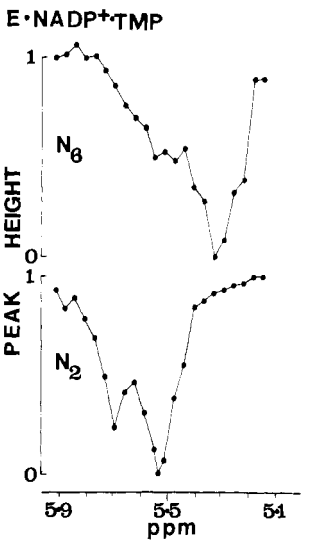

FIGURE 5: Intensities of the N2 and N6 proton resonances of free $NADP^+$ in the presence of the enzyme–$NADP^+$–trimethoprim complex as a function of irradiation frequency. The main peak in each case arises from the direct saturation effect, and the shoulders are from saturation transfer.

at 5.52 ppm. In free $NADP^+$ (Figure 4d) irradiation of the N1′ resonance leads, if anything, to a small increase in the intensity of the N2 signal. As discussed in detail elsewhere (Albrand et al., 1979), the decrease in intensity of the free N2 proton resonance on irradiating the bound N1′ resonance arises from a transferred nuclear Overhauser effect, indicating that the N2 and N1′ protons of bound $NADP^+$ are in close proximity to one another.

We have also examined the ternary complexes formed by the enzyme–$NADP^+$ complex with the substrate folate and with trimethoprim. The chemical shifts of the proton resonances of $NADP^+$ in these complexes were readily determined by transfer of saturation experiments at 11 °C and are given in Table I. The dissociation rate constants for $NADP^+$ were measured by the method illustrated in Figure 3; for the enzyme–$NADP^+$–trimethoprim complex $k_{off} = 4.2$ s^{-1}. The shifts in the folate ternary complex are generally similar to those seen with methotrexate, with two striking exceptions: the N4 proton resonance is 0.78 ppm further upfield, and the N5 resonance is 0.62 ppm further downfield in the folate than in the methotrexate ternary complex. With trimethoprim, on the other hand, the chemical shifts of all the nicotinamide ring protons are substantially different from those in the methotrexate ternary complex. In the trimethoprim ternary complex they are only 0.15–0.30 ppm downfield of their positions in free $NADP^+$, compared to 0.83–1.06 ppm downfield in the presence of methotrexate. Since the chemical shift differences between bound and free resonances were small, the irradiation frequency had to be rather close to the resonance whose intensity was being monitored, and direct saturation effects became important. The saturation transfer effects were indicated in the plot of signal intensity against irradiation frequency by marked shoulders on the negative "peak" produced by the direct saturation effect, as illustrated in Figure 5. These effects were very reproducible and clearly distinct from the completely symmetrical peak given in a control experiment by free $NADP^+$. A clear peak indicative of saturation transfer is seen for the A8 proton, 0.46 ppm from the free signal. The exchange rate must therefore be such that separate signals are observed for the bound and free states when their resonance frequencies differ by 0.46 ppm, and any other proton resonance which was shifted by ≥0.46 ppm on binding would also show

Table II: ^{1}H Chemical Shifts of NHDP$^+$, APADP$^+$, and TNADP$^+$, Free and Bound to Dihydrofolate Reductase

	chemical shift[a] (ppm from dioxane)							
proton	NHDP$^+$[b]	E-NHDP$^+$[b]	E-NHDP$^+$-methotrexate[c]	APADP$^+$[d]	E-APADP$^+$[d]	TNADP$^+$[d]	E-TNADP$^+$[d,e]	E-NADP$^+$[b]
A2	4.432	3.57	3.55	4.350	3.68	4.365	3.68	3.64
A8	4.601	4.31	4.10	4.666	4.27	4.658	4.27	4.42
N2	5.610	6.43	6.37	5.598	6.27	5.515	5.71 (5.68)	6.42
N4	5.134	6.57	6.16	5.107	5.98	5.057	5.39 (5.34)	6.60
N5	4.490	5.57	5.26	4.394	5.09	4.324	[f]	5.52
N6	5.443	6.31	6.30	5.350	5.72	5.215	(5.42)	6.31
A1′	2.394	3.02	2.97	2.347	3.01	2.316	3.01	3.03
N1′	2.375	2.02	[f]	2.347	[f]	2.197	[f]	2.04

