Selectivity in the Binding of NAD(P)$^+$ Analogues to NAD- and NADP-Dependent Pig Heart Isocitrate Dehydrogenases. A Nuclear Magnetic Resonance Study†

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ABSTRACT: The coenzyme selectivity of pig heart NAD-dependent and NADP-dependent isocitrate dehydrogenase has been investigated by nuclear magnetic resonance through the use of coenzyme analogues. For both isocitrate dehydrogenases, more than 10-fold lower maximal activity is observed with thionicotinamide adenine dinucleotide [sNAD(P)$^+$] than with NAD(P)$^+$ or acetylpyridine adenine dinucleotide [acNAD(P)$^+$] as coenzyme. Nuclear Overhauser effect measurements failed to reveal any differences in the adenine–ribose conformations among the enzyme-bound analogues. The 2′-phosphate resonance of the enzyme-bound NADP$^+$ analogues showed the same change in chemical shift observed for the natural coenzyme and revealed the same lack of pH dependence in the range from pH 5.4 to 8.2. NADP-dependent isocitrate dehydrogenase exhibits only small differences in Michaelis constants for the coenzymes with various nicotinamide substituents, reflecting a predominant role for the adenosine moiety in binding. The conformation of the bound nicotinamide–ribose of the natural coenzymes was appreciably different from that of the coenzyme, sNAD(P)$^+$, which shows low catalytic activity. For both isocitrate dehydrogenases, sNAD(P)$^+$ bound to the enzymes exhibits a mixture of syn and anti conformations while only the anti conformation can be detected for NAD(P)$^+$. Chemical shifts of NAD(P)$^+$ enriched with ^{13}C in the carboxamide indicate that interaction of this group with the enzymes may play a role in positioning the nicotinamide ring to participate in catalysis. Our results suggest that, although interaction of the nicotinamide moiety with the enzymes contributes relatively little to the energy of interaction in the binary complex, the enzymes must correctly position this group for the catalytic event. This orientation may be promoted through specific interactions with the carboxamide.

Pyridine nucleotide coenzymes exist in solution as rapidly interchanging mixtures of conformers in which the nicotinamide ring is either syn or anti with respect to the adjacent ribose. Dehydrogenases are able to transfer hydrogen from one side of the nicotinamide ring with extremely high selectivity. This selectivity is believed to be related to the ability to preferentially bind one of the nicotinamide–ribose conformers in a catalytically active complex (You et al., 1978). In addition, many dehydrogenases show great specificity between coenzymes with and without a 2′-phosphoryl group on the adenine ribose. The nicotinamide ring is symmetrical except for the substitution of a carboxamide at the 3-position. The kinetics with coenzymes having substitutions at this position and others have been investigated for many dehydrogenases, with activity frequently being retained (Anderson, 1982). X-ray crystallography may not always allow the delineation of the nicotinamide binding region. For example, the nicotinamide moiety could not be located in the complex with isocitrate dehydrogenase from *Escherichia coli* (Hurley et al., 1991). For glutathione reductase, the crystal structures of complexes with reduced thionicotinamide and acetylpyridine coenzymes showed reduced occupancy, particularly at the nicotinamide moiety, compared with the natural coenzyme, NADPH (Pai et al., 1988). To explore the relationship between activity and conformation, we have used ^{1}H, ^{13}C, and ^{31}P NMR[1] spectroscopy to compare the bound states of coenzyme analogues of NADP$^+$ and NAD$^+$ with isocitrate dehydrogenases.

Mammalian mitochondria contain two forms of isocitrate dehydrogenase, an NADP-dependent form (EC 1.1.1.42) and an NAD-dependent form (EC 1.1.1.41). Both catalyze reactions with the same stereochemistry (Chen & Plaut, 1962; Nakamoto & Vennesland, 1960). Both have been found to bind their coenzyme in the same conformation with the nicotinamide ring anti to the ribose (Ehrlich & Colman, 1985; Ehrlich & Colman, 1990). The selectivity of the NADP-dependent isocitrate dehydrogenase for its coenzyme presumably arises from a positively charged residue(s) close to the 2′-phosphate which keeps NADP(H) in the dianionic form below the pK of the free compound (Mas & Colman, 1984). The only dehydrogenase for which selectivity among coenzyme analogues has been studied by NMR is dihydrofolate reductase for which differences were found between the bound acetylpyridine or thionicotinamide analogue and NADP$^+$ (Hyde et al., 1980a,b). Isotopic substitution of ^{13}C in the carboxamide allowed distinction between three conformational states in the ternary complex of NADP$^+$, methotrexate, and dihydrofolate reductase (Way et al., 1975). NAD-dependent isocitrate

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; sNAD$^+$, thionicotinamide adenine dinucleotide; sNADP$^+$, thionicotinamide adenine dinucleotide 2′-phosphate; acNAD$^+$, acetylpyridine adenine dinucleotide; acNADP$^+$, acetylpyridine adenine dinucleotide 2′-phosphate; cNADP$^+$, nicotinamide adenine dinucleotide 2′,3′-cyclic monophosphate; amNADP$^+$, 3-aminopyridine adenine dinucleotide 2′-phosphate.

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dehydrogenase has three subunits with different primary sequences (Huang & Colman, 1990) and might have more than one type of coenzyme site. In the present study of isocitrate dehydrogenases, only the binary coenzyme–enzyme complexes were investigated. For both enzymes, evidence for a single bound conformer was found with the natural coenzymes, while a mixture of bound conformers was found with some coenzyme analogues.

