^{1}H Nuclear Magnetic Resonance Studies of the Conformation and Environment of Nucleotides Bound to Pig Heart NADP$^+$-Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The binding of coenzymes, NADP$^+$ and NADPH, and coenzyme fragments, 2′-phosphoadenosine 5′-(diphosphoribose), adenosine 2′,5′-bisphosphate, and 2′-AMP, to pig heart NADP$^+$-dependent isocitrate dehydrogenase has been studied by proton NMR. Transferred nuclear Overhauser enhancement (NOE) between the nicotinamide 1′-ribose proton and the 2-nicotinamide ring proton indicates that the nicotinamide–ribose bond assumes an anti conformation. For all nucleotides, a nuclear Overhauser effect between the adenine 1′-ribose proton and 8-adenine ring proton is observed, suggesting a predominantly syn adenine–ribose bond conformation for the enzyme-bound nucleotides. Transferred NOE between the protons at A2 and N6 is observed for NADPH (but not NADP$^+$), implying proximity between adenine and nicotinamide rings in a folded enzyme-bound form of NADPH. Line-width measurements on the resonances of free nucleotides exchanging with bound species indicate dissociation rates ranging from <7 s^{-1} for NADPH to ≈ 1600 s^{-1} for adenosine 2′,5′-bisphosphate. Substrate, magnesium isocitrate, increases the dissociation rate for NADPH about 10-fold but decreases the corresponding rate for phosphoadenosine diphosphoribose and adenosine 2′,5′-bisphosphate about 10-fold. These effects are consistent with changes in equilibrium dissociation constants measured under similar conditions. The ^{1}H NMR spectrum of isocitrate dehydrogenase at pH 7.5 has three narrow peaks between δ 7.85 and 7.69 that shift with changes in pH and hence arise from C-4 protons of histidines. One of those, with pK = 5.35, is perturbed by NADP$^+$ and NADPH but not by nucleotide fragments, indicating that this histidine is in the region of the nicotinamide binding site. Observation of nuclear Overhauser effects arising from selective irradiation at δ 7.55 indicates proximity of either a nontitrating histidine or an aromatic residue to the adenine ring of all nucleotides. In addition, selective irradiation of the methyl region of the enzyme spectrum demonstrates that the adenine ring is close to methyl side chains. The substrate magnesium isocitrate produces no observable differences in these protein–nucleotide interactions. The alterations in enzyme–nucleotide conformation that result in changes in affinity in the presence of substrate must involve either small shifts in the positions of amino acid side chains or changes in groups not visible in the proton NMR spectrum.

The NADP$^+$-dependent isocitrate dehydrogenase [*threo*-D$_S$-isocitrate: NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] from pig heart catalyzes the oxidative decarboxylation of isocitrate in the presence of divalent metal ions. The enzyme belongs to the A stereospecific class with hydride transfer involving the *pro*-R hydrogen of the reduced nicotinamide product (Nakamoto & Vennesland, 1960). Direct measurements of nucleotide binding demonstrate that the reduced coenzyme, NADPH, binds at last 1 order of magnitude more tightly than the oxidized coenzyme (Ehrlich & Colman, 1975). Nucleotides containing adenosine 2′-phosphate compete with NADP$^+$ and NADPH (Ehrlich & Colman, 1978; Mas & Colman, 1985).

The environment of bound coenzymes and analogues has previously been examined by chemical modification, ^{31}P nuclear magnetic resonance, and optical spectroscopic techniques. Ethoxyformic anhydride modifies a histidyl residue with pK = 5.67 that can be protected by NADP$^+$, NADPH, or nucleotide fragments containing the adenosine 2′-phosphate (Ehrlich & Colman, 1978). Periodate-oxidized NADPH has been shown to be an affinity label for NADP-dependent isocitrate dehydrogenase, but the product is not a Schiff base with lysine, and the residue modified was not identified (Mas & Colman, 1983). The ^{31}P NMR spectra of NADP$^+$, NADPH, and Rib-P$_2$-Ado-P[1] show that the chemical shifts of the 2′-phosphate of the bound nucleotides are identical for all three compounds (Mas & Colman, 1984) and that this chemical shift remains almost constant between pH 5 and pH 8. It is probable that the 2′-phosphoryl group of the enzyme-bound nucleotide is ionized throughout this pH range and is not perturbed by proton dissociation from an enzymic group.