[a] Free coenzyme, ±0.002 ppm; bound coenzyme, ±0.06 ppm. [b] 11 °C. [c] 40 °C. [d] 2 °C. [e] Values in parentheses are from fast-exchange experiments at 32 °C. [f] Not detected.

separate signals (provided that all portions of the coenzyme exchange at the same rate). Saturation transfer effects would also be observed, provided that the relaxation rates of the protons in the free state are similar. From measurements of the spin–lattice relaxation time, T_1, and the results for the binary complex (see above), the magnitude of the saturation transfer effect on the nicotinamide proton resonances would be expected to be ~80% of that on the A8 proton resonance, so that 0.46 ppm can be taken as an upper limit to their change in chemical shift on binding. The precision with which these small shifts can be measured is less than that for the other complexes, but a substantial difference in the environment of the nicotinamide ring of NADP$^+$ between the trimethoprim and methotrexate ternary complexes is evident.

Binding of NADP$^+$ Analogues. Three representative structural analogues of NADP$^+$ have been studied. Nicotinamide hypoxanthine dinucleotide phosphate (NHDP$^+$, in which the 6-amino group on the adenine ring of NADP$^+$ has been replaced by an oxo substituent), like other analogues modified in the adenine ring, shows very similar binding behavior to that of NADP$^+$ itself (Birdsall et al., 1980a,b). The chemical shifts of its nicotinamide, hypoxanthine, and ribose anomeric protons in its binary and ternary complexes with dihydrofolate reductase could readily be determined by saturation transfer experiments and are compared with those of NADP$^+$ in Table II. Although there are some small differences, overall the chemical shifts are very similar to those of NADP$^+$.

Modification of the nicotinamide ring of the coenzyme to give the analogues in which the carboxamide group is replaced by a thioamide (TNADP$^+$) or an acetyl group (APADP$^+$) has much larger effects on binding (Birdsall et al., 1980a,b). The complexes of both these analogues have substantially larger dissociation rate constants than does that of NADP$^+$ (Hyde et al., 1980), and the best results in the saturation transfer experiments were obtained at 2 °C. For APADP$^+$, quite clear-cut effects were observed; the chemical shifts are given in Table II. However, for TNADP$^+$ the chemical shift change on binding appeared to be much smaller for many of the protons. A very clear saturation transfer effect could be seen for the A2 proton, the bound peak being 0.6 ppm upfield of the resonance of this proton in free TNADP$^+$. As before, this serves as an upper limit to the shifts of the other protons. (We have measured the T_1 values of the protons of free TNADP$^+$, in case an anomalously short T_1 might prevent us from observing saturation transfer; however, the values are very similar to those for NADP$^+$.) For the N2 and N4 protons, shoulders indicative of saturation transfer were observed on the peak due to direct saturation (as illustrated for the enzyme–NADP$^+$–trimethoprim complex in Figure 5). To confirm that the observed "shoulders" did indeed correspond to the resonance frequencies of the protons of the bound coenzyme, we examined the binding of TNADP$^+$ to the enzyme at 35 °C, at which temperature the exchange of TNADP$^+$ between bound and free states has become fast on the NMR time scale. By measuring the chemical shifts of the proton resonances of TNADP$^+$ as its concentration was increased (in the presence of a fixed concentration of enzyme) and fitting the data to eq 5, we could extrapolate back to give a value for the chemical shift of the proton in the fully formed complex. Where a comparison was possible, these values were in excellent agreement with those obtained from the saturation transfer experiments at low temperature. Both sets of values for TNADP$^+$ are given in Table II; the shift of N5 was too small to detect. It is notable that for TNADP$^+$, and to a lesser extent for APADP$^+$, the changes in chemical shift of the nicotinamide proton resonances on binding are substantially smaller than those observed for NADP$^+$. For example, the N4 proton resonance shifts downfield 1.5 ppm in NADP$^+$, 0.9 ppm in APADP$^+$, and only 0.3 ppm in TNADP$^+$. In the case of TNADP$^+$, all the nicotinamide protons undergo much smaller chemical shift changes on binding (0.1–0.3 ppm vs. 0.9–1.5 ppm), but in APADP$^+$ there are still substantial chemical shift changes ranging from 0.4 ppm for N6 to 0.9 ppm for N4. In contrast, the A2 proton is very little affected, while the A8 proton shows a rather larger chemical shift change in the two coenzyme analogues than in NADP$^+$ itself.