EXPERIMENTAL PROCEDURES

Materials. NADP-dependent isocitrate dehydrogenase was isolated from pig hearts using procedures summarized by Ehrlich and Colman (1985). NAD-dependent isocitrate dehydrogenase was purified from pig hearts following the method of Ramachandran and Colman (1977) as scaled up by Ehrlich and Colman (1990). For proton NMR experiments, the enzymes were dialyzed against the appropriate buffer, followed by exchange into D_2O by repeated concentration in an Amicon Centricon-30 device using buffers that had been lyophilized and then redissolved in D_2O. Final concentrations were 10–20 mg/mL. The NADP-dependent isocitrate dehydrogenase concentration was obtained using $E^{1\%}_{280}$ = 10.8 (Johanson & Colman, 1981). The NAD-dependent isocitrate dehydrogenase concentration was obtained from the optical density at 280 nm using an absorbance of 1.55 for a 1 mg/mL solution (Shen et al., 1974) and an average subunit molecular weight of 40 000.

MOPS, MES, NAD^+, $NADP^+$, $sNAD^+$, $sNADP^+$, $acNAD^+$, $acNADP^+$, $amNAD^+$, deaminoNAD$^+$, and DL-isocitrate were obtained from the Sigma Chemical Co. Lactate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, and alcohol dehydrogenase were obtained from Boehringer. D_2O (99.8% D) was obtained from Aldrich Chemical Co. Ligands were dissolved in the appropriate buffers prepared in D_2O.

Kinetic Measurements. NADP-dependent isocitrate dehydrogenase was assayed in 0.03 M triethanolamine chloride, pH 7.4, containing 1 mM $MnSO_4$, 4 mM DL-isocitrate, and 100 μM $NADP^+$. Coenzyme analogues were tested by omission of the natural coenzyme and additions of varying concentrations of the analogues. NAD-dependent isocitrate dehydrogenase was assayed in 0.03 M Tris·HCl (pH 7.2) containing 2 mM $MnSO_4$, 40 mM DL-isocitrate, and 1 mM NAD^+. Michaelis constants for coenzymes were determined by substituting varying concentrations of the analogues for the natural coenzyme.

Synthesis of ^{13}C-Labeled NAD^+ and $NADP^+$. Synthesis of coenzymes enriched to 90% ^{13}C in the carboxamide was performed using a combination of published techniques (Way et al., 1975; Ott, 1981; Ault, 1979). $Cu^{13}CN$ was formed from $Na^{13}CN$ (MSD Isotopes) by mixing $CuSO_4$ (29.2 mmol) with sodium bisulfate (14.6 mmol) at 60 °C and then quickly mixing a solution of NaCN (20 mmol, 1 g) with the brown solution. Precipitated CuCN was collected with 89% yield. Nicotinic acid was made by refluxing CuCN (17.8 mmol) with 1.5-fold molar excess 3-bromopyridine (Ott, 1981). After 1 h, basic ethanol was added and the mixture was refluxed for an additional 3 h. The solution was dried, taken up in 2 N NaOH, and separated from bromopyridine on a Dowex AG1x8 column (Bio-Rad) run in 0.25 mM formic acid. The remaining Cu was removed by passage through a Chelex 100 column (Bio-Rad). The UV absorbing fractions were concentrated, applied to another Dowex AG1x8 column and, after a water wash, the column was eluted with formic acid. Nicotinic acid (yield 64%) was checked by melting point and proton NMR.

Nicotinamide was made using the procedure given by Ault (1979). Nicotinic acid (0.87 g) was refluxed with 10 mL of thionyl chloride, and the mixture was dried on a steam bath. Cold NH_4OH (15 mL) was added. The solution was repeatedly extracted with chloroform to yield 0.24 g of nicotinamide.

NAD^+ and $NADP^+$ were prepared by enzymatic exchange of nicotinamide with $sNAD^+$ and $sNADP^+$, respectively, in place of the $acNADP^+$ used by Way et al. (1975). A 5-fold excess of nicotinamide (144 μmol to 28.8 μmol of $NADP^+$) was used with 256 mg of crude NADase (Sigma). The mixture was incubated in 0.1 M potassium phosphate (pH 7.5) at 37 °C. The formation of NAD^+ was monitored by adding aliquots to an NAD-dependent isocitrate dehydrogenase assay mixture (Ramachandran & Colman, 1977) from which the coenzyme was omitted. This approach takes advantage of the kinetic result that $sNAD^+$ is a poor substrate for isocitrate dehydrogenase so that the initial change in absorbance at 340 nm is mostly due to formation of NADH from NAD^+. The exchange reaction was stopped after 12 min by the addition of trichloroacetic acid. After centrifugation, the supernatant was concentrated, adjusted to pH 5.5, and applied to a Dowex AG1x2 column (formate form). The column was eluted with a gradient from deionized water to 1 M formic acid. The formation of $NADP^+$ from $sNADP^+$ was monitored using an assay solution containing 3.3 mM glucose 6-phosphate, 2.0 mM $MnSO_4$ in triethanolamine chloride buffer (pH 7.4). The conversion of $NADP^+$ to NADPH was initiated using glucose-6-phosphate dehydrogenase. The exchange reaction was treated as before and applied to a Dowex AG1x2 column and eluted with a gradient from 0.1 M ammonium formate to 1.0 M ammonium formate. Fractions were tested by obtaining absorption spectra of aliquots before and after reduction with cyanide. Yields were 35–48% on the basis of $sNAD(P)^+$.

NMR Measurements. ^{1}H NMR spectra were measured in a Bruker WM-250 spectrometer at 250.13 MHz using quadrature phase detection, a 12-bit, digitizer and spectral width of 3521 Hz. Spectra were accumulated in 8K memory. Samples in 5-mm tubes were not spun in order to avoid protein denaturation. Dioxane (1–2 mM) was added as an internal reference standard (taken as 3.71 ppm). Peaks were assigned by comparison with the literature (Oppenheimer, 1982).