UV difference and fluorescence spectroscopies indicate that NADP$^+$, NADPH, Rib-P$_2$-Ado-P, and Ado(2′,5′)P$_2$ all perturb tryptophanyl residues on the enzyme (Mas & Colman, 1985). The changes seen in difference spectra obtained with binary complexes of enzyme and NADPH, NADP$^+$, Rib-P$_2$-Ado-P, and Ado(2′,5′)P$_2$ are similar. Sulfate ion decreases the affinity of the enzyme for nucleotides but does not cause spectral perturbation. NADP$^+$, Rib-P$_2$-Ado-P, and Ado-(2′,5′)P$_2$ also produces an enhancement in tryptophan fluorescence, which is eliminated by addition of metal isocitrate.

The present study has two major aims: to examine the conformation of nucleotides bound to NADP-dependent isocitrate dehydrogenase and to investigate and compare the environments of different nucleotides bound to the enzyme. Nucleotide conformation is probed by measuring intramolecular ^{1}H–^{1}H nuclear Overhauser effects (Noggle &

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[1] Abbreviations: Rib-P$_2$-Ado-P, 2′-phosphoadenosine 5′-(diphosphoribose); Ado(2′,5′)P$_2$, adenosine 2′,5′-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; ICDH, NADP$^+$-dependent isocitrate dehydrogenase; NOE, nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid.

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Schrimer, 1971). These measurements in the presence of exchange of ligands between free and enzyme-bound states were reported by Albrand et al. (1979), and the theory has been developed by Clore & Gronenborn (1982, 1983). This technique has been used to compare binding of nucleotide analogues to dihydrofolate reductase (Feeney et al., 1983), to compare conformations of NAD^+ and $NADP^+$ bound to a number of A and B stereospecific dehydrogenase (Levy et al., 1983), to examine the conformation of $Co(NH_3)_4ATP$ bound to protein kinase (Rosevear et al., 1983), and to elucidate the conformations of NAD^+ bound to sorbitol dehydrogenase (Gronenborn et al., 1984a) and of $NADP^+$ bound to glucose-6-phosphate dehydrogenase (Gronenborn et al., 1984b).

The enzyme environment of the bound nucleotide can be probed by use of intermolecular nuclear Overhauser effects between protons on the enzyme and those on the nucleotide or by perturbations in the protein NMR spectrum (Balaram et al., 1972a,b). Nuclear Overhauser effects have been used to investigate the environment of ATP on creatine kinase (James, 1976; Vasák et al., 1979) and Mg-ATP on adenylate kinase (Smith & Mildvan, 1982).

The current study explores alterations in the environment and/or conformation of the nucleotides bound to isocitrate dehydrogenase upon addition of the substrate magnesium isocitrate. In favorable cases, the nucleotide spectra at different nucleotide/enzyme ratios yield information on the affinity and kinetics of binding, and these are determined in the presence and absence of isocitrate. A preliminary version of part of this work has been presented (Ehrlich & Colman, 1984).

Experimental Procedures

Materials. $NADP^+$-dependent isocitrate dehydrogenase was isolated from pig hearts by a scaled-up procedure following the method of Bacon et al. (1981). The enzyme was concentrated to 25–30 mg/mL in an Amicon ultrafiltration cell with a PM-10 membrane. For NMR experiments, the enzyme was dialyzed against the appropriate buffer followed by dialysis against three changes of 10 mL each of the appropriate buffers prepared in D_2O. Enzyme activity was measured spectrophotometrically (Colman, 1968), and enzyme concentration was determined with $E^{1\%}_{280} = 10.8$ (Johanson & Colman, 1981a). Activity was assayed before and after the NMR experiments, and the specific activity remained constant at 30–35 units/mg of protein. At pH 5–6 over a period of 12 h some precipitation occurred with the loss of about 20% of the initial protein. MOPS, MES, DL-isocitrate, $NADP^+$, NADPH, Rib-P_2-Ado-P, Ado(2′,5′)P_2, and 2′-AMP were all obtained from Sigma Chemical Co. D_2O (99.8% D) was obtained from Aldrich Chemical Co. NADPH was dissolved directly in the appropriate buffer immediately prior to use. Other nucleotides were dissolved in buffers prepared in D_2O, the pH was adjusted, and then they were lyophilized and redissolved in D_2O.