Binding of NADPH. The reduced form of the coenzyme binds to the enzyme very much more tightly than the oxidized form, having a binding constant of 1×10^8 M^{-1} at pH 6.0, 25 °C (Dunn et al., 1978). In order to observe saturation transfer effects, it is necessary, as for NADP$^+$ in its ternary complex, to work at ~40 °C. Although the enzyme–NADPH complex is quite stable under these conditions, the 4 or 5 equiv of free NADPH$^+$ are not. We have therefore carried out the experiments with NADPH at pH* 7.0 and 40 °C. At this pH, the free coenzyme was stable for the several hours needed to find the correct irradiation frequency for saturation transfer, though a small amount (~10%) of decomposition was noted by the end of the experiment. As discussed by Birdsall et al. (1980a), the indications are that the binding of NADPH is very similar at pH 6.5 and pH 7.0.

Under these conditions, significant saturation transfer was observed for most of the adenine and nicotinamide protons; the chemical shifts of the resonances of bound coenzyme obtained in this way are given in Table III. The N5 and N1′ proton resonances could not be located, since the signals of these protons in free NADPH are obscured by the large water signal. In NADPH there are of course two N4 protons; in principle they would be expected to have different chemical shifts in the complex, although they are almost magnetically equivalent in the free coenzyme. We were only able to find

Table III: ^{1}H Chemical Shifts of NADPH, Free and in Its Binary Complex with Dihydrofolate Reductase

proton	chemical shift[a] (ppm from dioxane) NADPH	E-NADPH
A2	4.50	3.57
A8	4.68	4.53
N2	3.21	3.57
N4	−0.89	−0.25
N5	*b*	*b*
N6	2.22	2.98
A1′	2.45	2.94
N1′	*b*	*b*

[a] At 40 °C and pH* 7.0. [b] Not detected.

Table IV: Mononucleotide Chemical Shifts

proton	chemical shift[a] (ppm from dioxane) adenosine 2′,5′-diphosphate	hypoxanthine 2′,5′-diphosphate
A2	4.504	4.472
A8	4.806	4.778
A1′	2.472	2.481

proton	chemical shift[a] (ppm from dioxane) NMN^+	NMNH	$APMN^+$ [b]	$TNMN^+$ [b]
N2	5.812	3.422	5.771	5.729
N4	5.243	−0.681	5.343	5.253
N5	4.552	1.282	4.610	4.470
N6	5.582	2.511	5.747	5.513
N1′	2.455	1.133	2.495	2.439

[a] ±0.002; determined by using either the mononucleotide or a nucleotide pyrophosphatase digest of the appropriate dinucleotide, at a concentration of 5×10^{-4} M in 50 mM phosphate, 500 mM KCl, pH* 6.5, at 11 °C. [b] $APMN^+$, acetylpyridine mononucleotide; $TNMN^+$, thionicotinamide mononucleotide.

one chemical shift for these two protons; irradiation within a range of 80 Hz centered on a position 0.64 ppm to low field of the N4 proton resonance of free $NADP^+$ led to a significant transfer of saturation to the latter signal. It is not possible to establish whether one or both N4 protons of the bound coenzyme have their resonances at or near this position. If only one of the N4 proton signals is at this position, the other must be within 0.2 ppm of its position in the free coenzyme and thus not detectable by transfer of saturation. In any event, both N4 proton resonances of NADPH show much smaller changes in chemical shift on binding to the enzyme than does the N4 proton resonance of $NADP^+$. On the other hand, the chemical shifts of the adenine protons are similar in the oxidized and reduced coenzymes.

