Acknowledgments

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A Nuclear Magnetic Resonance Study of Nicotinamide Adenine Dinucleotide Phosphate Binding to *Lactobacillus casei* Dihydrofolate Reductase†

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ABSTRACT: The binding of $NADP^+$ to dihydrofolate reductase (EC 1.5.1.3) in the presence and absence of substrate analogs has been studied using ^1H and ^{13}C nuclear magnetic resonance (NMR). $NADP^+$ binds strongly to the enzyme alone and in the presence of folate, aminopterin, and methotrexate with a stoichiometry of 1 mol of $NADP^+$/mol of enzyme. In the ^{13}C spectra of the binary and ternary complexes, separate signals were observed for the carboxamido carbon of free and bound [^{13}CO]$NADP^+$ (enriched 90% in ^{13}C). The ^{13}C signal of the $NADP^+$–reductase complex is much broader than that in the ternary complex with methotrexate because of exchange line broadening on the binary complex signal. From the difference in line widths (17.5 ± 3.0 Hz) an estimate of the dissociation rate constant of the binary complex has been obtained (55 ± 10 sec^{-1}). The dissociation rate of the $NADP^+$–reductase complex is not the rate-limiting step in the overall reaction. In the various complexes studied large ^{13}C chemical shifts were measured for bound [^{13}CO]$NADP^+$ relative to free $NADP^+$ (upfield shifts of 1.6–4.3 ppm). The most likely origin of the bound shifts lies in the effects on the shieldings of electric fields from nearby charged groups. For the $NADP^+$–reductase–folate system two ^{13}C signals from bound $NADP^+$ are observed indicating the presence of more than one form of the ternary complex. The ^1H spectra of the binary and ternary complexes confirm both the stoichiometry and the value of the dissociation rate constant obtained from the ^{13}C experiments. Substantial changes in the ^1H spectrum of the protein were observed in the different complexes and these are distinct from those seen in the presence of NADPH.

Dihydrofolate reductase catalyses the NADPH-linked reduction of dihydrofolate to tetrahydrofolate, and is the target of a potent group of inhibitors of considerable chemotherapeutic interest (Blakley, 1969; Hitchings and Burchall, 1965). We are undertaking a detailed study of ligand binding to dihydrofolate reductase from a methotrexate-resistant strain of *Lactobacillus casei.*

We have already reported (Roberts et al., 1974) studies of the binding of the coenzyme (NADPH) and of a fragment of the substrate (*p*-aminobenzoyl-L-glutamate) to the

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enzyme, using nuclear magnetic resonance (NMR) spectroscopy. It was possible to demonstrate the 1:1 stoichiometry of the binding of NADPH to the enzyme, and to measure the binding constants and bound chemical shifts for *p*-aminobenzoylglutamate and related compounds.

In the present work, our aim was to obtain information on the binding of the oxidized form of the coenzyme ($NADP^+$) to the enzyme in the presence and absence of substrate and substrate analogs. It is clearly important to establish the way in which the binary and ternary complexes involving $NADP^+$ differ from the corresponding complexes involving NADPH. Steady-state kinetic studies (J. G. Dann and G. C. K. Roberts, unpublished work) have shown that $NADP^+$ is a powerful product-inhibitor of the reaction, and have provided some evidence for the formation of the "dead-end" ternary complex dihydrofolate-$NADP^+$-reductase; in addition there is the possibility that release of $NADP^+$ might be rate limiting in the overall reaction.

If a ligand is strongly bound ($K_a \geq 10^5\ M^{-1}$) to an enzyme, separate NMR absorption signals are usually obtained for the free and bound states. In ^1H NMR studies it is often difficult to identify the resonances from the strongly bound ligand in the 1:1 complex because of the presence of the spectrum of the protein. This was the case, for example, in our studies of NADPH binding to dihydrofolate reductase (Roberts et al., 1974). Even if resonances from the bound ligand can be detected, one is faced with the difficult problem of assigning them to the individual protons of the ligand. The only general solution to this problem is to examine selectively deuterated ligands in the presence of deuterated protein. In ^{13}C NMR work, on the other hand, the problem can be circumvented by selectively enriching sites in the ligand with ^{13}C. For this reason we have prepared $NADP^+$ with 90% ^{13}C enrichment of the 3-carboxamido carbon on the nicotinamide ring. There is no difficulty in distinguishing the resonance of this carbon from those of the protein carbonyl ^{13}C resonances (natural abundance only 1.1%), and of course there is no assignment problem since the site of enrichment is known. An additional advantage of this approach is that it is possible to have a nuclear probe at a site which is not accessible for proton NMR studies. Furthermore, the absence of a directly bonded proton ensures that line broadening from dipolar interactions is minimized; thus in the absence of exchange effects, narrow ^{13}C signals for enzyme-bound [^{13}CO]$NADP^+$ are anticipated.