Time-dependent nuclear Overhauser effects (NOE's) were measured with the radio frequency pulse sequence t_1–t_2–$\pi/2$. A delay time, t_1, was chosen so that the sum of this time and the time, t_2, of selective irradiation was 1.0–1.5 s. Saturation of the free nucleotide resonances was complete for times, t_2 > 0.05 s using power levels of 23–25 dB below 0.2 W. Blocks of 32 scans were accumulated alternating between irradiation of specific nucleotide peaks and irradiation at either 3 ppm or −1.5 ppm. The procedure was repeated until 256–1024 scans were obtained and the free induction decay with irradiation at each specific peak was subtracted from the free induction of the off-resonance control. NOE's are expressed as a decimal fraction of the intensity of the peak obtained from the Fourier transform of the control. In the presence of enzymes, all NOE's are negative. Control experiments, performed with nucleotide samples identical to those used for the measurements with isocitrate dehydrogenase but from which enzyme was omitted, did not show any NOE's. To detect NOE's between the enzymes and various coenzymes, the region from 7 to 8 ppm was irradiated at 10–25-Hz intervals and the Fourier transform treated as for intracoenzyme resonances.

The observed rate of change of NOE arising from irradiation of either the resonance of a proton on the free compound or an averaged resonance from free and enzyme-bound compounds (in the case of fast exchange on the cross-relaxation scale) is given by

$$\frac{d(NOE)}{dt} = -(P_b\sigma_b + P_f\sigma_f) \quad (1)$$

where σ_b and σ_f are the cross-relaxation rates for bound and free molecules and P_b and P_f are the fractions of bound and free molecules (Clore & Gronenborn, 1983). For a sample of fixed composition, the cross-relaxation rates may be determined from the slope of the observed experimental intensities at short irradiation times. At longer times, effects of spin diffusion from distal spins will contribute to the observed NOE's. For $t \leq 1.0$ s, the NOE's for free nucleotides are positive and <0.02 so that only contributions from the bound compounds need be considered. If the correlation times for two sets of protons (i,j) and (k,l) are the same, the distances between them can be calculated from the cross-relaxation rates for the pairs:

$$r_{ij}/r_{kl} = (\sigma_{kl}/\sigma_{ij})^{1/6} \quad (2)$$

The protons in the nicotinamide ring of NAD^+ are fixed with $r(N5,N6) = r(N4,N5) = 2.48$ Å. The cross-relaxation rate between these protons is used to scale the rates between other protons. If more than one conformational species is present, eq 2 cannot be used: the observed NOE's are the sum of the r^6 contributions from the species present which may have different interproton distances.

^{31}P NMR measurements were made at 101 MHz in a Bruker WM-250 spectrometer using 1.5–2.0-mL samples in 10-mm-diameter tubes. Chemical shifts were determined by reference either to an external standard of phosphoric acid or to a concentric 5-mm tube containing phosphoric acid. Samples contained 10% D_2O for the field frequency lock. Samples were titrated by small additions of 0.1 M NaOH or 1.0 M MES. Exchange between bound and free nucleotide was followed by saturating the resonance of the bound nucleotide with a DANTE sequence of pulses and following the time-dependent change in the amplitude of the free nucleotide. The relaxation time (T_1) of the free nucleotide was measured using a 180°–90° pulse sequence.

^{13}C measurements were made at 62.87 MHz in a Bruker WM-250 spectrometer using either 2-mL samples in 10-mm tubes or 0.4–0.5-mL samples in 5-mm tubes. Dioxane or glycerol was used as an internal reference with a chemical shift of 67.3 ppm for dioxane from tetramethylsilane.

RESULTS

Kinetic Properties of Coenzyme Analogues with NAD and NADP-Dependent Isocitrate Dehydrogenases. Measurements of Michaelis constants and maximum velocities for coenzyme analogues relative to NAD^+ or $NADP^+$ are presented in Table I. For NADP-dependent isocitrate dehydrogenase, the apparent affinity of the enzyme for all analogues having an intact 2′-phosphate is comparable. The maximum velocity is substantially reduced upon substitution of the oxygen in the carboxamide by sulfur ($sNADP^+$). As previously found, the absence of the 2′-phosphate greatly increases the K_m and decreases the catalytic rate (Ehrlich & Colman, 1978). The activity with the 2′,3′-cyclic phosphate derivative (cNADP) is also low, demonstrating that a fully ionized phosphate is important, as was surmised from the NMR titration of the ^{31}P resonance (Mas & Colman, 1984). The 3-aminopyridine analogue is not used as a coenzyme, but it binds weakly, as demonstrated by competitive inhibition (K_I = 1.1 mM).

Table I: Kinetic Properties of Nucleotide Analogues with Isocitrate Dehydrogenases

enzyme	nucleotide	K_m (μM)	relative V_{max}
NADP-dependent isocitrate dehydrogenase	$NADP^+$	4.7	1.0
	$acNADP^+$	7.1	0.15
	$sNADP^+$	26.0	0.012
	$cNADP^+$	2500	0.031
	$amNADP^+$	1100[a]	
NAD-dependent isocitrate dehydrogenase	NAD^+	50	1.0
	$acNAD^+$	120	0.56
	$sNAD^+$	1300	0.04

[a] Inhibition constant.

For the NAD-dependent isocitrate dehydrogenase, $acNAD^+$ is only a slightly poorer substrate than is the natural coenzyme (NAD), but both the K_m and turnover are reduced 25-fold with $sNAD^+$. As reported by Plaut et al. (1979), the deamino analogue is inactive.

Conformation of Coenzyme Analogues. The conformations of coenzyme analogues bound to isocitrate dehydrogenases were obtained by measuring the nuclear Overhauser effects arising from irradiation of ring and ribose protons. Figure 1A shows the proton NMR spectrum of $sNAD^+$ in the presence of NAD-dependent isocitrate dehydrogenase. As expected from the previous experiments using NAD^+ (Ehrlich & Colman, 1990), irradiation of the N2 proton results in an NOE for the N1′ proton. Irradiation of the N6 ring proton also results in a decrease in the amplitude of the N1′ proton. In contrast, for $acNAD^+$ (Figure 1B) under the same conditions, only irradiation of the N2 proton results in an NOE at the N1′ position. Moreover, for irradiation of the N1′ proton, an NOE is found for both the N2 and N6 protons in the case of $sNAD^+$ but only for the N2 proton in the case of $acNAD^+$.

