catechol, 120-80-9; resorcinol, 108-46-3; *p*-hydroquinone, 123-31-9; *o*-toluidine, 95-53-4; heme, 14875-96-8; sulfate, 14808-79-8.

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Conformations of Nicotinamide Coenzymes Bound to Dehydrogenases Determined by Transferred Nuclear Overhauser Effects†

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ABSTRACT: Transferred nuclear Overhauser enhancement was used to examine the conformation of NAD^+ and $NADP^+$ bound to glucose-6-phosphate dehydrogenase and glutamate dehydrogenase and of NAD^+ bound to lactate dehydrogenase. The results demonstrate that the conformation of the nicotinamide–ribose bond is anti for dehydrogenases with A stereospecificity and syn for dehydrogenases with B stereospecificity. In those dehydrogenases that bind both NAD^+ and $NADP^+$, significant differences occur in the conformations of the bound nicotinamide coenzymes.

Dehydrogenases catalyze the stereospecific transfer of hydrogen atoms between their substrates and the coenzymes NAD or NADP. These enzymes fall into two classes, A stereospecific and B stereospecific, depending on whether the hydrogen transfer involves the *pro-R* or the *pro-S* hydrogen of the reduced nicotinamide coenzyme, respectively (You, 1982). X-ray crystallographic studies on NAD-utilizing dehydrogenases have shown that in alcohol dehydrogenase, malate dehydrogenase, and lactate dehydrogenase, all of which are A stereospecific, the conformation of the nicotinamide–ribose bond of bound NAD^+ is anti, whereas in glyceraldehyde-3-phosphate dehydrogenase, the only B-stereospecific enzyme examined, this conformation is syn (Rossmann et al., 1975) (Chart I). The NADPH-utilizing dihydrofolate re-

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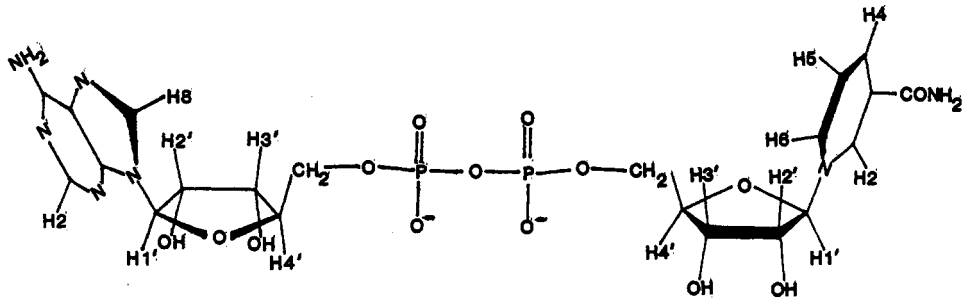
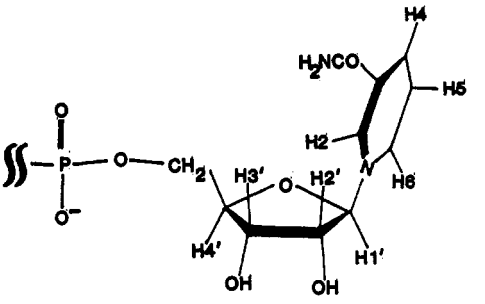
0006-2960/83/0422-2792$01.50/0

Chart I





ductase is also A stereospecific (You, 1982), and the conformation of the nicotinamide–ribose bond in the enzyme-bound NADPH is anti (Matthews et al., 1979). You et al. (1978) suggested that the hydrogen transfer stereospecificity of dehydrogenases is the direct result of this difference in conformation of the nicotinamide–ribose bond in enzyme-bound coenzymes. As glyceraldehyde-3-phosphate dehydrogenase provides the only example of an enzyme for which the syn conformation has been observed, it is clearly desirable to examine other enzymes which can be tested for the suggestion of You et al. (1978).