NMR Measurements. ^1H NMR spectra were measured in a Bruker WM-250 spectrometer at 250.13 MHz using quadrature phase detection, a 12-bit digitizer, and a spectral width of 3521 Hz. Spectra were accumulated in 8K memory, and a pulse width of 5.0 μs (≈90°) was used. Samples (0.25–0.35 mL) were contained in 5-mm tubes that were not spun so as to avoid protein denaturation. Dioxane (1 mM) was added as an internal reference (taken as 3.71 ppm).

Time-dependent nuclear Overhauser effects (NOE) were measured with the sequence t_1–t_2–$\pi/2$–acquisition. During t_2, selective irradiation of a proton resonance of the nucleotide or region of the proton spectrum is applied (21–27 db below 0.2 W), while t_1 is a relaxation delay and the acquisition time is 1.16 s. The sum of t_1 and t_2 was kept constant (usually 0.5 s) while the irradiation time t_2 was varied. Saturation of the free nucleotide resonances was complete for $t_2 \geq 0.05$ s.

To detect NOE between protein and nucleotide, the region from 0 to 8.0 ppm was irradiated at 10–25-Hz intervals. Blocks of 32 scans were accumulated, and the procedure was repeated until 320–1024 scans were obtained. Free induction decays were subtracted from a control irradiated at −1.5 ppm. NOE's are expressed as a decimal fraction of the intensity obtained with the control. In the presence of enzyme, all NOE's are negative (sign omitted).

Line widths were obtained from the peak widths at half-maximum amplitude ($\Delta\nu_{1/2}$). Under conditions of slow exchange, only peaks corresponding to free nucleotide are observed. The rate constant for release of bound nucleotide from the enzyme, k_{off}, was determined from the following (Pople et al., 1959):

$$\frac{1}{T_2^*} = \pi(\Delta\nu_{1/2}) \tag{1}$$

and

$$\frac{1}{T_2^*} = \frac{1}{T_{2F}} + \frac{P_b k_{off}}{P_f} \tag{2}$$

where T_2^* is the empirical transverse relaxation time, T_{2F} is the transverse relaxation time of a proton in free nucleotide, and P_f and P_b are the fractions of free and bound nucleotides. When exchange is fast on the chemical shift scale

$$\frac{1}{T_2^*} = \frac{1}{T_{2F}} + \frac{P_b}{T_{2B} + 1/k_{off}} \tag{3}$$

where T_{2B} is the relaxation time of the proton in the enzyme-bound nucleotide. In the intermediate region, both line width and peak position vary with nucleotide/enzyme ratio, and a more complex analysis is necessary (Feeney et al., 1979). For fast exchange in the presence of a chemical shift difference between sites, the additional contribution to the relaxation rate (Pople et al., 1959) is

$$\frac{1}{T_{2\,\text{exchange}}} = \frac{4\pi^2 P_b(1 - P_b)^2}{k_{off}}(\Delta\nu)^2 \tag{4}$$

For titration of histidine residues, enzyme was dialyzed into 5 mM MES or 5 mM MOPS buffer of the desired pH containing 0.5 mM EDTA and 0.15 M Na_2SO_4. The pH was measured with a combined electrode (Radiometer) and a Radiometer Model 26 pH meter. The readings were not corrected for D_2O since histidine pK values determined in D_2O with uncorrected electrode readings agree with those obtained in H_2O (Meadows, 1972). Spectral resolution was enhanced by convolution difference (Campbell et al., 1973). Titration curves were analyzed by a nonlinear least-squares computer program following Marquardt's algorithm (Marquardt, 1963; Mas & Colman, 1984). The pH region (8–6) in which distinct peaks could be followed was used to obtain δ_u and δ_p, the shifts for the unprotonated and protonated species, and the pK values obtained were used to draw theoretical titration curves for the region where peaks appear to cross.