These qualitative findings are further explored by examining the time dependence of the NOE's for coenzyme analogues in the presence of NAD-dependent isocitrate dehydrogenase. Figure 2 shows the time dependence of several NOE's for $sNAD^+$ (A) and $acNAD^+$ (B). The cross relaxation rates are determined from the slopes of plots such as those shown in Figure 2. Data are presented in Table II for comparison with data from previous experiments on NAD^+ (Ehrlich & Colman, 1990). The data are presented as relative rates with respect to the cross-relaxation rate between the fixed protons N5 and N6 in order to compare experiments done with different ratios of bound to free nucleotide. The major difference between $sNAD^+$ and the other analogues is the magnitude of the cross-relaxation rates between N1′ and N6 and between N2′ and N2. The relative NOE's are the same within experimental error for the adenosine rings of all three analogues. The presence of cross-relaxation between N1′ and both N2 and N6 can only come from the existence of two different bound conformations (anti and syn) of $sNAD^+$, as the N1′ proton cannot be close to both ring protons in any one structure. The possibility of a small portion of the anti conformation characterized by proximity of N6 and N1′ as well as N2 and N2′ cannot be ruled out for $acNAD^+$, but the amount is certainly less than for $sNAD^+$.

A similar contrast between the bound conformations of $sNADP^+$ and $acNADP^+$ on the NADP-dependent isocitrate dehydrogenase can be inferred from the time-dependent NOE's shown in Figure 2C,D. Relative cross-relaxation rates are summarized in Table III. The substantial cross-relaxation between N2 and N2′ and between N6 and N1′ protons is

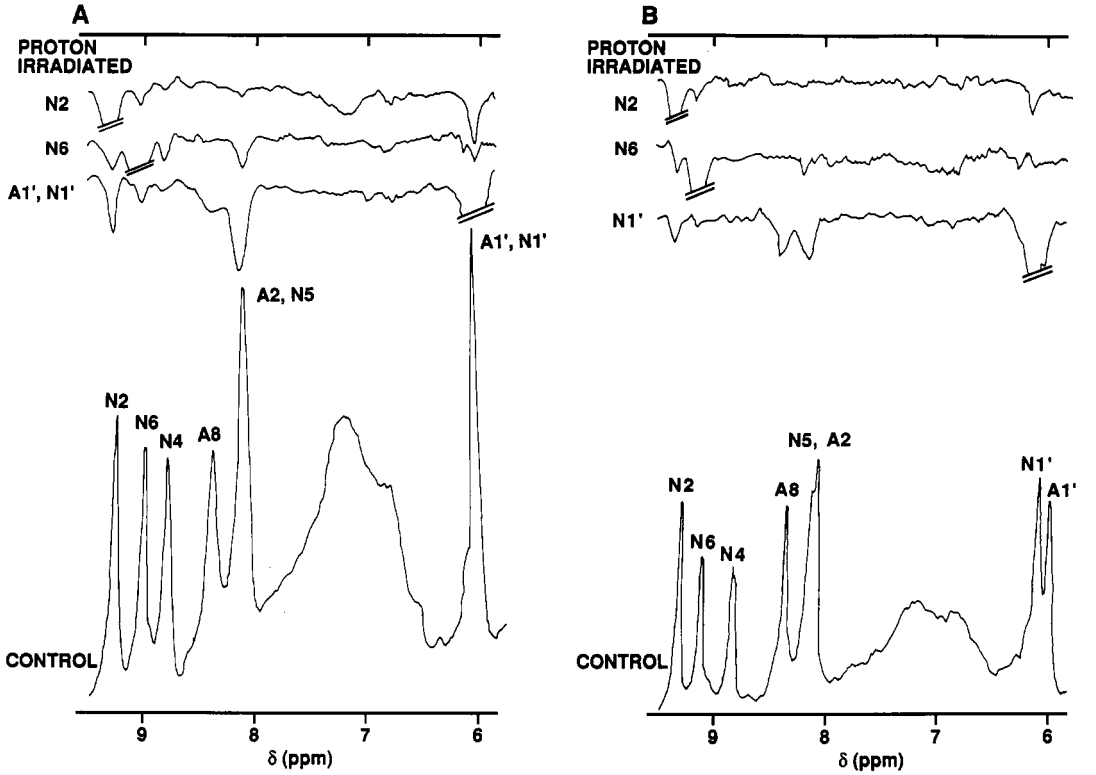

FIGURE 1: Spectra of coenzyme analogues bound to NAD-dependent isocitrate dehydrogenases. (A) sNAD$^+$ and NAD-dependent isocitrate dehydrogenase in Na/0.05 M MOPS (pH 7.0). The control is obtained with 3.5 mM sNAD$^+$ and 0.53 mM NAD-dependent isocitrate dehydrogenase with radio frequency irradiation for 0.1 s at 3 ppm. Difference spectra between the control and spectra obtained with irradiation at the positions of the indicated peaks are shown with an expansion of 2. For all spectra, 480 scans were obtained. (B) acNAD$^+$ and NAD-dependent isocitrate dehydrogenase in Na/0.05 M MOPS (pH 7.0). All samples contained 2.2 mM acNAD$^+$ and 0.38 mM NAD-dependent isocitrate dehydrogenase. Spectra (960 scans) are labeled as for A.