Materials and Methods

Enzyme. Dihydrofolate reductase from *Lactobacillus casei* MTX/R was purified as described by Dann et al. (1975). The enzyme was lyophilized twice from D_2O to remove exchangeable protons and then dissolved in D_2O to give an enzyme concentration of 0.82–1.26 m*M* in 50 m*M* potassium phosphate–500 m*M* KCl, pH* (meter reading, uncorrected for the deuterium isotope effect on the glass electrode) 6.5. The enzyme concentration was determined by spectrophotometric assay (Dann et al., 1975).

Other Materials. $NADP^+$, folic acid (Sigma Chemical Co.), methotrexate, aminopterin (Nutritional Biochemicals Corp.), and trimethoprim (Wellcome Laboratories) were used without further purification. All ligands were dissolved in D_2O buffer (as above) or in D_2O adjusted to pH* 6.5, and volumes of up to 100 μl were added to 2.0 ml (in ^{13}C experiments) or 1.0 ml (in ^1H experiments) of enzyme solution.

Preparation of [Carboxamido-^{13}C]$NADP^+$. (i) [*carboxamido*-^{13}C]Nicotinamide was synthesized by a modification of the procedure of Murray et al. (1948) and Murray and Williams (1958). Halogen–metal interconversion between 3-bromopyridine and *n*-butyllithium (in ether at −75°) gave 3-pyridyllithium. This was immediately carbonated (without isolation) using 90% $^{13}CO_2$ (Prochem Ltd.) to give [*carbonyl*-^{13}C]nicotinic acid. Treatment with anhydrous thionyl chloride gave [*carbonyl*-^{13}C]nicotinoyl chloride, which on reaction with ammonia gas in tetrahydrofuran gave [*carboxamido*-^{13}C]nicotinamide (yield, based on $^{13}CO_2$, 50%).

(ii) [*carboxamido*-^{13}C]$NADP^+$ was prepared by an exchange reaction of [^{13}C]nicotinamide with acetylpyridine-$NADP^+$ (Sigma Chemical Co.) catalyzed by calf spleen nicotinamide adenine dinucleotidase (Sigma Chemical Co.), using a modification of the method of Kaplan and Ciotti (1956a,b). We are most grateful to Drs. W. J. LeQuesne and D. J. Thomas of the Radiochemical Centre (Amersham) for giving us details of their improved procedure for this reaction. The [^{13}C]$NADP^+$ was isolated by preparative paper chromatography and ion-exchange chromatography; no isotopic dilution occurred, and the yield was 10% (the [^{13}C]nicotinamide was recovered and the exchange reaction repeated).

NMR Spectroscopy. ^1H and ^{13}C NMR spectra were obtained at 100 and 25.2 MHz, respectively, using a Varian Associates XL-100-15 spectrometer equipped with Fourier transform facilities controlled by a VDM 620 i computer. The field-frequency lock was obtained from the deuterium in the solvent. For ^1H spectra, 500 transients were accumulated with an acquisition time of 1.00 sec; the resonance of the residual water protons was irradiated selectively and continuously with a second radiofrequency field. For ^{13}C spectra, approximately 32,000 transients were accumulated, using an acquisition time of 0.80 sec, and a total interval between pulses of 2.4 sec. Control experiments indicate that the enzyme is stable under these conditions for times longer than those required for these experiments. In both cases, the signal-to-noise ratio was improved by multiplying the free induction decay by an exponential weighting function; the effects of various time constants for this weighting function were carefully investigated to ensure that the measured line widths were not influenced by the weighting functions. The sample temperature was controlled by a V4343 variable temperature unit, and measured with a thermocouple; the sample temperature for each experiment is indicated in the appropriate figure caption.

