Use of Transferred Nuclear Overhauser Effect Measurements To Compare Binding of Coenzyme Analogues to Dihydrofolate Reductase†

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ABSTRACT: Transferred nuclear Overhauser effect measurements have been made on complexes of $NADP^+$ and thio-$NADP^+$ with *Lactobacillus casei* dihydrofolate reductase to provide information about the glycosidic bond conformations in these complexes. Both $NADP^+$ and thio$NADP^+$ are shown to have very similar anti conformations about their adenine glycosidic bonds when bound to the enzyme. However, their nicotinamide glycosidic bond conformations are very different: while $NADP^+$ binds in an exclusively anti conformation, thio$NADP^+$ binds with a distribution of syn/anti conformations very similar to that observed in nicotinamide mononucleotides in free solution (~50:50). Thus for thio$NADP^+$, binding to the enzyme does not significantly perturb the potential function for rotation about the nicotinamide glycosidic bond. Earlier NMR studies [Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980) *Biochemistry 19*, 3738] had indicated that large downfield ^1H shifts of the nicotinamide ring protons (0.61–1.36 ppm) are detected on binding $NADP^+$ while only very small shifts (<0.1 ppm) are observed in complexes with thio$NADP^+$. The chemical shift and conformational findings are best explained if the thionicotinamide ring extends into solution making essentially no contacts with the enzyme.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate by using NADPH[1] as coenzyme. It is of considerable pharmacological interest, being the target for antifolate drugs such as trimethoprim (antibacterial) and methotrexate (antineoplastic). Considerable efforts are being made to determine the conformation of substrates, inhibitors, and coenzyme in their complexes with the enzyme, by using X-ray crystallography (Matthews et al., 1977, 1978, 1979, Baker et al., 1981; Filman et al., 1982; Volz et al., 1982) and NMR spectroscopy (Feeney et al., 1975; Cayley et al., 1979, 1980; Albrand et al., 1979; Hyde et al., 1980a,b; Gronenborn et al., 1981b). In our recent studies of the binding of coenzyme analogues to *Lactobacillus casei* dihydrofolate reductase, we noted that the changes in chemical shift of the nicotinamide protons on binding were very different for $NADP^+$ and its thionicotinamide analogue thio$NADP^+$ (Hyde et al., 1980a). Large downfield chemical shifts (0.61–1.36 ppm) accompanied the binding of $NADP^+$, whereas for thio$NADP^+$ the largest chemical shift change was only 0.1 ppm. These observations indicate that in these two coenzymes the nicotinamide ring binds to the enzyme in different ways. However, chemical shift information alone is insufficient to characterize the difference in mode of binding, and we have been exploring more direct methods for obtaining conformational information on these complexes.

In this paper, we report nuclear Overhauser effect (NOE) (Noggle & Schirmer, 1971) measurements aimed at determining the conformation about the glycosidic bond of coenzymes bound to dihydrofolate reductase. These measurements have been made by using the transferred NOE (TNOE) method we have described earlier (Albrand et al., 1979; Cayley et al., 1979; Hyde et al., 1980a), in which NOE effects between nuclei in a ligand bound to the enzyme are transferred to the more easily detected nuclei in excess free ligand by virtue of the exchange of ligand molecules between the bound and free states. In these experiments the negative ^1H–^1H NOE effects (Balaram et al., 1972) arising from nuclei in close proximity in the enzyme–ligand complex are readily distinguished from the positive NOE effects encountered in small molecules. TNOE effects can also be detected when the ligand exchanges sufficiently rapidly between free and bound states for averaged resonances to be observed (Bothner-By & Gassend, 1972; Clore & Gronenborn, 1982).

Experimental Procedures

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as previously described (Dann et al., 1976). $NADP^+$ and thio$NADP^+$ were from Sigma. Samples for NMR contained approximately 1 mM enzyme and 5 mM coenzyme in 0.4 mL of ^2H_2O with 500 mM KCl and 50 mM potassium phosphate, pH* 6.5 (pH* indicates a meter reading uncorrected for the isotope effect on the glass electrode). For NOE measurements, a selective radio-frequency pulse was applied for 0.5–1.5 s, followed by a 5-ms delay and the nonselective observation pulse. The selective irradiation was applied at 10–20-Hz intervals over a wide spectral range, covering the ribose and aromatic proton resonances of the coenzyme. The changes in intensity were measured from changes in peak heights. Quadrature detection was employed, with a spectral width of 4.2 kHz and an acquisition time of 0.975 s; 200–2000 transients were averaged for each spectrum. The ^1H chemical shifts are referenced to dioxane used as an internal reference: downfield shifts are given positive values.