Dehydrogenases are also distinguished by their coenzyme specificity. Despite their great structural similarity, NAD and NADP serve quite different metabolic roles in all forms of life. This difference is reflected in the fact that the great majority of dehydrogenases are either NAD or NADP specific. A few dehydrogenases, however, utilize both nicotinamide coenzymes. For these enzymes, it is of interest to determine what factors regulate their reactivity with NAD and NADP. In this connection, it is important to examine differences in the conformations of the enzyme–coenzyme complexes. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* reacts with both NAD^+ and $NADP^+$, and these two coenzymes induce different conformational changes in the enzyme, as monitored by using three different fluorescent probes (Haghighi & Levy, 1982). It is not known whether the conformations of the two bound coenzymes also differ.

Although structure elucidations based on NMR results are not as accurate as those obtained from X-ray studies, the NMR technique has advantages of greater speed and lower cost. Further, NMR uniquely allows determination of conformations in solution (Jardetzky & Roberts, 1981). Transferred nuclear Overhauser enhancement (TRNOE) is one of several NMR techniques which have recently been used to investigate interactions of proteins with their ligands (Balaram et al., 1972; Bothner-By & Gassend, 1973; James, 1976; Cayley et al., 1979; Gronenborn et al., 1981; Clore et al., 1981; Gronenborn & Clore, 1982; Clore & Gronenborn, 1982; Feeney et al., 1983). Several A-stereospecific enzymes have been studied by using TRNOE. Gronenborn & Clore (1982) found that the conformation of the nicotinamide–ribose bond is anti in NAD^+ bound to alcohol dehydrogenases from yeast and liver, and Feeney et al. (1983) showed similar results for $NADP^+$ bound to dihydrofolate reductase. No studies have been reported yet in which TRNOE measurements have been used to compare the conformations of bound nicotinamide coenzymes in A- and B-stereospecific dehydrogenases or to compare the conformations of NAD^+ and $NADP^+$ bound to dehydrogenases that can utilize both coenzymes. The results of such studies are the subject of this report.

Materials and Methods

Glucose-6-phosphate dehydrogenase (G6PD) from *L. mesenteroides* was obtained from Worthington Biochemicals and shown to be homogeneous by polyacrylamide gel electrophoresis. Rat liver glutamate dehydrogenase (GDH) was purified to homogeneity and kindly supplied by Dr. J. Ellis Bell of the University of Rochester. Beef heart lactate dehydrogenase (LDH), NAD^+, and $NADP^+$ were obtained from Sigma Chemical Co. D_2O, 99.8 atom % D, was from Aldrich, and D_2O, 100% D, low in paramagnetic impurities, was from Stohler Isotope Chemicals. The enzymes were dialyzed extensively against buffers prepared in 99.8% D_2O and, finally, against buffer in 100% D_2O. Buffers were chosen in which dissociation constants for coenzymes had been determined: 0.03 M potassium phosphate, pH 7.6, for glucose-6-phosphate dehydrogenase (Haghighi & Levy, 1982), 0.11 M potassium phosphate, pH 7.0, containing 10 μM ethylenediaminetetraacetate for glutamate dehydrogenase (Dalziel & Egan, 1972), and 0.1 M potassium phosphate, pH 6.8, for lactate de-

Table I: TRNOE Measurements for Nicotinamide Coenzymes Bound to Dehydrogenases

irradiated resonance	obsd resonance	TRNOE (%)[a] G6PD[b]–NADP$^+$	G6PD[b]–NAD$^+$	PxyG6PD[c]–NAD$^+$	GDH[d]–NADP$^+$	GDH[d]–NAD$^+$	LDH[e]–NAD$^+$
H_N1'	H_N2	6	6	4	~5	6	23(A)
H2′	H_N2	-	0	0	0	*g*	0
H3′	H_N2	13(S)	21(S)	11(S)	25(S)	20(S)	0
H5′,5″[f]	H_N2	0	8	0	~12	18	0
H_N1'	H_N6	22(S)	22(S)	20(S)	19(S)	25(S)	<5
H2′	H_N6	-	0	0	0	0	<5
H3′	H_N6	~5	11(A?)	0	10	0	16(A)
H5′,5″[f]	H_N6	0	0	0	<5	0	0
H_A1'	H_A8	0	<5	0	<5	0	0
H2′	H_A8	21	33	19	22	25	18
H3′	H_A8	<5	20	0	<5	0	5
H5′,5″[f]	H_A8	9	10	9	~12	0	<5