Data Analysis. In the equilibrium

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \qquad (1)$$

if the rate of exchange of the ligand, I, between the free and bound states is slow on the NMR time-scale, then separate NMR absorption bands are observed for the two states. Two clearly resolved signals will be observed if $\tau \geq 4/(2\pi\Delta\nu)$ where $\Delta\nu$ is the chemical shift difference for a ligand nucleus between the free and bound states (Pople et al., 1959). Under these circumstances, the chemical shift of a ligand nucleus in the bound state can be measured directly from the spectrum. The line width, *W*, of the resonance from the bound state depends on the effective spin–spin re-

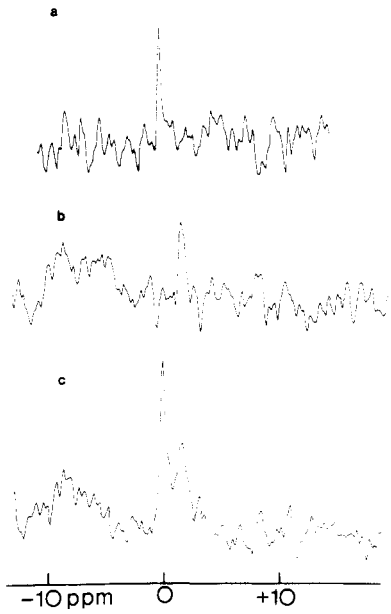

FIGURE 1: The ^{13}C NMR spectra at 25.2 MHz of (a) a 1 mM solution of [^{13}CO]NADP$^+$; (b) [^{13}CO]NADP$^+$ in the presence of dihydrofolate reductase (molar ratio 1:1); (c) [^{13}CO]NADP$^+$ in the presence of dihydrofolate reductase (molar ratio 3:1). The broad resonance between −2 and −12 ppm in (b) and (c) arises from the protein carbonyl carbons. Sample temperature, 10°.

laxation time, $T^*_{2,\text{bound}}$ (including inhomogeneity contributions), and on the lifetime, τ, as follows:

$$W = \frac{1}{\pi T_{2,\text{obsd}}^{\text{bound}}} \tag{2}$$

$$\frac{1}{T_{2,\text{obsd}}^{\text{bound}}} = \frac{1}{T^*_{2,\text{bound}}} + \frac{1}{\tau} \tag{3}$$

If $\tau > T^*_{2,\text{bound}}$, then the line width will decrease with increasing temperature, whereas if $T^*_{2,\text{bound}} > \tau$ the exchange lifetime becomes the dominant contribution, and the line width will increase with increasing temperature. In the latter case, it is possible to determine τ from line width measurements, and, hence, since $1/\tau = k_{-1}$, the dissociation rate constant in the equilibrium (eq 1). Similar information can be obtained from measurements of the resonance from ligand molecules in the free state. For this resonance, the corresponding equation to (3) above is

$$\frac{1}{T_{2,\text{obsd}}^{\text{free}}} = \frac{P_B}{1 - P_B}\frac{1}{\tau} + \frac{1}{T^*_{2,\text{free}}} \tag{4}$$

where P_B is the fraction of ligand molecules in the bound state. Under the conditions of the present experiments, $[E]_{\text{total}} \gg 1/K_a$, so that $P_B = [E]_{\text{total}}/[I]_{\text{total}}$.

Results

^{13}C Studies of the Binding of [carboxamido-^{13}C]NADP$^+$ to Dihydrofolate Reductase. When 1 equiv of [*carboxamido*-^{13}C]NADP$^+$ ([^{13}CO]NADP$^+$) is added to a 1.26 mM solution of dihydrofolate reductase a single broad ^{13}C absorption band is observed at 1.6 ppm to high field of the signal from free [^{13}CO]NADP$^+$ (see Figure 1); there is no difficulty in distinguishing the signal of the [^{13}CO]NADP$^+$ from the protein carbonyl ^{13}C spectrum. The addition of a further equivalent of [^{13}CO]NADP$^+$ results in the appearance of a second broad ^{13}C absorption at the same chemical shift value as that of free NADP$^+$. Further additions of [^{13}CO]NADP$^+$ increased the intensity and narrowed the line width of this signal but did not cause any change in its chemical shift (Figure 1). The appearance of two well-resolved separate ^{13}C absorption bands (chemical shift difference = 40 Hz) indicates that the free and bound states of NADP$^+$ are in slow exchange on the NMR time scale (lifetime $\tau \geq 4/(2\pi\Delta\nu)$, i.e., $\tau \geq 0.016$ sec). When 1 equiv of methotrexate is added to the NADP$^+$–reductase complex the ^{13}C signal corresponding to bound NADP$^+$ is shifted upfield (2.62 ppm from free NADP$^+$, see Figure 2b) and *both* the free and bound NADP$^+$ signals are considerably narrower. The difference in line widths for the bound NADP$^+$ signal in the binary and ternary complexes is $\Delta W = 17.5 \pm 3.0$ Hz. It is argued below that this reduction in line width arises from an increased lifetime of the NADP$^+$ in the ternary as compared to the binary complex. The ^{13}C spectra of [^{13}CO]NADP$^+$ bound to the enzyme in the presence of various inhibitors and substrates have been measured. In all cases NADP$^+$ is in slow exchange on the NMR time scale and the bound chemical shifts can be determined directly from the spectrum. Aminopterin which has the same pteridine ring structure as methotrexate produces only a slightly different bound shift from that observed for methotrexate (2.90 ppm from free NADP$^+$; Fig-