Nomenclature. The structure of $NADP^+$ is given in Chart I. Protons on the nicotinamide side of the molecule are labeled with the prefix N and those on the adenine side with the prefix A. Thus the nicotinamide aromatic protons are labeled N2, N4, N5, and N6, and the nicotinamide ribose protons are labeled N1′, N2′, N3′, N4′, N5′, and N5″. Likewise, the adenine aromatic protons are labeled A2 and A8, and the adenine ribose protons are A1′, A2′, A3′, A4′, A5′, and A5″.

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[1] Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; thio-$NADP^+$, thionicotinamide adenine dinucleotide phosphate; NOE, nuclear Overhauser enhancement; TNOE, transferred nuclear Overhauser enhancement; NMR, nuclear magnetic resonance.

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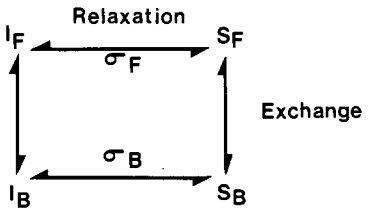
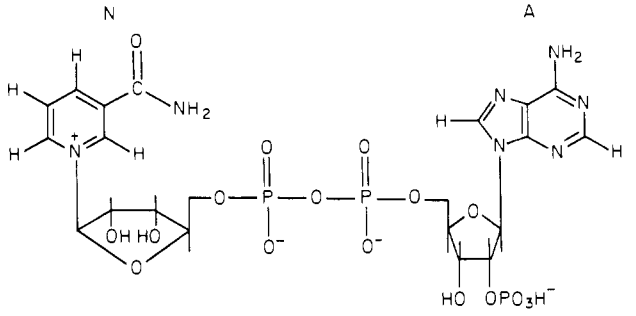


FIGURE 1: Scheme showing exchange and relaxation processes relating magnetization of two nuclei I and S in a ligand binding to a protein. The two nuclei interconvert between their bound and free states by exchange.

Chart I



Theory

The ^1H–^1H nuclear Overhauser effect is expressed as $f_I(S)$, the fractional change in intensity of the resonance of proton I when that of proton S is saturated. It is given by (Noggle & Schirmer, 1971)

$$f_I(S) = \sigma_{IS}/\rho_I \quad (1)$$

where σ_{IS} is the rate of cross-relaxation between protons I and S and ρ_I is the total spin–lattice relaxation rate of proton I. Positive $f_I(S)$ indicates an increase in intensity.

In the present case, I and S are protons on a ligand that is exchanging between two states: bound to the protein and free in solution (denoted by subscripts B and F, respectively). This situation is shown schematically in Figure 1. Now, if the rate of exchange of the ligand between the two states is greater than the difference in proton relaxation rates between them, protons I and S will each be characterized by relaxation rates that are the weighted average of those in the two states. The NOE effect is thus (Noggle & Schirmer, 1971; Clore & Gronenborn, 1982)

$$f_I(S) = \frac{X_F\sigma_{IS,F} + X_B\sigma_{IS,B}}{X_F\rho_{I,F} + X_B\rho_{I,B}} \quad (2)$$

where X_B and X_F are the mole fractions of the ligand in the bound and free states. This equation will hold whether separate or averaged resonances are observed for protons I and S in the two states, provided only that the exchange rate is faster than the relaxation rates.

Equation 2 can be simplified in cases where $\sigma_{IS,F} = 0$ (i.e., no NOE effect is observed for the ligand in the absence of the protein). For proteins, assuming $\sigma_{IS,B} \neq 0$ and $\rho_{I,B} \gg \rho_{I,F}$, eq 2 reduces to

$$f_I(S) = \sigma_{IS,B}/\rho_{I,B} \quad (3)$$

Now, if the effect on resonance I of irradiating the signal of another proton, T, is measured, $f_I(T)$ will be given by an equation analogous to eq 3. Then

$$f_I(S)/f_I(T) = \sigma_{IS,B}/\sigma_{IT,B} = r_{IT}^6/r_{IS}^6 \quad (4)$$

where the r_{ij}'s are internuclear distances (Noggle & Schirmer, 1971). Equation 4 holds provided that (i) the correlation time(s) of the I–S and I–T vectors are the same and (ii) indirect cross-relaxation effects are negligible. Thus, if the conditions for the applicability of eq 2–4 can be shown to hold, the TNOE method can provide quantitative conformational information about a ligand bound to a protein without the necessity for direct observation of the signals of the bound ligand.