Results

Binding of $NADP^+$ to Isocitrate Dehydrogenase. The numbering of protons in $NADP^+$ is indicated in Figure 1, which is drawn with adenine in the syn conformation and nicotinamide in the anti conformation. Figure 2 shows the

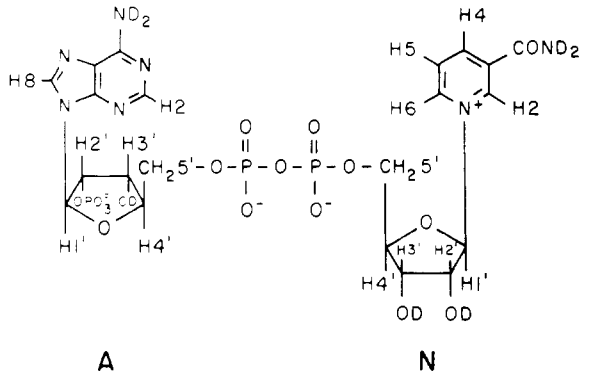

FIGURE 1: Numbering system for the protons of $NADP^+$ drawn with adenine (A) in the syn conformation and nicotinamide (N) in the anti conformation.

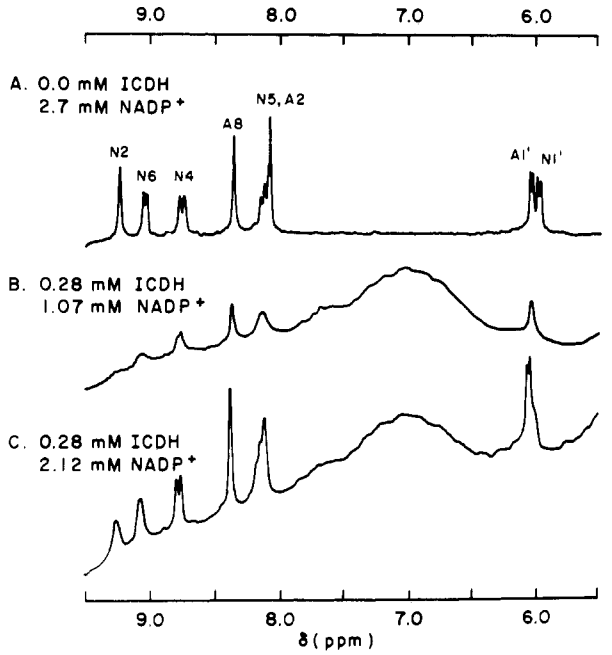

FIGURE 2: ^1H NMR spectra of $NADP^+$. Spectra were obtained at 25 °C on a Bruker WM-250 at 250.13 MHz with spectral width 3521 Hz, 8K memory, and 90° pulse. All spectra result from 500 or more accumulations. $NADP^+$ and enzyme concentrations are as indicated. Assignments of peaks are by comparison with the literature (Oppenheimer, 1982).

aromatic region of the ^1H NMR spectrum of $NADP^+$ obtained in Na–0.05 M MOPS (pH 7.5) containing 0.15 M Na_2SO_4 and 0.5 mM EDTA. In the presence of enzyme (Figure 2B,C) the lines are broadened, but there are only small changes in chemical shifts.

Figure 3 shows the excess line widths of the A2 and A8 resonances in the presence of enzyme as a function of the ratio of bound nucleotide to free nucleotide. The concentration of bound nucleotide was calculated from a dissociation constant of 0.15 mM for the enzyme–$NADP^+$ complex. This value gives the best fit to the NMr data and is consistent with the value of 0.11 mM determined in the presence of 0.1 M Na_2SO_4 (Mas & Colman, 1985). From the increase in line width of A8 with P_b, eq 3, the exchange rate, k_{off}, is ≥ 86 s^{-1}; this value of k_{off} is a lower limit if $T_{2B} = 0$. A similar analysis based upon the N4 resonance gives $k_{off} \geq 78$ s^{-1}. From Figures 2 and 3 it can be seen that resonance A2 is broadened more than A8. While assignment of the position of A2 bcomes difficult at low nucleotide/enzyme ratios because of overlap of A2 with N5, if the position of A2 is taken to coincide with the observed maximum, then A2 is shifted 40 Hz (0.16 ppm) downfield.