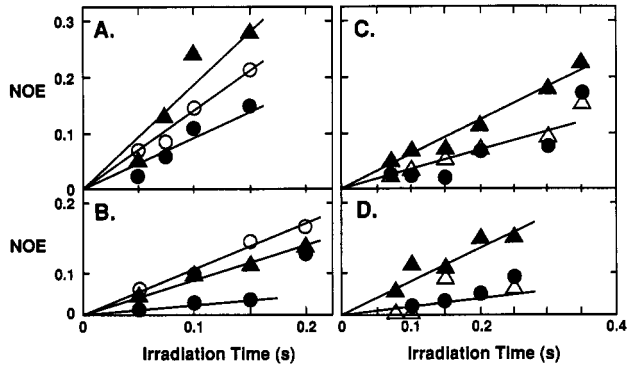

FIGURE 2: Time-dependent NOE's. NOE's were measured as the ratio of the peak change in the difference spectrum to the corresponding peak in the control spectrum with increasing irradiation times. Sample conditions were as in Figure 1. Enzyme–coenzyme complexes and irradiated peaks are the following. (A) sNAD$^+$ and NAD-dependent isocitrate dehydrogenase: irradiation of N1′ and measurement of N2 (▲), irradiation of N1′ and measurement of N6 (●), irradiation of N2′ and measurement of N6 (○). (B) acNAD$^+$ and NAD-dependent isocitrate dehydrogenase: symbols are identified as in (A). (C) sNADP$^+$ and NADP-dependent isocitrate dehydrogenase: positions of irradiation and measurement are as in (A) with the addition of irradiation at N2′ and measurement of the N2 peak (△). (D) acNADP$^+$ and NADP-dependent isocitrate dehydrogenase. Symbols are as in (C).

indicative of the presence of sNAD$^+$ bound to the enzyme in the syn conformation. NOE experiments do not indicate any differences among the analogues in the bound conformations with respect to adenosine–ribose geometry. A nonzero cross-relaxation between A1′ and A8 is observed which has been previously attributed to the syn conformer (Ehrlich & Colman, 1985) but could arise from conformations with the anti range. The aminopyridine analogue, which is an inhibitor but not a substrate, shows equal contributions from syn and anti

Table II: Relative Cross-Relaxation Rates between Protons of NAD$^+$ Analogues Bound to NAD-Dependent Isocitrate Dehydrogenase[a]

proton		analogue		
irradiated	observed	NAD$^+$	acNAD$^+$	sNAD$^+$
N1′	N2	1.94	1.05	1.44
N1′	N6	<0.07	0.26	0.57
N2′, N3′	N2	<0.13	0.26	0.58
N2′, N3′	N6	1.70	1.31	1.09
A1′	A8	<0.26	<0.11	<0.25
A2′	A8	0.82	0.73	0.91

[a] Cross-relaxation rates for the indicated protons are ratios of the observed rates to the rates observed for the N5–N6 proton pair. Cross-relaxation rates are determined from slopes of time-dependent NOE plots such as are shown in Figure 2.

conformers at the nicotinamide–ribose moiety and a substantial amount of syn conformer at the adenosine–ribose bond. Thus, the enzyme-bound forms of this inactive nucleotide may not be different from the forms present in solution in the absence of enzyme.

Effect of Coenzymes on the Histidine of NADP-Dependent Isocitrate Dehydrogenase. Addition of NADP$^+$ to NADP-dependent isocitrate dehydrogenase has been found to cause a shift in one of the three peaks in the range 7.5–8.0 ppm that have been ascribed to histidine (Ehrlich & Colman, 1985). In the absence of nucleotides, these are found at 7.86, 7.79, and 7.69 ppm at pH 7.0. With NADP$^+$, acNADP$^+$, and sNADP$^+$, these are found at 7.85, 7.73, and 7.69 ppm with a maximum error in chemical shift of 0.01 ppm. Thus, when bound to the enzyme, all nucleotides are able to shift a (presumably) specific histidine on the enzyme. This shift has been ascribed to interaction with the nicotinamide ring since none of the analogues of NADP$^+$ that lacked this ring caused

Table III: Relative Cross-Relaxation Rates between Protons of NADP+ Analogues Bound to NADP-Dependent Isocitrate Dehydrogenase[a]

proton		analogue			
irradiated	observed	NADP+	acNADP+	sNADP+	amNADP+
N1′	N2	1.85	1.26	1.37	0.83
N1′	N6	<0.13	<0.26	0.57	1.13
N2′, N3′	N2	0.19	<0.23	0.59	1.08
N2′, N3′	N6	1.35	1.18	0.52	1.08
N2	N1′	1.72	1.55	1.02	0.83
N6	N1′	0.25	0.05	0.76	0.65
A1′	A8	0.48	0.47	0.46	0.89

[a] Cross-relaxation rates for the indicated protons are ratios of the observed rates to the rates observed for the N5–N6 proton pair. Cross-relaxation rates are determined from slopes of time-dependent NOE plots such as are shown in Figure 2.

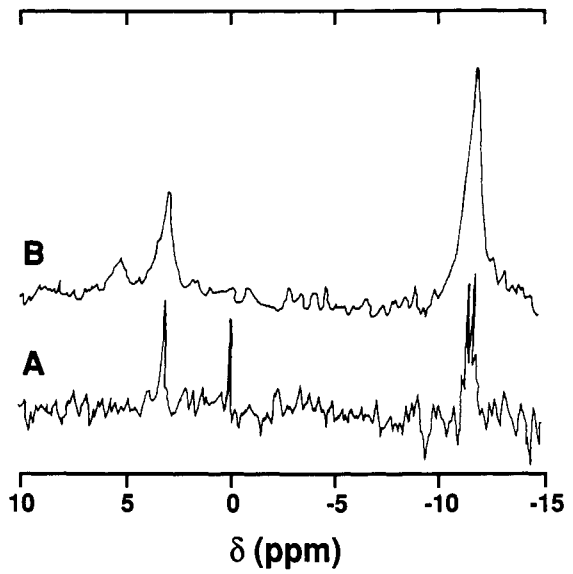

FIGURE 3: Phosphorus NMR spectra of sNADP+. Samples are in Na/0.05 MOPS (pH 7.5) containing 10% glycerol. (A) 0.31 mM sNADP+, 13 908 scans. The peak at 0 ppm arises from a capillary tube containing 85% phosphoric acid. (B) The spectrum of 0.62 mM sNADP+ in the presence of 0.32 mM NADP-dependent isocitrate dehydrogenase, 20 000 scans.

the shift in the 7.79 ppm histidine peak (Ehrlich & Colman, 1985).