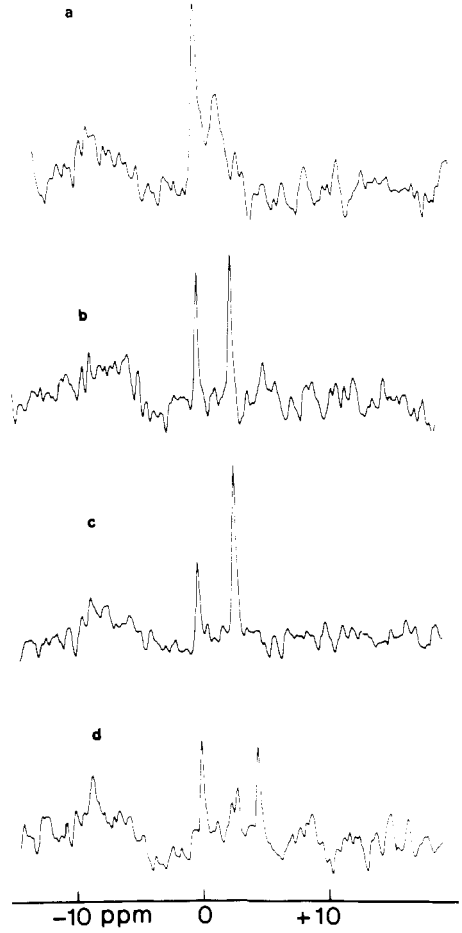

FIGURE 2: The ^{13}C NMR spectra at 25.2 MHz of (a) [^{13}CO]NADP$^+$ in the presence of dihydrofolate reductase (molar ratio 3:1); (b) the [^{13}CO]NADP$^+$-dihydrofolate reductase-methotrexate complex; (c) the [^{13}CO]NADP$^+$-dihydrofolate reductase-aminopterin complex; (d) the [^{13}CO]NADP$^+$-dihydrofolate reductase-folate complexes. In all spectra the sharp resonance at 0 ppm corresponds to free [^{13}CO]NADP$^+$ and the sharp signals at higher fields to bound [^{13}CO]NADP$^+$. The broad resonance between −2 and −12 ppm arises from the protein carbonyl carbons. Sample temperature, 10°.

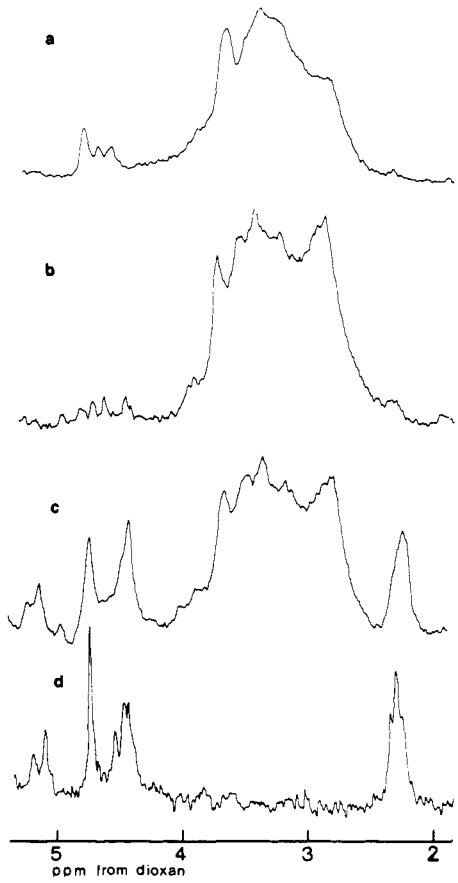

FIGURE 3: The aromatic region of the 100 MHz proton NMR spectra of (a) dihydrofolate reductase; (b) dihydrofolate reductase in the presence of 1 equiv of $NADP^+$; (c) dihydrofolate reductase in the presence of 4 equiv of $NADP^+$; (d) $NADP^+$ (1 m*M*). Sample temperature, 14°.