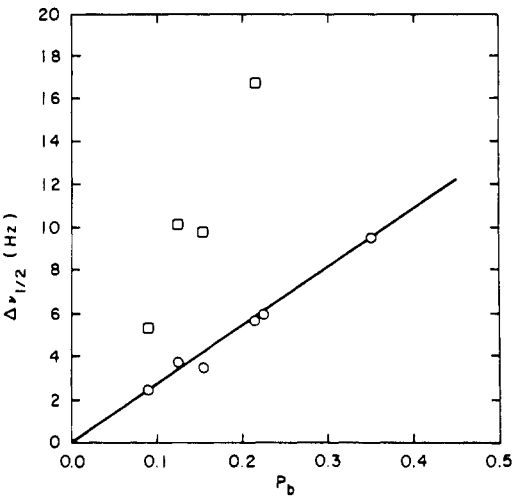

FIGURE 3: Broadening of peaks of $NADP^+$ as a function of proportion of nucleotide bound (P_b). The broadening ($\Delta\nu_{1/2}$) of A2 (□) and A8 (○) is the difference between the measured line widths at half-maximum amplitude of nucleotide plus enzyme and of free nucleotide.

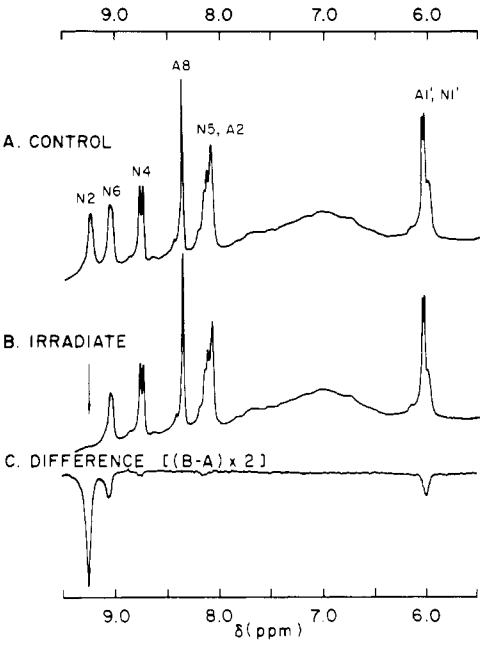

FIGURE 4: Transferred nuclear Overhauser effect in $NADP^+$. The samples contain 5.3 mM $NADP^+$ and 0.47 mM ICDH. In (B), resonance N2 is irradiated for 0.3 s. Spectra result from 512 scans.

With this value and the observed broadening, eq 4 gives an exchange rate $k_{off} = 107 \pm 18$ s^{-1}. A similar analysis shows a small shift in N6 of 17 ± 5 Hz (0.068 ppm) and a corresponding $k_{off} = 121 \pm 40$ s^{-1}. Comparion of the values obtained from eq 4 with those found from eq 3 gives $T_{2B} \approx 0.003$ s.

Conformation of $NADP^+$ Bound to Isocitrate Dehydrogenase. The conformation of $NADP^+$ bound to isocitrate dehydrogenase was investigated by means of the transferred nuclear Overhauser effect (Clore & Gronenborn, 1982, 1983). A given proton of the free nucleotide is irradiated. During the irradiation period, the nucleotide exchanges between free and bound states. In the bound state, cross-relaxation occurs between the irradiated proton and other protons of the bound nucleotide, which is then manifested by a reduction in the amplitudes of those protons in the NMR spectrum of free nucleotide. Figure 4 shows spectra obtained upon irradiation of the N-2 proton of $NADP^+$. For ligands bound to large molecules, the NOE's are negative. In Figure 4 a reduction