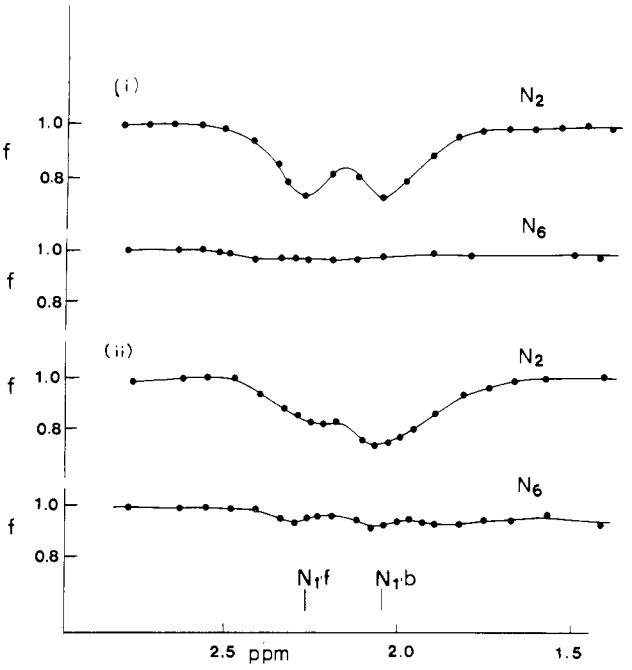


FIGURE 2: TNOE effects on N2 and N6 proton signals of $NADP^+$ in the presence of dihydrofolate reductase measured at (i) 20 °C and (ii) 3 °C. The fractional intensity (f) of the N2 and N6 signals is shown as a function of the frequency of the saturation pulse.

Results

The ^1H NMR spectra of complexes of $NADP^+$ or (at low temperature) thio$NADP^+$ with dihydrofolate reductase show separate signals for bound and free coenzyme. The positions of the resonances of the bound coenzyme can be identified by saturation transfer experiments (Hyde et al., 1980a). In these experiments a change in intensity of a resonance of the free ligand is observed following irradiation at the resonance frequency of the corresponding proton in the bound coenzyme. This irradiation can also produce smaller changes in intensity of the resonances of other protons of the free coenzyme, due to the transferred NOE effect.

An example, for the enzyme–$NADP^+$ complex, is shown in Figure 2. Selective irradiation at the resonance frequency of the N1′ proton of the free coenzyme leads to a decrease in the intensity of the free N2 proton signal due to the TNOE effect. The mechanism is as follows (cf. Figure 1): saturation of the free N1′ proton resonance produces a decrease in intensity of the bound N1′ proton signal by transfer of saturation; this in turn causes a decrease in the bound N2 proton signal due to a negative NOE effect, and this effect is finally transferred to the resonance of the free N2 proton by the exchange process.

At 20 °C, the resonance of the N2 proton of free $NADP^+$ shows TNOE effects of almost equal magnitude (fractional intensity changes of −0.29 and −0.32) when the irradiation is centered on the N1′(free) and N1′(bound) proton resonance frequencies, respectively (see Figure 2). Thus, with reference to Figure 1, the observed effect is the same for the pathway $S_F \rightarrow S_B \rightarrow I_B \rightarrow I_F$ as for the pathway $S_B \rightarrow I_B \rightarrow I_F$, where

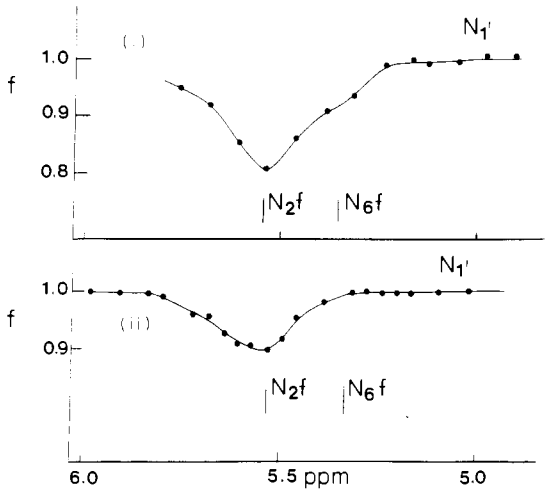


FIGURE 3: TNOE effects on N1′ proton signals of NADP+ in the presence of dihydrofolate reductase measured at (i) 20 °C and (ii) 3 °C.