ure 2c). However, the addition of folate to the $NADP^+$–reductase complex causes much larger shifts. For this system the ^{13}C spectrum (Figure 2d) clearly indicates the presence of two different ternary complexes: in one of these complexes the position of the [^{13}CO]$NADP^+$ signal is similar to that seen in the methotrexate ternary complex (2.6 ppm) while the other complex has a more intense signal at much higher fields (4.33 ppm from free $NADP^+$). Addition of aminopterin to the folate–$NADP^+$–reductase system results in the ^{13}C spectrum expected for the $NADP^+$–reductase–aminopterin complex. Experiments with dihydrofolate were complicated by enzyme-catalyzed decomposition of the substrate during the long periods of spectrum accumulation. Control experiments showed that for folate no decomposition occurred over time periods substantially longer than those used for obtaining the spectra.

^1H Studies of the Binding of $NADP^+$ to Dihydrofolate Reductase. The results of the ^1H studies of the binding of $NADP^+$ to the reductase are fully consistent with the ^{13}C observations. Addition of 1 equiv of $NADP^+$ to the reductase produces marked changes in the His C2H, aromatic, and methyl regions of the protein ^1H spectrum (compare Figure 3a and b). These changes cannot be described in detail, though the chemical shifts of at least four of the six His C2H resonances are affected.

The changes in the protein spectrum confirm that the $NADP^+$ is binding to the enzyme. However, no identifiable resonances from the bound $NADP^+$ could be detected, suggesting that their line widths are excessively broadened by exchange. If we assume that the natural line widths of

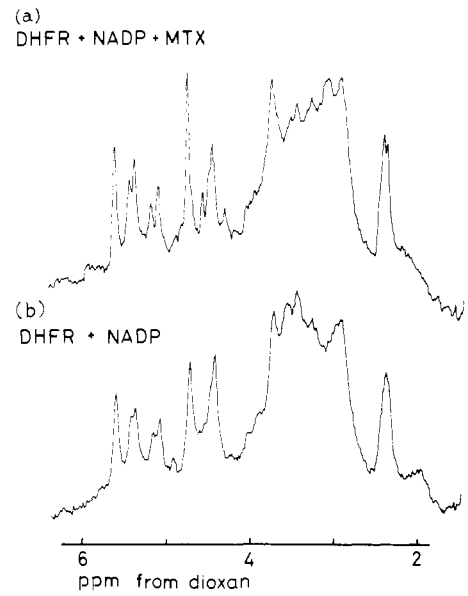

FIGURE 4: The aromatic region of the 100 MHz proton NMR spectra of (a) dihydrofolate reductase in the presence of 1 equiv of methotrexate and 4 equiv of $NADP^+$; (b) dihydrofolate reductase in the presence of 4 equiv of $NADP^+$. Sample temperature, 14°.

the sharpest bound $NADP^+$ proton signals (those of the 2 and 8 protons of the adenine ring) are comparable with those of the protein histidine C2H resonances (~5 Hz) then the additional line broadening from the exchange process (~18 Hz, estimated from the ^{13}C spectra of the $NADP^+$–reductase complex) would result in observed line widths of ~23 Hz; such broad lines would be very difficult to detect in the presence of the NH and His C2H resonances from the protein. When additional $NADP^+$ is added no further changes in the protein spectrum are seen, and at molar ratios ($NADP^+$–reductase) of 2:1 or greater broad resonances are discernible in the spectrum at the chemical shift positions of the adenosine 2, 8, and 1′ and nicotinamide 2, 4, 6, and 1′ protons in free $NADP^+$ (Figure 3c). As the concentration of $NADP^+$ is increased, the chemical shifts of these resonances do not change, remaining at positions identical with those seen for $NADP^+$ in the absence of enzyme. This is strong evidence that for the ^1H spectrum as for the ^{13}C spectrum, $NADP^+$ is in slow exchange, and that the observed $NADP^+$ ^1H resonances arise only from molecules in the free state.