The dissociation rate of NADPH from the ternary enzyme–NADPH–methotrexate complex is much too slow for saturation transfer experiments to be possible, even at elevated temperatures.

Mononucleotide Chemical Shifts. In solution $NADP^+$ exists, for part of the time, in a conformation in which the adenine and nicotinamide rings are "stacked", giving rise to ring-current shifts of the resonances of protons on these rings [see, e.g., Jardetzky & Wade-Jardetzky (1966), Sarma & Mynott (1972), and Zens et al. (1976 and references cited therein)]. In contrast, in the enzyme–NADPH–methotrexate complex, the coenzyme is in an extended conformation with the two rings well separated (Matthews et al., 1978, 1979). The changes in chemical shift on binding will therefore have a contribution from this change in conformation, and this contribution might vary from one coenzyme to another if the degree of stacking or the magnitude of the ring-current varies. Before we can make a detailed comparison of the chemical shift changes on binding, we must subtract out this contribution, since the variations in it reflect differences in behavior of the free rather than of the bound coenzymes. In order to isolate this contribution, we need to compare the chemical shifts of the free coenzyme with those of the corresponding mononucleotides. The mononucleotide chemical shifts determined by using dilute solutions of the coenzyme which had been treated with snake venom nucleotide pyrophosphatase are given in Table IV. Comparison of the adenine (for $APADP^+$ and $TNADP^+$) or the nicotinamide (for $NHDP^+$) proton chemical shifts in these solutions with those of the appropriate mononucleotide showed excellent agreement, confirming that there was not residual stacking. In Table V, the chemical shifts of the bound coenzymes (from Tables I–III) are expressed relative to those of the appropriate mononucleotide (Table IV), so as to make them independent of any differences in conformation or ring-current shifts between the various coenzymes.

Discussion

The changes in chemical shift of the proton resonances of a ligand when it binds to a protein provide a valuable indication of the environment of the bound ligand and thus of its mode of binding. Hitherto, experiments of this sort have been largely confined to relatively weakly binding ligands for which exchange between the free and bound states is fast on the NMR time scale, because of the difficulty in resolving and assigning the resonances of tightly bound ligands. The experiments described here [together with similar ones on trimethoprim binding to dihydrofolate reductase, (Cayley et al., 1979) and the work of Bendall et al. (1977) on papain] show that the saturation transfer experiment provides a simple and widely applicable method by which the chemical shifts of the resonances of tightly bound ligands can be determined.

The resonances of the adenine ring protons A2 and A8 show an upfield shift on binding, while the A1′ proton signal shifts downfield. The changes in chemical shift are large, and yet

Table V: ^{1}H Chemical Shift Changes on Coenzyme Binding to Dihydrofolate Reductase

proton	chemical shift[a] (ppm) E-NADP	E-$NADP^+$-methotrexate	E-$NADP^+$-folate	E-$NADP^+$-trimethoprim	E-$NADPH^+$ [b]	E-$APADP^+$	E-$TNADP^+$	E-$NHDP^+$	E-$NHDP^+$-methotrexate
A2	−0.86	−0.86	−0.83	−0.84	−0.93	−0.82	−0.82	−0.90	−0.92
A8	−0.39	−0.50	−0.47	−0.61	−0.28	−0.54	−0.54	−0.47	−0.68
N2	0.61	0.53	0.56	0.10	0.15	0.50	−0.03	0.62	0.56
N4	1.36	0.92	0.14	0.09	0.64	0.64	0.11	1.33	0.92
N5	0.97	0.79	1.41	0.01		0.48		1.02	0.71
N6	0.73	0.73	0.69	−0.07	0.47	−0.03	0.09	0.73	0.72
A1′	0.56	0.48	0.53	0.56	0.47	0.54	0.54	0.55	0.49
N1′	−0.42	−0.45	−0.43					−0.44	