[a] 0, no TRNOE observed; -, not measured; (S) and (A) indicate syn and anti conformations, respectively. All NOE values are negative. [b] G6PD = *L. mesenteroides* glucose-6-phosphate dehydrogenase. For the NAD$^+$ experiment, enzyme concentration = 1.0 mM (all enzyme concentrations are stated in terms of the concentration of subunits), and ratio of free to bound coenzyme = 10, based on K_D = 7.2 mM. For the NADP$^+$ experiment, enzyme concentration = 0.20 mM, and ratio of free to bound coenzyme = 13, based on K_D = 6.5 μM. [c] PxyG6PD = *L. mesenteroides* glucose-6-phosphate dehydrogenase covalently modified with phosphopyridoxyl groups (1.2 mol/mol of enzyme dimer). Enzyme concentration = 0.21 mM, and ratio of free to bound NAD$^+$ = 12, based on K_D = 0.79 mM. [d] GDH = rat liver glutamate dehydrogenase. For both experiments, enzyme concentration = 0.23 mM, and ratio of free to bound coenzyme = 15, based on K_D = 0.5 mM for E-NAD$^+$ and K_D = 2.5 mM for E-NADP$^+$. [e] LDH = beef heart lactate dehydrogenase. Enzyme concentration = 0.59 mM, and ratio of free to bound NAD$^+$ = 20, based on K_D = 0.32 mM. [f] 5′ and 5″ are the two C5′ protons which are indistinguishable as their resonances are superimposed. [g] A significant TRNOE was observed in one of two independent experiments.

hydrogenase (Takenaka & Schwert, 1956). The pH (actual reading of the pH meter, uncorrected for the isotope effect) of the final dialysis buffer upon completion of dialysis was 7.2 for glutamate dehydrogenase and 7.0 for lactate dehydrogenase. Coenzymes were dissolved in the same buffers as the enzymes, lyophilized to dryness, and redissolved in D_2O. This procedure was repeated twice more. These solutions were sufficiently concentrated that their addition to the enzyme diluted it less than 4%.

^{1}H NMR spectra were measured at 360 MHz in 5-mm cells (0.25–0.4 mL) on a Bruker WM-360 WB spectrometer at 22 °C. The residual solvent peak served as the reference chemical shift, which was assumed to be 4.8 ppm. Enzyme concentrations were 0.2–1.0 mM (concentrations of subunits). Coenzyme solutions in D_2O were added gradually, and spectra were examined to find the lowest free to bound coenzyme ratio with averaged signals that were sufficiently narrow; this ratio was generally 10–20. Temperature effects of NAD(P) proton shifts and the lack of observation of TRNOE from irradiation of multiple frequencies which would cover bound ligand resonances indicate that fast exchange was operative in all cases.

Typical TRNOE acquisition parameters were the following: 4K data points, acquisition time = 0.41 s, 800 scans. Repetitive cycling through free induction decays (FIDs) corresponding to different decoupling frequencies, each of 80 scans, suppressed degradation from long-term instabilities. During processing, the spectra were zero filled to 8K, and 5-Hz exponential line broadening was applied.