Dissociation Rates for Coenzyme Analogues. Dissociation rates of nucleotides from enzyme complexes may be obtained from measurements of line widths obtained with several ratios of nucleotide to enzyme (Ehrlich & Colman, 1985; Pople et al., 1959). The dissociation rate for NADP+ from the NADP-dependent isocitrate dehydrogenase is 15 ± 4 s^{-1} in 0.05 M Tris·HCl. This value is consistent with the results of Ehrlich and Colman (1985) taking into account the decreasing dissociation rate with decreasing ionic strength. The dissociation rates measured for acNADP+ and sNADP+ were 20 ± 6 and 67 ± 7 s^{-1}, respectively.

Phosphorus NMR Study of Coenzyme Binding. Mas and Colman (1984) have shown that the binding of NADP+ and NADP+ fragments having the 2′-phosphate is accompanied by a change in the ^{31}P resonance of the 2′-phosphate from the position of the free analogue. This shift, unlike that of the free compounds, does not vary in the pH range from 5.5 to 8.0. Figure 3 shows the ^{31}P NMR spectrum of sNADP+ in the presence and absence of the NADP-dependent isocitrate dehydrogenase. The spectrum in the presence of the enzyme shows the normal 2′-phosphate resonance at 3.1 ppm and an additional peak at 5.4 ppm. The peaks are broadened due to

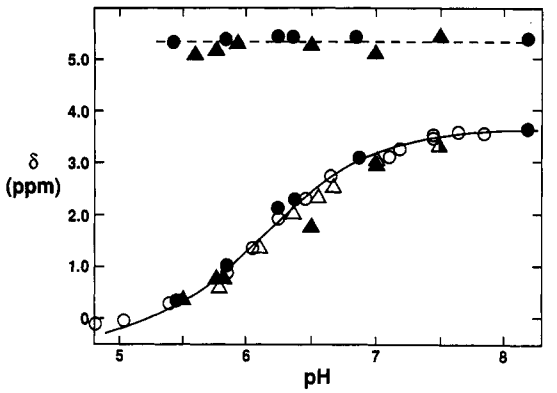

FIGURE 4: Phosphorus chemical shifts as a function of pH. Buffers used were Na/MOPS (pH 6.2–8.4) and NaMES (pH 4.8–6.5) containing 10% glycerol. Chemical shifts for acNADP+ (△, ▲) and sNADP+ (○, ●) were obtained in the presence (▲, ●) and absence (△, ○) of NADP-dependent isocitrate dehydrogenase as a function of pH. The line is a nonlinear least-squares fit for the peak of sNADP+ in the absence of isocitrate dehydrogenase with chemical shift in the doubly ionized form of 3.64 ± 0.05 and chemical shift in the singly ionized form of −0.48 ± 0.15 with a pK of 6.13 ± 0.04. The dashed line is drawn arbitrarily.

exchange and/or slow rotation of the enzyme–nucleotide complex. Similar spectra were obtained over the pH range from 5.4 to 8.2. The peak positions are plotted in Figure 4. It can be seen that the peak which occurs at high field is titrated with pK 6.1 and is identical with that of free sNADP+. The downfield peak shows no shift over the same pH range. These results indicate that sNADP+ binds in the same manner as NADP+ with the 2′-phosphate doubly ionized in the bound form. Similar results were obtained for acNADP+, and these are also given in Figure 4. Thus, the conformations of the adenosine moiety in the vicinity of the 2′-phosphate are similar for all three coenzyme analogues.

Although we have concluded that the doubly ionized form of the 2′-phosphate is necessary for proper binding, Wong and Blanchard (1989) have shown that an enzyme, glutathione reductase, that normally uses NADP+ can also use cNADP+ in which the phosphate is singly ionized. This analogue is also a substrate for isocitrate dehydrogenase (Table I). Phosphorus NMR spectra were obtained for cNADP+ in the presence and absence of isocitrate dehydrogenase. The chemical shift of the 2′,3′-cyclic phosphate is the same in both samples and does not change with pH in the region 5.5–8.0. Thus, there is no evidence that the enzyme can specifically interact with the cyclic phosphate.

NMR of ^{13}C-Labeled Carboxamide. The ^{13}C spectra of NAD+ and NADP+ enriched with ^{13}C in the carboxamide carbon are shown in Figure 5. The chemical shift of the NAD+ resonance is altered slightly from that of free NAD+ in the presence of NAD-dependent isocitrate dehydrogenase. A larger difference is seen between the chemical shifts of free NADP+ and NADP+ bound to NADP-dependent isocitrate dehydrogenase. Since all of the coenzyme is not bound, the shifts must be corrected for the amount of free and bound coenzyme. Chemical shifts as a function of coenzyme:enzyme ratio are shown in Figure 6. The curves given are calculated on the basis of previously measured binding constants for NAD+ and NADP+ to the two types of isocitrate dehydrogenase (Ehrlich & Colman, 1981; Mas & Colman, 1985) and give values for the chemical shifts of the bound species. In the presence of 60 mM sodium sulfate, the same chemical shift for the bound species was calculated for NADP-dependent isocitrate dehydrogenase (data not shown), but the dissociation constant was higher as was observed by Ehrlich and Colman