N1′ ≡ S and N2 ≡ I. It follows that the rate of exchange of the coenzyme between the two states must be much faster than the relaxation rate of the N1′ proton in the bound state—a condition for the applicability of eq 2 to this system. The estimated dissociation rate constant of NADP+ from this complex is 29 s^{-1} at 20 °C (calculated from measured values of 23 s^{-1} at 11 °C and 33 s^{-1} at 25 °C; Hyde et al., 1980a,b). By contrast, at 3 °C, where the estimated dissociation rate constant is 18 s^{-1}, irradiation at the resonance position of N1′(free) has a distinctly smaller effect on the intensity of the N2(free) signal than irradiation at the N1′(bound) resonance frequency. At this temperature, therefore, eq 2 is not applicable. [The usefulness of this criterion for the validity of eq 2 in systems where separate signals are observed for the bound and free ligand is confirmed by the calculations reported by Clore & Gronenborn (1982).]

While saturation of the N1′ proton resonances has a substantial effect on the intensity of the N2 proton signal [Figure 2; $f_{N2}(N1') = -0.32$ at 20 °C], it has very much less effect on the N6 proton resonance [$f_{N6}(N1') < -0.04$ at 20 °C]. This clear selectivity immediately rules out a nonspecific cross-relaxation ("spin-diffusion") mechanism for these effects. A similar selectivity is seen in the converse experiment, shown in Figure 3; irradiation at the N2(free) proton resonance frequency leads to a decrease in intensity of the N1′(free) signal, but irradiation at the N6(free) frequency does not.

When similar NOE measurements were made on NADP+ in the absence of the enzyme, only very small effects were observed: $f_{N1'}(N2)$ and $f_{N1'}(N6)$ were both <+0.02 at 20 and 3 °C. These negligible NOE effects in the free state allow us to use the simplified analysis outlined under Theory and justify the use of eq 3. The additional conditions concerning correlation times and indirect cross-relaxation effects are also satisfied. For the NADP+–enzyme complex, the correlation times for the N2–N1′ and N6–N1′ vectors will obviously be very similar. The observed NOE effects on N2 and N6 from irradiation of N1′ and N5′ and N5″ are very selective: no significant indirect cross-relaxation involving other nuclei was observed.

Nicotinamide Glycosidic Bond Conformation. The distances of the N1′ proton from the N2 and N6 protons depend on the dihedral angle about the glycosidic bond, as seen in Chart II. It is clear from the results shown in Figure 3 that $f_{N1'}(N2) \gg f_{N1'}(N6)$ for NADP+ at both 20 and 3 °C. Since eq 3 has been shown to be applicable, this indicates qualitatively that the conformation of NADP+ bound to dihydrofolate reductase is of the anti type shown in Chart II. This is confirmed by the observation of a substantial TNOE on the N6 proton resonance on irradiating at the frequency of the N5′ and *N* N5″ signals (Table I). Quantitative measurement of $f_{N1'}(N2)$ is straightforward, but at 20 °C there is some overlap in the resonances of N2 and N6, making an accurate estimate of $f_{N1'}(N6)$ difficult. It is clear from the results at 3 °C where the resonances for the free coenzyme are much sharper, due to a decreased exchange contribution (Hyde et al., 1980a), that $f_{N1'}(N6)$ is very small, but at 20 °C, we can only set an upper limit of 0.03. Thus

$$\sigma_{N1'N2,B}/\sigma_{N1'N6,B} = r_{N1'N6}^6/r_{N1'N2}^6 > 7$$

Thus $r_{N1'N6}/r_{N1'N2} > 1.38$, and χ (the dihedral angle about the glycosidic bond, defined with $\chi = 0°$ when C2–N1–C1′–O4′ are syn planar) must be within the range 190–280°. This encompasses the usual range of anti conformations ($\chi \sim 250°$), and the absence of a precise value for the very small TNOE between N1′ and N6 means we can only say that the conformation about this bond is somewhere in the anti range. However, the results indicate that no significant amounts of the syn conformation are present.