The theory of TRNOE measurements has been discussed by Clore & Gronenborn (1982). Usually TRNOE is measured by using the sequence $(D1–D2–PW–ACQ)_n$ where D1 is an optional relaxation delay, used to minimize spin-diffusion effects (the latter can give a nonspecific TRNOE due to the transmission of the NOE along a strongly coupled spin system), D2 is a period of selective proton irradiation when the NOE builds up, PW is the nonselective observing pulse, and ACQ is the period for acquisition of the FID (in which all protons cross-relaxing with the preirradiated proton will change their integral intensities). Clore and Gronenborn used a sequence where D1 = 0 and the decoupling period and acquisition time had comparable duration, 1.5 and 1.0 s (Gronenborn et al., 1981) or 0.5 and 0.5 s (Gronenborn & Clore, 1982; Clore & Gronenborn, 1982), respectively. We applied the latter parameters when complexes of glucose-6-phosphate dehydrogenase with NAD$^+$ and NADP$^+$ were investigated, but we found that substantial nonspecific TRNOE can occur. Variation of the parameters D1, D2, and DP (decoupler power) was systematically examined. It was established that in our case, nonspecific TRNOE can be effectively eliminated when D1 = 1.0 s, D2 = 0.4 s, and ACQ = 0.41 s. Optimum decoupling power can change for different enzyme concentrations and free to bound ratios of coenzymes (ratios of 10–40, in our study).

The NOE in a pure 2.4 mM solution of NAD$^+$ was measured to minimize the possibility of experimental error. Small and positive NOEs were found; e.g., $f_{H_A8}(H_A2') = +0.03$ or $f_{H_N2}(H_N1') = +0.04$.[1] Positive values of NOE confirm that the uncomplexed coenzyme fulfills the extreme narrowing condition. The observed NOEs were small because of the concentration of sample and the lack of degassing of the solution. TRNOE is detected by using difference spectroscopy, where a control spectrum irradiated outside of the spectral region is subtracted from the spectrum irradiated at a particular position to yield negative NOE values.

Results and Discussion

The TRNOE measurements are summarized in Table I. Two aspects of the results should be noted: differences among the dehydrogenases in the conformations of the nicotinamide–ribose bonds of the bound coenzymes; differences between the conformations of bound NAD$^+$ and bound NADP$^+$ in the two dehydrogenases that bind both coenzymes.

Irradiation of H_N1' (see Figure 1) gives a significant TRNOE on H_N6 for both coenzyme complexes of glucose-6-

[1] The protons are labeled with the subscript N or A to designate nicotinamide or adenine, respectively. Unprimed numbers indicate aromatic protons on the bases; primed numbers indicate ribose protons. When the subscript is omitted, the signals of ribose protons attached to the two bases are indistinguishable.

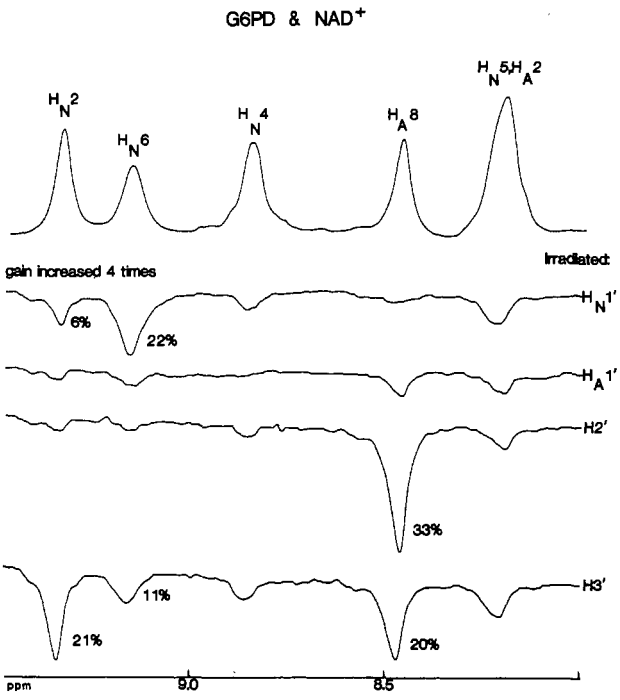


FIGURE 1: 360-MHz ^1H NMR spectra of aromatic protons from NAD^+ complexed with G6PD. The upper spectrum was obtained without preirradiation; the remaining traces show difference spectra (the control spectrum was irradiated at −1.0 ppm) corresponding to preirradiation of the sugar protons as indicated on the right-hand side of the figure. TRNOE values are shown.