Further evidence that the ^1H spectrum is in the same exchange region as the ^{13}C spectrum is provided by the effects of methotrexate. Addition of 1 equiv of methotrexate to a solution containing enzyme and 4 equiv of $NADP^+$, either in the presence or absence of 1 m*M* EDTA, leads to a marked sharpening of the "free" $NADP^+$ resonances (Figure 4).

As noted above, if the line widths of the ^1H resonances of $NADP^+$ are dominated by exchange, as these results indicate, then the lines should become broader with increasing temperature. Attempts were made to observe this effect, and some indications of line broadening were seen, but over the limited temperature range accessible to us (10–35°; limited by thermal denaturation of the protein) the changes were too small ($\Delta W \sim 2$ Hz) to be analyzed.

Discussion

Stoichiometry of Binding and Dissociation Rate Constants. The observation that the ^{13}C spectrum obtained on

Table I: ^{13}C Chemical Shifts at 25.2 MHz of [^{13}CO] $NADP^+$ Bound to Dihydrofolate Reductase.

Complex	Chemical Shift[a] (ppm)
$NADP^+$–reductase	1.60
$NADP^+$–reductase–methotrexate	2.62
$NADP^+$–reductase–aminopterin	2.90
$NADP^+$–reductase–folate	2.6 4.33
$NADP^+$–reductase–trimethoprim	3.60

[a] Chemical shifts measured relative to free [^{13}CO] $NADP^+$; positive shifts upfield.

adding 2 equiv of $NADP^+$ to the enzyme consists of one shifted resonance and one resonance at the position of free $NADP^+$ suggests that the stoichiometry of binding is 1 mol of $NADP^+$/mol of enzyme. It is, however, possible that $NADP^+$ binds to a second site but that this second binding leads to little or no chemical shift of the ^{13}C resonance. This latter possibility is made much less likely by the results of the proton studies. All the changes produced by $NADP^+$ in the protein spectrum are complete on addition of 1 equiv of $NADP^+$. Furthermore all the observed proton resonances of $NADP^+$ appear at frequencies identical with those of free $NADP^+$ when 2 or more equivalents of $NADP^+$ are added, and show no shifts on increasing $NADP^+$ concentration. It is highly unlikely that *all* the $NADP^+$ protons would have the same chemical shifts in the bound and free states. Thus the ^1H and ^{13}C data show that there is only a single strong ($K_a > 10^2\ M^{-1}$) binding site for $NADP^+$ on the enzyme. This is also true for NADPH binding to the *L. casei* enzyme (Roberts et al., 1974), although more than one binding site for coenzyme has been reported for the *Escherichia coli* enzyme (Poe et al., 1974; Williams et al., 1973).

The appearance of separate ^{13}C signals for free and bound $NADP^+$ indicates that the $NADP^+$ is in slow exchange on the NMR time scale. A lower limit for the exchange lifetimes ($\tau \geq 4/(2\pi\Delta\nu)$) may be estimated from the chemical shift difference, $\Delta\nu$, between the bound and free states. The calculated values are $NADP^+$–reductase, $\tau \geq 0.016$ sec; $NADP^+$–reductase–methotrexate, $\tau \geq 0.01$ sec; $NADP^+$–reductase–aminopterin, $\tau \geq 0.019$ sec; $NADP^+$–reductase–trimethoprim, $\tau \geq 0.007$ sec.

Addition of methotrexate to a solution of the enzyme containing 2 equiv of $NADP^+$ leads to a marked sharpening of both the free and bound ^{13}C resonances and of the free proton resonance for $NADP^+$. The line width differences observed on the free $NADP^+$ resonances point strongly to an exchange process being the origin of this effect rather than differences in relaxation rates resulting from changes in correlation times or trace paramagnetic impurities. (The latter is unlikely in view of the lack of effect of EDTA.) Studies of other dihydrofolate reductases (for example, Perkins and Bertino, 1966; Hillcoat et al., 1967; Poe et al., 1974) have shown that the coenzyme binds more strongly in the ternary than in the binary complex. Thus in the present experiment if the dissociation rate of $NADP^+$ from the ternary complex is much less than that from the binary complex a decreased exchange contribution to the line width could result. If we assume that there is no exchange contribution to the line width for the ternary complex, then we can calculate the dissociation rate constant of the binary complex from the decrease in line width (17.5 Hz) produced by adding methotrexate. From eq 2 a value of $55 \pm 10\ sec^{-1}$ is obtained. Identical values, within experimental error, are obtained from analyzing the ^{13}C and ^1H line width changes of free $NADP^+$ (eq 4) lending support to this interpretation of the line width effects.