Chart II

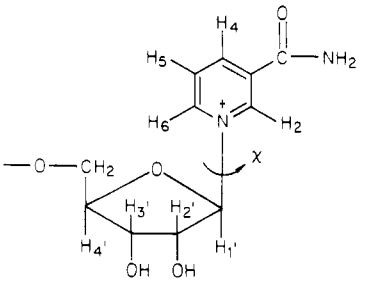


Table I: TNOE Effects for NADP+ and ThioNADP+ in Their Complexes with Dihydrofolate Reductase[a]

obsd nucleus	irradiated nucleus	fractional NOE values: NADP+ 20 °C	NADP+ 3 °C	thioNADP+ 3 °C
N1′	N2(free)	−0.21	−0.10	−0.14
N1′	N2(bound)			−0.14
N1′	N6(free)	<−0.03	<−0.02	−0.13
N1′	N6(bound)			−0.13
N2	N1′(free)	−0.29	−0.17	−0.17
N2	N1′(bound)	−0.32	−0.26	
N6	N5′ and N5″	−0.28		
N6	N5 (free)			−0.16
N6	N1′(free)	<−0.04	−0.04	−0.08
A8	N3′(free)	−0.18		−0.26
A8	N5′(free) and N5″(free)	−0.31		−0.38

[a] Fractional NOE values accurate to ±0.02.

The TNOE results obtained with the thioNADP+–enzyme complex are quite different from those obtained with the NADP+–enzyme complex. The effects of irradiating at the N1′(free) proton frequency on the N2(free) and N6(free) signals in the two complexes are compared in Figure 4, and the TNOE results for thioNADP+ are shown in more detail in Figure 5. It is apparent that *both* N2 and N6 are affected by irradiation at N1′ in the thioNADP+ complex.

Because of the smaller chemical shift differences between the bound and free states for the protons of thioNADP+, we cannot use the same test for the applicability of eq 2 as for NADP+. However, since the dissociation rate constant of

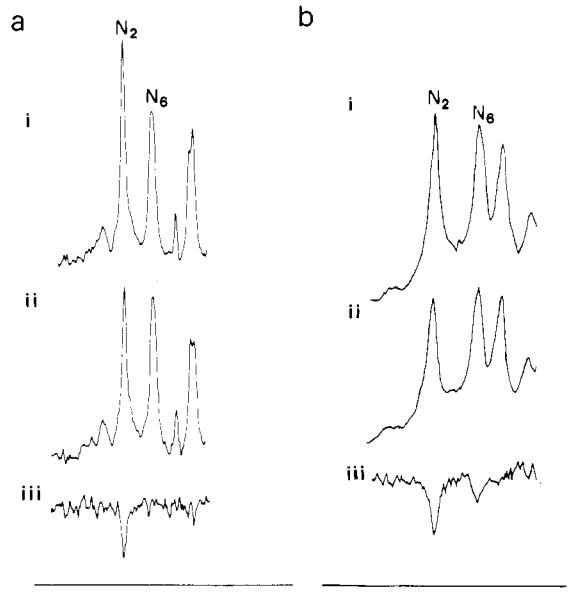


FIGURE 4: (a) (i) Part of the aromatic region of the ^1H NMR spectrum (270 MHz) of $NADP^+$ in the presence of dihydrofolate reductase (5 equiv of $NADP^+$/1 equiv of enzyme) with irradiation 230 Hz from the frequency of N1′ in free $NADP^+$. (ii) As in (i) but with irradiation at the frequency of N1′ in free $NADP^+$. (iii) The difference between (i) and (ii). (b) (i) Part of the aromatic region of the ^1H NMR spectrum (270 MHz) of thio$NADP^+$ in the presence of dihydrofolate reductase (5 equiv of thio$NADP^+$/1 equiv of enzyme) with irradiation 190 Hz from the frequency of N1′ in free thio$NADP^+$. (ii) As in (i) but with irradiation at the frequency of N1′ in free $NADP^+$. (iii) The difference between (i) and (ii).