[a] Shifts (positive downfield) in the indicated complex (Tables I–III), relative to the appropriate mononucleotide (Table IV). [b] pH* 7.0.

they vary relatively little from one complex to another. Some significant differences can be seen in the resonance position of A8—for example, formation of a ternary complex produces an upfield shift, and the change in shift of this resonance of NADPH is smaller than that of A8 in the oxidized coenzymes—but these are relatively small (~0.2 ppm). In general, it appears that the adenosine moiety binds to the enzyme in much the same way in all these complexes. Further evidence for this is presented in the following paper (Hyde et al., 1980), where we show that the ^{31}P resonance of the 2′-phosphate group also has the same position in a number of different complexes. The variations in binding constant between the different coenzymes are thus not accompanied by any major changes in this part of the coenzyme binding site.

The crystal structure of the enzyme–NADPH–methotrexate complex (Matthews et al., 1978) shows that the imidazole ring of histidine-64 is stacked almost parallel to the plane of the adenine ring, over the N1 and C2 atoms. The ring current of this protonated imidazole can account for the observed upfield shift of the A2 proton resonance (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments). The C2 proton resonance of His-64 has been identified (Wyeth et al., 1980) and found to show a corresponding large upfield shift (~0.8 ppm) in a variety of coenzyme complexes (A. Gronenborn, B. Birdsall, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments). Since these ring-current shifts are sensitive to small changes in geometry, we can conclude that this particular structural feature is present in all the coenzyme complexes.

In contrast, the environment of the nicotinamide ring, as indicated by the proton chemical shifts, varies markedly from one complex to another. When $NADP^+$ binds to the enzyme, all four nicotinamide proton resonances undergo very large downfield shifts, ranging from 0.61 ppm for N2 to 1.36 ppm for N4 (Table V). We have earlier shown that the ^{13}C resonance of [*carboxamido*-^{13}C]$NADP^+$ shifts substantially upfield on binding to the enzyme (Way et al., 1975). The ^1H shift changes seen with $NHDP^+$ are very similar to those of $NADP^+$, being within 0.05 ppm for all the protons except A8 (0.08 ppm). However, modification of the nicotinamide ring, as in $APADP^+$ and $TNADP^+$, leads to substantially smaller shifts. The effects of these two coenzyme analogues on inhibitor binding are also quite different from those of $NADP^+$ (Birdsall et al., 1980a,b). The change in chemical shift of the N2 proton resonance of $APADP^+$ on binding is similar to that seen with $NADP^+$, but the N4 and N5 resonances shift only about half as much as in $NADP^+$, and the shift of the N6 signal is essentially zero (after correction for the shift difference between the free coenzyme and the mononucleotide—the "unstacking" shift). For $TNADP^+$, all the nicotinamide protons show very small (<0.1 ppm) shifts on binding (again after correction for the unstacking shift). On the basis of these chemical shift changes, it is clear that the mode of binding of $TNADP^+$ is quite different from that of $NADP^+$, while that of $APADP^+$ is less so. This is also reflected in their binding behavior (Birdsall et al., 1980a,b) and ^{31}P NMR spectra (Hyde et al., 1980).

It is particularly interesting that the chemical shift changes accompanying binding are quite different for the oxidized and reduced coenzymes. Although the shift of the N6 proton is similar, the N2 and N4 protons show much smaller changes in shift in NADPH than in $NADP^+$. These shift differences are clearly consistent with earlier indications [Feeney et al., 1977; Kimber et al., 1977; Hood et al., 1979; see also Birdsall et al., (1980a)] of a significant difference in conformation between the NADPH and $NADP^+$ complexes.