The equilibrium constant for the formation of the binary $NADP^+$–reductase complex is $\sim 2 \times 10^5\ M^{-1}$ (R. Bjur, unpublished work); combining this value with that for k_{-1} calculated above, we obtain $k_1 \sim 1.1 \times 10^7\ M^{-1}\ sec^{-1}$. This is only slightly less than that expected for a diffusion-limited reaction (Eigen and Hammes, 1963).

Kinetic studies of the reduction of dihydrofolate to tetrahydrofolate by *L. casei* dihydrofolate reductase have shown that the turnover rate for the enzyme under conditions similar to those of the NMR experiment, is 3 sec^{-1} (J. G. Dann and G. C. K. Roberts, unpublished work). Thus it is clear that the faster dissociation rate of the $NADP^+$–reductase binary complex cannot be the rate-limiting step in the overall reaction.

^{13}C Chemical Shifts of Bound [^{13}CO]NADP$^+$. Table I summarizes the chemical shift observed for bound [^{13}CO]$NADP^+$ in the various complexes studied; in all cases large shifts to high field (increasing shielding) relative to free $NADP^+$ are observed. At present it is not possible to identify the interaction(s) responsible for these shifts with any certainty, but their direction and magnitude allows us to eliminate some possibilities.

It is known that in solution some fraction of the $NADP^+$ molecules exist in a folded conformation in which the adenine and nicotinamide rings are stacked (Weber, 1957; Velick, 1961) whereas when bound to lactate dehydrogenase (Chandrasekhar et al., 1973) and probably also to alcohol dehydrogenase (Eklund et al., 1974) the coenzyme (NAD^+) is in an open, unstacked conformation. Previous studies of the ^{13}C spectrum of $NADP^+$ (Birdsall and Feeney, 1972) have shown that the unstacking at low pH is accompanied by a small (0.31 ppm) *downfield* shift of the carbonyl ^{13}C resonance. If such a change in the conformation of $NADP^+$ occurs on binding to dihydrofolate reductase, the accompanying chemical shift effects clearly do not make a major contribution to the total observed change in chemical shift.

Similarly, since a hydrogen bonding interaction between the carbonyl group of $NADP^+$ and a neutral donor (e.g., NH) on the enzyme would be expected to lead to a large (~5 ppm) *downfield* shift of the carbonyl resonance (Stothers, 1972), this interaction alone cannot explain the observed shifts.

One possibility for the observed upfield shifts is that they arise from aromatic ring currents: a 2 ppm upfield shift would result if the carbonyl carbon was placed ~3 Å (i.e., van der Waals contact) above the center of the plane of an aromatic ring. The large shift (4.3 ppm) seen in the ternary $NADP^+$–folate–reductase complex, however, cannot readily be explained in this way.

The most likely origin for the bound shifts lies in the effects of electric fields from nearby charged groups on the ^{13}C shielding. The C=O bond is easily polarizable, and thus large electric field shifts will be observed; the observed upfield shift would arise from the carbonyl group being oriented toward a positive charge or away from a negative charge (Buckingham et al., 1962; Batchelor, 1975; Batchelor et al., 1975). In this connection it is interesting to note that in the crystal structure of lactate dehydrogenase His-195 is close to the carbonyl group of NAD^+ in the binary

complex, and moves close enough to form a hydrogen bond in the ternary complex (Adams et al., 1973).

For the $NADP^+$–reductase–folate system at least two ^{13}C signals from bound species are observed. This striking observation appears to indicate the existence of two forms of the ternary complex in slow exchange with each other and with free folate. It is difficult to estimate the precise proportions of the complexes in the mixture because of relaxation time differences but the ratio cannot be far from one (see Figure 2d). Stopped-flow kinetic studies of ternary complexes with substrates have also given evidence for the existence of more than one form of the ternary complex which interconvert slowly (R. W. King and G. C. K. Roberts, unpublished work). From the NMR spectrum, the rate of interconversion must be less than 65 sec^{-1}; the kinetic experiments indicate that it is much slower than this.

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