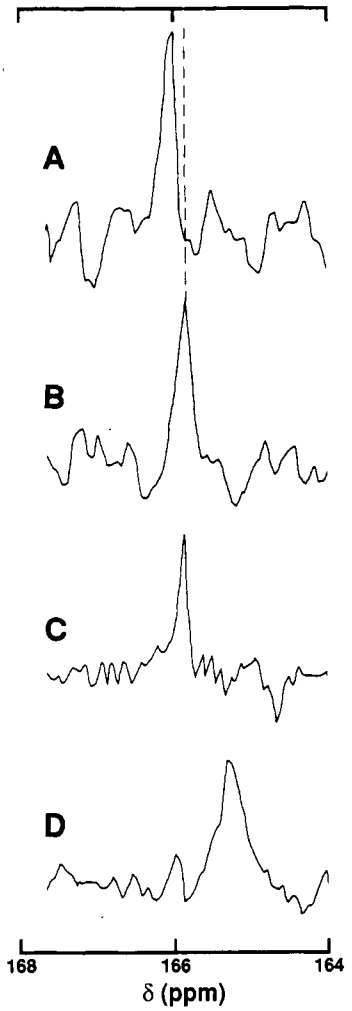

FIGURE 5: ^{13}C spectra of NAD^+ and $NADP^+$ enriched at the 4-carboxamide position of the nicotinamide ring: (A) NAD^+, 0.12 mM in 0.05 M MES buffer (pH 6.1) containing 20% glycerol, 5700 scans; (B) NAD^+ (0.11 mM) plus NAD-dependent isocitrate dehydrogenase (0.30 mM subunits) in the same buffer as in (A) 5614 scans; (C) $NADP^+$ (0.2 mM) in 0.05 triethanolamide chloride containing 10% glycerol (pH 7.5); (D) $NADP^+$ (0.18 mM) plus NADP-dependent isocitrate dehydrogenase (0.36 mM in subunits) in the same buffer as in (C), 42 000 scans.

(1985) and Mas and Colman (1985).

Chemical shifts for coenzyme–enzyme complexes have previously been published for dihydrofolate reductase (Way et al., 1975). In order to compare the results obtained for isocitrate dehydrogenase with those for other dehydrogenases, measurements were made for NAD^+ in the presence of mitochondrial malate dehydrogenase, lactate dehydrogenase, and liver alcohol dehydrogenase. A change in chemical shift could only be detected for malate dehydrogenase. The position of resonance depends upon the ratio of [NAD] to [malate dehydrogenase] consistent with δ_{bound} = 165.39 ppm. With lactate dehydrogenase, the observed resonance remained at the same position even when the ratio of coenzyme to enzyme was more than stoichiometric and the resonance showed little alteration from that of free coenzyme. The absence of an observed resonance in the case of alcohol dehydrogenase may be due to unfavorable exchange rates with NAD^+ or exchange among different bound conformations. Similar measurements were made with $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase, as well as glutamate dehydrogenase. The carboxamide resonance of $NADP^+$ bound to glucose-6-phosphate dehydrogenase shifted upfield by about 0.16 ppm. Glucose-6-phosphate dehydrogenase can use either NAD^+ or

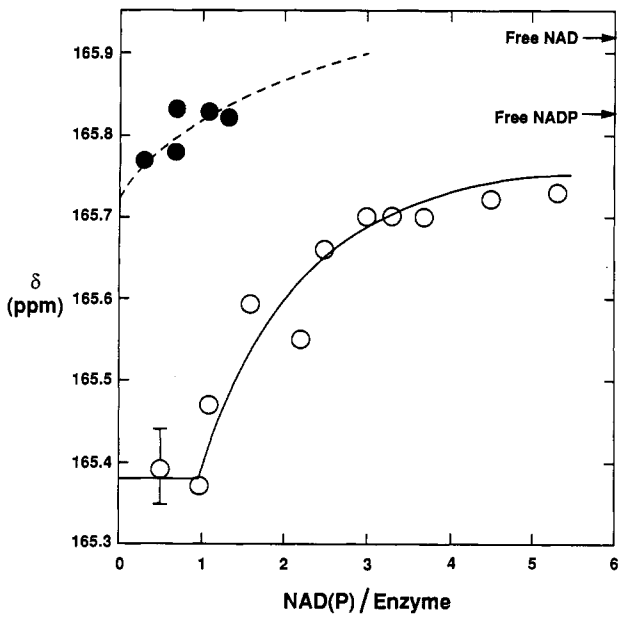

FIGURE 6: ^{13}C chemical shifts for NAD^+ (●) and $NADP^+$ (○) in the presence of NAD and NADP-dependent isocitrate dehydrogenase, respectively. Spectral conditions are as in Figure 5, but the ratio of coenzyme to enzyme was varied. The curves are fits to the data with lines representing (- - -) bound shift for NAD^+ of 165.73 ppm and dissociation constant 0.06 mM and (—) bound shift for $NADP^+$ of 165.38 ppm with dissociation constant less than 10 μM.

$NADP^+$ as coenzyme. The carboxamide chemical shift for NAD^+ bound to glucose-6-phosphate dehydrogenase was the same as for bound $NADP^+$, indicating similar environments of the nicotinamide rings. No resonance was detected for glutamate dehydrogenase at coenzyme:enzyme ratios of 1:1. At higher values, a resonance corresponding to free coenzyme was observed. Although data were obtained on only a few enzymes, the observations suggest that the environment of the carboxamide moiety may be different in various dehydrogenases.

DISCUSSION

Both NAD and NADP-dependent pig heart isocitrate dehydrogenases show greater catalytic activity with NAD(P)$^+$ and acNAD(P)$^+$ than with sNAD(P)$^+$. In the case of NAD-dependent isocitrate dehydrogenase, the affinity as indicated by the Michaelis constant is reduced more than 20-fold. The binding of analogues to NADP-dependent isocitrate dehydrogenase depends on the existence of a doubly ionized phosphate group as predicted by the NMR titrations of Mas and Colman (1984) and supported by the failure of $cNADP^+$ to be an effective coenzyme. The importance of the adenosine moiety in binding to this enzyme has also been shown by the strong binding of fragments of the coenzyme that retain the 2′-phosphate (Ehrlich & Colman, 1978; Mas & Colman, 1984). The reduced activity of analogues such as sNAD(P)$^+$ and $cNADP^+$ indicates that these compounds, even when bound, may not maintain the nicotinamide moiety in the proper configuration for hydride transfer from isocitrate to occur.