The only detailed information we have on the environment of the nicotinamide ring relates to the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979). This is, unfortunately, one complex for which the saturation transfer method cannot be used, since the dissociation of the coenzyme is very slow (Birdsall et al., 1980a). Examination of the crystal structure of this complex does, however, give an indication of the nature of the amino acid residues which interact with the nicotinamide ring, although their relative positions must differ from one complex to another to explain the observed chemical shift differences. The nicotinamide ring binds in a hydrophobic pocket, the only "specific" (as opposed to "hydrophobic") interactions it makes being three hydrogen bonds between the carboxamide group and the peptide backbone (Matthews et al., 1979). Among the residues in contact with the nicotinamide are Trp-5 and Trp-21 and the peptide bond between Gly-98 and Gly-99, while the phenyl ring of Phe-103 is also nearby. There are thus a number of potential sources of magnetic anisotropy near the nicotinamide protons. These, together with possible effects of hydrogen bonding of the carboxamide group on electron distribution in the ring, seem the most likely origins of the large changes in chemical shift of the nicotinamide protons (the nearest charged group, Asp-26, is some 5 Å away).

If, as it appears, hydrogen bonding to the carboxamide group is important in orienting the nicotinamide ring in the binding site, then modifications which drastically affect the hydrogen-bonding ability of this group, as in $APADP^+$ and $TNADP^+$, might be expected to lead to an altered orientation of the ring within the binding "pocket". Any rotation of the ring within the ring-current "field" of the neighboring aromatic rings would lead to marked changes in chemical shift of the nicotinamide protons. An alternative possibility is that these different chemical shifts reflect not simply a different orientation with the same binding subsite but interaction of the nicotinamide ring with a quite different region of the protein, as has been observed for the pyridine and 3-iodopyridine analogoues of NAD^+ on alcohol dehydrogenase (Samama et al., 1977). There is clear evidence (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) for the latter possibility in the case of the enzyme–trimethoprim–$TNADP^+$ complex, but the choice between the two cannot yet be made for the binary complex.

One of the aromatic rings in contact with the nicotinamide, that of Trp-21, can be specifically modified by *N*-bromosuccinimide (Liu & Dunlap, 1974; Freisheim et al., 1977). This modification decreases the affinity of the enzyme for NADPH very much more than that for $NADP^+$ (Thomson et al., 1980). Furthermore, one of the resonances in the ^{19}F spectrum of the 6-fluorotryptophan-containing enzyme (Kimber et al., 1977) is affected markedly by NADPH but much less so by $NADP^+$. Although this ^{19}F resonance has not been specifically assigned, these observations taken together do suggest that the relationship between the nicotinamide ring and Trp-21 differs for the oxidized and reduced coenzymes. Of the protons on the nicotinamide ring, N2 is the closest to Trp-21, and this different relationship would explain the markedly different effect on binding to the enzyme on the shift of this proton in NADPH as compared to that in $NADP^+$.

The addition of methotrexate to the enzyme–$NADP^+$ complex to give the ternary complex has a very selective effect on the coenzyme ^1H chemical shifts. The resonance of the N4 proton is shifted upfield by 0.44 ppm and that of the neigh-

boring N5 proton is shifted upfield by 0.18 ppm, while the effects on N2 and N6 are very small; there is also a substantial upfield shift of the carboxamido ^{13}C resonance (Way et al., 1975). In the ternary complex containing NADPH, the 4-carbon of the nicotinamide lies over the pteridine ring (Matthews et al., 1978). Assuming that this is also true for $NADP^+$ (although perhaps not with precisely the same geometry), the simplest explanation of the shifts of the N4 and N5 protons is that they are due to the magnetic anisotropy of the pteridine ring of methotrexate.