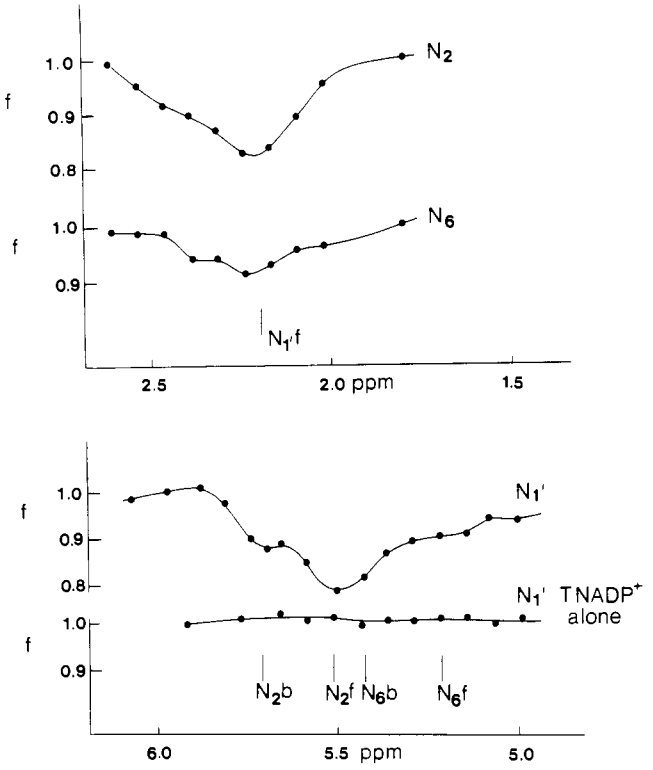


FIGURE 5: TNOE effects on N2, N6, and N1′ proton signals of thio$NADP^+$ in the presence of dihydrofolate reductase at 3 °C. The absence of NOE effects on N1′ when no enzyme is present is also shown.

thio$NADP^+$ from the complex is an order of magnitude greater than that of $NADP^+$ (Hyde et al., 1980b), we can be confident that this equation is indeed valid even at 3 °C. At this temperature, no detectable NOE effects were observed between

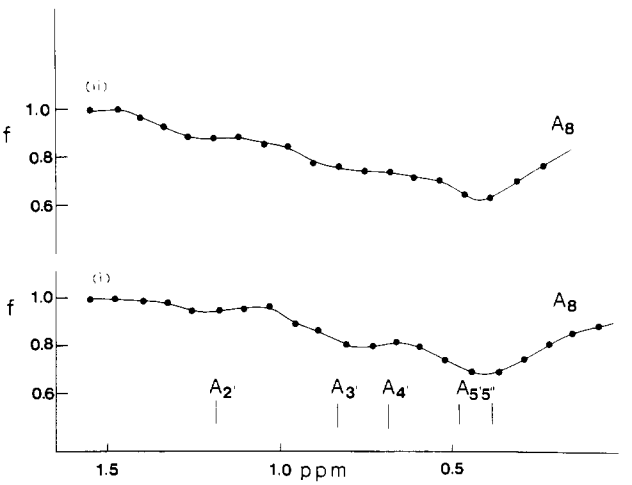


FIGURE 6: TNOE effects on A8 for (i) $NADP^+$ (at 20 °C) and (ii) thio$NADP^+$ (at 3 °C) in the presence of dihydrofolate reductase.

sugar protons and base protons for thio$NADP^+$ in the absence of enzyme (Figure 5). Therefore, eq 4 can be used for a quantitative analysis of the TNOE effects on thio$NADP^+$ summarized in Table I.

The TNOE effects can be "calibrated" in terms of internuclear distances by measuring the TNOE between two protons (such as N5 and N6) whose separation is independent of conformation. Thus

$$\frac{f_{N6}(N5)}{f_{N6}(N1')} = \frac{\sigma_{N6N5,B}}{\sigma_{N6N1',B}} = \frac{r_{N6N1'}{}^6}{r_{N6N5}{}^6} = 2.00$$

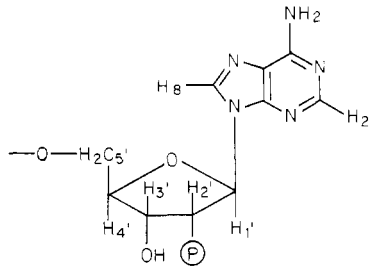
Since $r_{N6N5} = 2.48$ Å, this gives a value for $r_{N6N1'}$ of 2.78 Å, assuming that there is only a single conformation about the glycosidic bond of thio$NADP^+$ in the complex with the enzyme. Similarly

$$\frac{f_{N1'}(N2)}{f_{N1'}(N6)} = \frac{\sigma_{N1'N2,B}}{\sigma_{N1'N6,B}} = \frac{r_{N1'N6}{}^6}{r_{N1'N2}{}^6} = 1.15$$

and taking $r_{N6N1'} = 2.78$ Å, we calculate $r_{N2N1'} = 2.71$ Å. However, there is no single conformation about the glycosidic bond that is simultaneously consistent with both these distances. [With an estimated precision of ±0.02 in $f_I(S)$, the calculated distances have a precision of better than 0.02 Å.] It is therefore necessary to postulate a mixture of syn and anti conformations about this bond in bound thio$NADP^+$. For example, a mixture of 54% syn ($\chi = 60°$) and 46% anti ($\chi = 250°$) conformations would be entirely consistent with the observed TNOE effects. Similar mixtures of approximately equal amounts of syn and anti conformations about the nicotinamide glycosidic bond have been postulated for free nicotinamide mononucleotide (Egan et al., 1975) and for free NAD^+ (Zens et al., 1976).