phosphate dehydrogenase and glutamate dehydrogenase (Table I), indicating that these protons are proximate in the complex, but little or no TRNOE is observed for H_N2. Conversely, irradiation of H3′ gives a substantial TRNOE on H_N2 for these four complexes but little or none on H_N6. Precisely the opposite effect is seen for lactate dehydrogenase (Table I) and the alcohol dehydrogenases from yeast and horse liver (Gronenborn & Clore, 1982): the NAD^+ complexes of these enzymes show significant TRNOE values on the resonance of H_N2, but not on H_N6, when H_N1' is irradiated and, conversely, on H_N6 but not H_N2 upon irradiation of H3′. Similarly, there is a strong TRNOE between H_N1' and H_N2, but not H_N6, in the $NADP^+$ complex of dihydrofolate reductase (Feeney et al., 1983). These results demonstrate that the conformation of the nicotinamide–ribose bond is syn in the NAD^+ and $NADP^+$ complexes of glucose-6-phosphate dehydrogenase and glutamate dehydrogenase and anti in the NAD^+ complexes of lactate dehydrogenase and the two alcohol dehydrogenases, and in the $NADP^+$ complex of dihydrofolate reductase. The differences in conformation correlate with the known B stereospecificities of glucose-6-phosphate dehydrogenase and glutamate dehydrogenase and the A stereospecificities of alcohol dehydrogenase, lactate dehydrogenase, and dihydrofolate reductase (You, 1982). For *L. mesenteroides* glucose-6-phosphate dehydrogenase, the stereospecificity has only been verified for the reaction with NAD^+ (Arnold et al., 1976), and the glutamate dehydrogenase for which the stereospecificity for NAD^+ and $NADP^+$ was determined was not from rat liver (Levy & Vennesland, 1957; Nakamoto & Vennesland, 1960). It has been shown, however, that the stereospecificity for the same enzyme isolated from different sources is the same and that in those enzymes that utilize both NAD and NADP the stereospecificity is the same for both coenzymes (Bentley, 1970). Our results support the suggestion of You et al. (1978) that the stereospecificity of dehydrogenases is determined by the conformation of the nicotinamide–ribose bond in the bound coenzyme. As You et al. (1978) point out, the consequence of 180° rotation around the nicotinamide–ribose bond (anti and syn conformations) is that either the A or the B side of the nicotinamide ring faces the substrate.

Whereas the stereospecificity of H transfer between substrates and coenzymes in dehydrogenases is absolute (You, 1982), the TRNOE measurements indicate that there is a small contribution from the anti conformation of the nicotinamide–ribose bond in the coenzymes bound to glucose-6-phosphate dehydrogenase and glutamate dehydrogenase (Table I). This is most marked for the NAD^+–glucose-6-phosphate dehydrogenase complex. It may be significant, in this regard, that this complex displays the highest K_D among those examined in this study, K_D = 7.2 mM (Haghighi & Levy, 1982). During catalysis, however, NAD^+ binding to glucose-6-phosphate dehydrogenase is greatly enhanced, as suggested by fluorescence measurements (Grove et al., 1976; Haghighi & Levy, 1982) and by the fact that the apparent K_m for NAD^+ is decreased by increasing glucose 6-phosphate concentrations, with the K_m being 70-fold lower than the K_D (Olive et al., 1971; Haghighi & Levy, 1982). This tightened binding, if it involves the nicotinamide region of the coenzyme, could result in more restricted rotation about the nicotinamide–ribose bond. Support for this idea comes from TRNOE measurements made with the NAD^+ complex of glucose-6-phosphate dehydrogenase modified by the covalent attachment of a phosphopyridoxyl group at a unique lysine residue of the enzyme. This modified enzyme binds NAD^+ 9 times tighter (K_D = 0.79 mM) than the native enzyme (Haghighi & Levy, 1982). No TRNOE was observed from H3′ to H_N6 in this phosphopyridoxyl enzyme–NAD^+ complex, whereas a TRNOE from H_N1' to H_N6 was comparable to that in the NAD^+ complex of the native enzyme (Table I). The conformation of the nicotinamide–ribose bond in a catalytically active ternary complex is, presumably, entirely anti or syn, depending on the enzyme's stereospecificity.