The experiment shown in Figure 4 demonstrates that in the enzyme–$NADP^+$–methotrexate complex there is a nuclear Overhauser effect between the N1′ proton and the N2 proton but not between the N1′ proton and the N6 proton. The observation of a nuclear Overhauser effect between two protons is usually taken to imply that they are in close proximity (Noggle & Schirmer, 1971). We have calculated (Albrand et al., 1979) that in an indirect "transferred nuclear Overhauser effect" experiment of this sort, the observation of an effect places an upper limit of ~4 Å on the internuclear distance. The distance between N1′ and N2 or N6 is governed by the torsion angle about the glycosidic bond. In the anti conformation, N1′ and N2 are in close proximity, while in the syn conformation it is N6 which is near N1′ The observation of a nuclear Overhauser effect on N2 but *not* N6 is therefore consistent only with an anti conformation of $NADP^+$ in the complex, although because of the qualitative nature of the experiment we cannot specify the value of the torsion angle. The crystal structure of the enzyme–NADPH–methotrexate complex (Matthews et al., 1978) shows NADPH to have the anti conformation also. Thus, whatever the differences in the environment of the nicotinamide ring between $NADP^+$ and NADPH, the conformation about the glycosidic bond is, at least approximately, the same in both cases in the ternary complex.

Folate selectively shifts the same two proton resonances of $NADP^+$ that are affected by methotrexate but in a very different manner. It produces a much larger (1.22-ppm) upfield shift of the N4 signal, and a 0.44-ppm *downfield* shift of that of N5. The pteridine ring of folate will produce different ring-current effects from that of methotrexate (particularly if folate binds in the *keto* rather than the *enol* form; Hood & Roberts, 1978). However, the very large differences in the shift of the N4 and N5 resonances do suggest a distinctly different mutual orientation of the nicotinamide and pteridine rings in the folate complex as compared to that in the methotrexate complex. We have recently obtained conclusive evidence for this in studies of the stereochemistry of the reduction of folate by the enzyme (Charlton et al., 1979).

In contrast to these selective shifts, trimethoprim produces a large upfield shift of *all* the nicotinamide proton resonances of bound $NADP^+$; in fact, well over half the changes in chemical shift of these resonances of $NADP^+$ on binding to the enzyme–trimethoprim complex are accounted for by the unstacking of the coenzyme on binding. The shifts remaining after correcting for this effect are only marginally, if at all, outside the experimental error. The simplest explanation for this would be that trimethoprim completely displaces the nicotinamide ring from its binding pocket, so that the surrounding magnetically anisotropic groups no longer affect the nicotinamide proton chemical shifts. We have recently shown (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) that the enzyme–trimethoprim–$NADP^+$ complex exists as a mixture of two conformational states, in one of which the conformation is substantially different from that seen for NADPH in the crystal structure (Matthews et al., 1979). In the conformation we have proposed for trimethoprim bound to the enzyme (Cayley et al., 1979), there could well be an unfavorable steric interaction with a nicotinamide ring bound as in the crystal (Matthews et al., 1978, 1979).

It is clear from these experiments that the environment of the nicotinamide ring of the coenzyme is markedly affected by the addition of substrate or inhibitors, while that of the adenosine moiety remains at least approximately the same. The interactions responsible for the observed cooperativity in ligand binding (Birdsall et al., 1980a,b) must thus be localized at the nicotinamide end of the molecule. The marked effects of trimethoprim on the nicotinamide proton chemical shifts and the changes produced by substrate or inhibitors in the environment of the pyrophosphate group (Hyde et al., 1980; A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) clearly indicate that the cooperativity involves more than a simple direct interaction between the nicotinamide and pteridine rings. In addition, comparison of $NADP^+$, $APADP^+$, and $TNADP^+$ shows that very similar binding constants (Birdsall et al., 1980a) can be obtained with very different modes of binding. The proton chemical shifts are obviously a sensitive indicator of the environment of the nicotinamide ring; we are now attempting to account for them quantitatively on the basis of the magnetic anisotropy of the neighboring groups, using the crystal structure of the complex with NADPH and methotrexate as a parting point. In this way it should be possible to provide a detailed description of the way in which the binding of the nicotinamide ring differs from one complex to another.

Acknowledgments

We are most grateful to Gill Ostler for skillful technical assistance and to Dr. David Matthews for generously supplying us with the atomic coordinates derived from this crystallographic work.

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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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Received December 12, 1979.
‡ Supported by an MRC Research Studentship.

0006-2960/80/0419-3746$01.00/0