Adenine Glycosidic Bond Conformation. In the complexes of both $NADP^+$ and thio$NADP^+$ with the enzyme, the A8 proton resonance shows large TNOE effects on irradiation at the resonance frequencies of the A2′, A3′, and A5′ and A5″ protons. As shown in Figure 6, these effects are closely similar in the two complexes. The ribose proton resonances of the free and bound coenzyme are insufficiently well resolved for us to quantitate the TNOE effects from individual ribose protons, but for both $NADP^+$ and thio$NADP^+$, the maximum effect on the A8 signal is observed when the irradiation is centered on the frequency of the A5′(free) and A5″(free) signals. Saturation of the A1′(free) resonance did not affect the intensity of the A8 signal. These observations indicate that both $NADP^+$ and thio$NADP^+$ have a similar anti conformation

Chart III



(Chart III) about their adenine glycosidic bonds when they are bound to dihydrofolate reductase.

Discussion

From our previous NMR studies of the binding of coenzyme analogues to dihydrofolate reductase (Hyde et al., 1980a,b; Gronenborn et al., 1981a), it appears that structural changes in the nicotinamide moiety, while producing marked changes in the mode of binding of the nicotinamide ring, do not perturb the adenosine ring binding site. Thus in the case of thioNADP$^+$, the changes in chemical shift of the A2, A8, and A1′ proton resonances and of the 2′-phosphate ^{31}P resonance on binding are very similar to those of NADP$^+$. The present TNOE observations show that the conformation about the adenine glycosidic bond is the same in the two complexes and are thus entirely consistent with the idea that structural changes in the nicotinamide ring do not affect the mode of binding of the adenine end of the coenzyme.

By contrast, the chemical shift changes produced by binding are very much smaller for the nicotinamide protons of thioNADP$^+$ than for those of NADP$^+$ (<0.1 ppm for thioNADP$^+$ vs. 0.61–1.36 ppm for NADP$^+$; Hyde et al., 1980a). The nicotinamide ring of NADP$^+$ must be bound to a site and in an orientation in which its protons experience strong deshielding fields from local magnetic anisotropies. In the crystal structure of the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979; Filman et al., 1982), a number of carbonyl groups are found to be close to the nicotinamide ring, and these could be responsible for a large part of the shifts observed. The nicotinamide proton shifts are not the same for NADPH as for NADP$^+$ (Hyde et al., 1980a), indicating that the exact position or orientation of the oxidized nicotinamide ring in the binding site differs somewhat from that seen in the crystal even though a similar anti conformation is found in each case. The amide group on the nicotinamide ring of NADPH forms three hydrogen bonds to the enzyme; in particular, the carbonyl oxygen hydrogen bonds to the backbone NH of Ala-6 (Filman et al., 1982). These interactions are probably involved in stabilizing the anti conformation. If NADP$^+$ forms similar hydrogen bonds, then, when the amide carbonyl oxygen is replaced by sulfur, in thioNADP$^+$, such hydrogen bonds will be substantially weakened. This could be responsible for the marked change in the mode of binding of the nicotinamide ring as evidenced by the bound proton chemical shifts.

The chemical shifts alone do not provide unambiguous information as to the nature of this change. Magnetic anisotropy effects on chemical shifts are strongly dependent on geometry, so that a simple change in orientation of the ring within the binding site might suffice to explain the markedly smaller shifts in thioNADP$^+$. Alternatively, the thionicotinamide ring might occupy a quite different site on the enzyme from the nicotinamide ring, as observed crystallographically for the binding of other coenzyme analogues to alcohol dehydrogenase (Samama et al., 1977). Finally, it is conceivable that the thionicotinamide ring is simply displaced from the enzyme and is not interacting with it at all.

In addition to the differences in nicotinamide proton chemical shifts, the ^{31}P resonances of the pyrophosphate moiety of thioNADP$^+$ bound to the enzyme have different chemical shifts from those seen with NADP$^+$ (Hyde et al., 1980b). The ^{31}P chemical shift changes cannot be quantitatively interpreted but are likely to reflect changes in conformation about P–O bonds (Gronenborn et al., 1981b, and references cited therein). Since we have established that the adenine end of the coenzyme binds in the same way in both NADP$^+$ and thioNADP$^+$, this suggestion of a difference in conformation of the pyrophosphate between the two makes it likely that their mode of binding differs by more than just the orientation of the nicotinamide ring in its binding pocket.