Nuclear Overhauser effect measurements with both enzymes indicate that all active analogues may bind in the same configuration of the adenine–ribose bond as the natural coenzymes. The analogues bound to NAD-dependent isocitrate dehydrogenase exhibit a measurable NOE between the A2′ and A8 protons but none between the A1′ and A8 protons. This corresponds to proximity between the A2′ and A8 protons

indicative of an anti configuration for the adenine–ribose bond. For analogues bound to NADP-dependent isocitrate dehydrogenase, there is a measurable NOE between the A1′ and A8 protons which for $NADP^+$ was attributed to a syn conformation for the adenine–ribose bond but is also consistent with a mixture of syn and anti conformations (Rosevear et al., 1987). Measurements on NADP-dependent isocitrate dehydrogenase indicate that all analogues exhibit an NOE between the A2′ and A8 protons of the same magnitude as that between the A1′ and A8 protons, although the overlap of the A2′ peak with the HDO peak complicates this measurement. It is difficult to fit these measurements to a single conformation but Stewart et al. (1989) have identified an anti conformation with angle O4′–C1′–N1–C4 of 35° that exhibits NOE's between both A1′ and A8 and between A2′ and A8. The 3-aminopyridine derivative, which is not a substrate, has a greater NOE between A1′ and A8 and may bind to the enzyme in the same mixture of syn and anti configurations that exists in solution.

The nicotinamide–ribose configurations of NAD^+ and $NADP^+$ bound to isocitrate dehydrogenases have been found to be in the anti configuration as expected for dehydrogenases catalyzing stereospecific addition of the hydride to the A side of the coenzyme (Levy et al., 1983). The NOE evidence for this conformation comes from the cross-relaxation observed between N1′ and N2 protons and between N2′ and N6 protons. In contrast, a syn conformation would place the N1′ and N6 protons close (<3 Å) yielding an NOE between these protons and between the N2′ and N2 protons. The NOE data on $sNAD(P)^+$ with both isocitrate dehydrogenases indicate that the bound species contain a mixture of syn and anti configurations. It is hence likely that the poor catalytic activity observed with thionicotinamide derivatives is a result of failure of the nicotinamide ring to maintain the proper geometry with respect to the isocitrate binding site for the stereospecific reaction to occur.

Evidence that nucleotides can bind to dehydrogenases with the nicotinamide moiety in several different configurations, only one of which is catalytically productive, is provided by analyses of crystal structures of a number of dehydrogenases. For liver alcohol dehydrogenase, four different modes of nucleotide binding have been directly determined from X-ray crystal structures (Cedergren-Zeppezauer, 1983). The productive mode corresponds to a tight configuration with little room for nicotinamide motion (Eklund et al., 1984). The three unproductive modes have the nicotinamide near the adenine pocket, close to the subunit interface or rotated 180° so that the B-side of the ring faces the active site (Cedergren-Zeppezauer, 1983). While these configurations have been obtained in the presence of inhibitors, there are precedents for a dehydrogenase which binds coenzymes in configurations that include the completely opposite nicotinamide–ribose configurations. A modeling study on alcohol dehydrogenase has indicated that more subtle changes may play an important role in altering catalytic activity. The carboxamide side chain (or substitutions) at the 3-position of the nicotinamide also shows a preferred orientation with respect to the ring. The magnitude of rotation of this side chain (based on modeling) has been correlated with changes in catalytic activity (Beijer et al., 1990). Maximal activity is obtained when the side chain is rotated out of the plane of the nicotinamide ring.

The binding of analogues to glyceraldehyde-3-phosphate dehydrogenase has been modeled by Wallén and Branlant (1983) and compared with kinetic data. A favorable hydrogen bond to an enzyme side chain is maintained in the case of $sNAD^+$, but in the case of $acNAD^+$ either the carboxamide must be rotated to avoid contact with the pyrophosphate group or the entire nicotinamide ring must be rotated. The hydrogen bond is believed to be important for productive binding. An internal hydrogen bond between the amido group of NAD^+ and the pyrophosphate may also be important for maintenance of configuration (Skarżyński et al., 1987).

The X-ray crystal structure of dihydrofolate reductase has also indicated a role for positioning of the carboxamide group in forming the catalytic complex. As in the case of alcohol dehydrogenase, the carboxamide group of the bound coenzyme is rotated through 180° from the most stable position in the free coenzyme (Filman et al., 1982). This configuration is stabilized by hydrogen bonds to the enzyme involving both the carbonyl and amido groups. The ^{13}C chemical shift observed for the carbonyl carbon of $NADP^+$ bound to dihydrofolate reductase (Way et al., 1985) may arise from this interaction with the protein. In contrast, lactate dehydrogenase, which uses sNADH as a coenzyme with a relative maximum velocity of 0.3, shows little difference in chemical shift for the bound carbonyl from that of the free nucleotide. On the basis of the chemical shift observed for NAD^+ bound to malate dehydrogenase, a significant role in orienting the nicotinamide ring would be predicted for interactions between the carbonyl and protein groups.

For both isocitrate dehydrogenases, binding of the nicotinamide carboxamide in a preferred conformation is indicated by NOE and chemical shift changes and these changes may arise from interaction with group(s) on the enzyme. The reduced activity with thionicotinamide derivatives indicates that the nature of the enzyme–carbonyl interaction is critical for proper positioning of the nicotinamide ring in a catalytic complex. The role of the amido group in coenzyme–enzyme interaction cannot be ascertained from this study. Nevertheless, little change in activity is observed when the amido group is replaced by a methyl group in the acetylpyridine derivatives. The NOE experiments gave no evidence for interaction of the acetyl group with amino acid side chains on the enzyme, but coordination with groups containing exchangeable protons or oxygen cannot be ruled out.

The nuclear magnetic resonance experiments have revealed that an analogue, $acNAD(P)^+$, which is used almost as efficiently as the natural coenzymes shows little change in configuration from that of $NAD(P)^+$ when bound to isocitrate dehydrogenases, while analogues such as $sNAD(P)^+$ which are poor substrates or weakly binding inhibitors ($amNADP^+$) show altered configuration of the nicotinamide when bound to isocitrate dehydrogenases. These results support previous studies indicating that the interaction of dehydrogenases with the nicotinamide moiety of coenzymes may be of less significance in the energy of interaction in binary complexes than it is in correctly positioning the coenzyme for the catalytic event.

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