Significant differences in TRNOE values between the NAD^+ and $NADP^+$ complexes of both glucose-6-phosphate dehydrogenase and glutamate dehydrogenase can be seen (Table I). For glutamate dehydrogenase, this is evident in substantial differences in the TRNOE between H5′,5″ and H_A8, H2′ and H_N2, and H5′,5″ and H_N2'; with glucose-6-phosphate dehydrogenase, there are differences in the TRNOE between H3′ and H_A8 and between H3′ and H_N6, and possibly between H2′ and H_A8 and between H3′ and H_N2. In the case of glucose-6-phosphate dehydrogenase, these differences may be a reflection of the marked difference in the protein conformation of the NAD^+– and $NADP^+$–enzyme complexes, as revealed by fluorescent probes (Haghighi & Levy, 1982). It is likely that such conformational differences are exploited in mechanisms that allow dual nucleotide-specific dehydrogenases to regulate their interactions with NAD and NADP.

Glutamate dehydrogenases from animal tissues contain multiple binding sites for nicotinamide coenzymes and purine nucleotides. Extensive studies with beef liver glutamate dehydrogenase have shown that both $NADP^+$ and NADPH bind only to one site per subunit but that NADH binds also to a second, regulatory site (Fisher, 1973). Recent studies suggest that NAD^+ may also bind to a second site on the beef liver enzyme (Bayley & O'Neill, 1980; Smith & Bell, 1982). In the absence of the substrate analogue glutarate, however, both equilibrium dialysis (Dalziel & Egan, 1972) and circular dichroism measurements (Bayley & O'Neill, 1980) show that NAD^+ binds to a single site per subunit under conditions similar to those employed in our TRNOE measurements. This

argues strongly against the possibility that our TRNOE measurements on NAD^+ binding to the rat liver enzyme reflect a mixture of signals from two different binding modes. A second suggestion by Bayley & O'Neill (1980)—that NAD^+ binds preferentially to the regulatory rather than the catalytic site, in the absence of substrate or substrate analogues—has not yet been confirmed but can be addressed by TRNOE measurements that we plan to undertake.

It should be emphasized that there is no evidence for more than one nicotinamide coenzyme binding site per subunit of *L. mesenteroides* glucose-6-phosphate dehydrogenase. Kinetic (Olive et al., 1971) and binding studies (Grove et al., 1976; Haghighi & Levy, 1982) demonstrate that this enzyme has a single site per subunit that binds NAD^+, $NADP^+$, and NADPH.

Although TRNOE measurements have been interpreted in terms of quantitative bond angles and distances between protons (Clore & Gronenborn, 1982; Gronenborn & Clore, 1982; Feeney et al., 1983), in this preliminary study we make no attempt to interpret such distances quantitatively. Nevertheless, our results demonstrate that TRNOE measurements provide a facile method for distinguishing between dehydrogenases that are A and B stereospecific. Furthermore, differences in the TRNOE between the same proton pairs in NAD^+ and $NADP^+$ bound to the same enzyme [e.g., $f_{H_A8}(H3')$ for glucose-6-phosphate dehydrogenase] indicate that subtle conformational effects may be discernible with this technique. Thus, TRNOE can provide a useful probe for localizing changes in the conformation of bound ligands induced by binding of other ligands or other perturbations in solution.

Registry No. NAD, 53-84-9; NADP, 53-59-8; glucose-6-phosphate dehydrogenase, 9001-40-5; glutamate dehydrogenase NAD(P), 9029-12-3; lactate dehydrogenase, 9001-60-9; dehydrogenase, 9035-82-9.

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