The TNOE results reported here, which show that thioNADP$^+$ bound to the enzyme exists as a mixture of conformations about the glycosidic bond, in contrast to the anti conformation of NADP$^+$, certainly indicate a substantial alteration in the nicotinamide ring environment. In fact, the distribution of conformations about this bond in the enzyme–thioNADP$^+$ complex appears to be very similar to that in nicotinamide mononucleotide (NMN$^+$) in free solution, studied by Egan et al. (1975). The calculated apparent internuclear distance ratio $r_{N1'N6}/r_{N1'N2}$ is 0.99 for free NMN$^+$ and 1.02 for bound thioNADP$^+$, and in both cases a distribution involving roughly equal amounts of syn and anti conformations is the simplest that will fit the data. This suggests that binding to the enzyme has led to little perturbation of the potential function for rotation about this glycosidic bond, which is most easily explained if the thionicotinamide ring is in fact extending into solution, making essentially no contacts with the enzyme.

This single-atom structural change in the coenzyme thus produces a striking effect on its mode of binding. This is accompanied by only a 4.4-fold decrease in the binding constant (Birdsall et al., 1980). The implication that the oxidized nicotinamide ring makes only a marginal contribution to the binding energy of NADP$^+$ is supported by the observation that an analogue in which the entire nicotinamide ring has been replaced by a methoxy group binds virtually as tightly as NADP$^+$ (Birdsall et al., 1980).

Acknowledgments

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Registry No. NADP$^+$, 53-59-8; thioNADP$^+$, 19254-05-8; dihydrofolate reductase, 9002-03-3.

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Purification and Characterization of Dihydrofolate Reductase from Methotrexate-Resistant Human Lymphoblastoid Cells†

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ABSTRACT: Dihydrofolate reductase has been isolated from methotrexate-resistant WIL2 human lymphoblastoid cells. This subline produces ca. 150 times more enzyme than the parental drug-sensitive line. The reductase has been purified to homogeneity by methotrexate affinity chromatography, gel filtration, and preparative isoelectric focusing in a yield of 65%. The enzyme has a pI = 7.7 and a molecular weight of ca. 22000. The amino-terminal 27 amino acid residues have been determined, revealing the location of the single cysteine residue at position 6. Reaction of this cysteine with *p*-(hydroxymercuri)benzoate in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) results in a 5-fold increase in enzyme activity. Other agents including KCl, urea, and thiourea also cause enzyme activation. The K_m values for NADPH and dihydrofolate are 0.25 and 0.036 μM, respectively. Mercurial activation of the enzyme results in a 27-fold increase in the K_m for NADPH and a 35-fold increase in the K_m for dihydrofolate.

Dihydrofolate reductase (EC 1.5.1.3) (DHFR)[1] catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The latter serves as a coenzyme for a number of one-carbon transfer reactions in purine and pyrimidine biosynthesis, including that of thymidylate (Blakely, 1969; Huennekens et al., 1971). The reductase appears to be the major intracellular receptor for the action of 4-amino analogues of folic acid, such as methotrexate. Inhibition of the enzyme by methotrexate depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis. Thus, methotrexate has been employed extensively in the chemotherapeutic treatment of leukemias, lymphomas, psoriasis, and other clinical disorders (Bertino & Johns, 1972).

Dihydrofolate reductase has been purified and characterized from a number of bacterial and animal sources, including murine L1210 cells (Perkins et al., 1967; Reyes & Huennekens, 1967), porcine liver (Smith et al., 1979), bovine liver (Rowe & Russel, 1973; Kaufman & Kemerer, 1976), and chicken liver (Kaufman & Gardiner, 1966; Kaufman & Kemerer, 1977). Species differences are apparent in the properties of the various dihydrofolate reductases.

Despite the importance of dihydrofolate reductase as a target for chemotherapy of human neoplasms, there have been few studies of the human enzyme. Dihydrofolate reductase from human placenta (Jarabak & Bachur, 1971) has a molecular weight of 20000–22000, has two pH optima, and is activated by potassium chloride and by urea. The human enzyme has also been isolated from acute myelogenous and

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[1] Abbreviations: MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TKEM, 50 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol, pH 7.0; NaDodSO$_4$, sodium dodecyl sulfate; MeHgOH, methylmercuric hydroxide; pHMB, *p*-(hydroxymercuri)benzoate; FAH$_2$, dihydrofolic acid; DHFR, dihydrofolate reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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