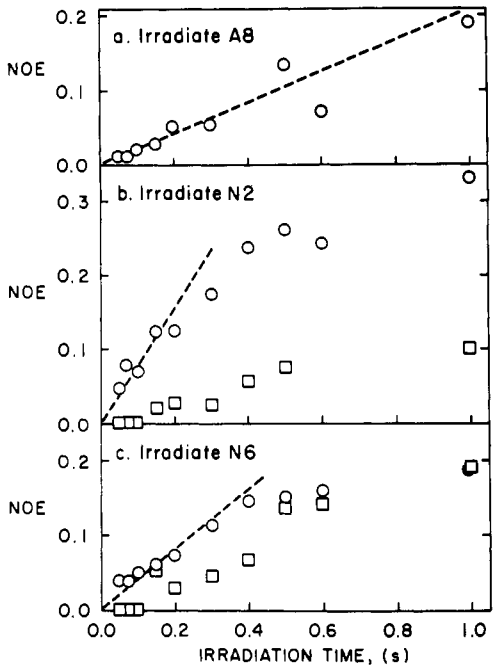

FIGURE 5: NOE's observed for $NADP^+$ as a function of irradiation time: (A) A8 is irradiated and A1′ is observed; (B) N2 is irradiated, and N1′ (○) and N5 (□) are observed; (C) N6 is irradiated and N1′ (□) and N5 (○) are observed. Values of NOE's have estimated errors of ±0.02.

in the amplitude of N1′, but not in the amplitude of the nearly overlapping resonance A1′, is observed. The effect seen on N6 arises from spillover of irradiation power to the region of N6. Clore & Gronenborn (1983) have shown that the observed NOE arising from irradiation of either the averaged free and bound resonance or the free resonance (in the case of fast exchange on the cross-relaxation scale) is given by

$$\left(\frac{d(NOE)}{dt}\right)_{t=0} = -(P_b\sigma_B + P_f\sigma_F) \quad (5)$$

where σ_B and σ_F are the cross-relaxation rates for bound and free molecules. Figure 5 illustrates plots of the NOE's for several resonances as a function of irradiation time. Where a lag is observed as in the NOE upon N1′ when N6 is irradiated (Figure 5C), the effect is due to spin–diffusion (Clore & Gronenborn, 1983), presumably through protons within the enzyme. Table I summarizes the effects observed for various combinations of resonances. For * ≤ 0.5 s, the NOE's observed for free nucleotide are positive and <0.03 so that the observed NOE depends primarily on σ_B. If the correlation times for sets of protons (i, j) and (k, l) are the same, the distances between them can be calculated from the cross-relaxation rates determined for these pairs:

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

The protons in the nicotinamide ring are fixed with r(N5, N6) = r(N5, N4) = 2.48 Å. Distances were calculated from eq 6 for all pairs showing NOE for short irradiation times (t < 0.15). The results tabulated in Table I indicate that N1′ is close to N2 and N3′ is close to N6. Thus, the conformation of the nicotinamide–ribose bond is anti in the $NADP^+$–isocitrate dehydrogenase complex. The distances (N1′, N2) and (N3′, N6) are almost equal, and the distance (N3′, N6) is greater than the distance (N2′, N6), which is consistent with a 3′-endo configuration of the nicotinamide ribose ring.

The distance between A1′ and A2′, 2.71 Å, is within the limits 2.9 ± 0.2 Å determined in crystal structures of adenosine

Table I: Cross-Relaxation between Proteins of $NADP^+$

irradiated resonance	obsd resonance	cross-relaxation rate, σ (s^{-1})[a]	r[b] (Å)
N2	N1′	7.9 ± 1.7	2.25 ± 0.11
N1′	N2	8.5 ± 1.5	
N6	N5	4.6 ± 0.7	2.48[c]
N5, A2	N6	4.6 ± 1.3	
N3′	N6	6.5 ± 0.7	2.34 ± 0.10
N5	N4	5.1 ± 1.4	2.51 ± 0.10
N4	N5	5.0 ± 1.6	
A8	A1′	2.5 ± 0.3	2.77 ± 0.17
A1′	A8	2.2 ± 0.9	
A2′	A1′	2.7 ± 1.0	2.71 ± 0.20

[a] Cross-relaxation rates between protons were calculated (eq 5) from the initial slopes of plots of NOE vs. time (Figure 5). Pairs (N2, N5), (N2, N4), (A2, A8), and (N6, N1′) either showed effects due to spillover causing direct saturation or lags indicative of spin–diffusion and r > 3.5 Å. [b] Calculated using eq 6 and assuming r(N5, N6) = 2.48 Å. [c] Assumed.

(Lai & Marsh, 1972) and AMP (Neidle et al., 1976) and the distance, 2.81 Å, found for NAD^+ bound to yeast alcohol dehydrogenase (Clore & Gronenborn, 1983). The observation of an NOE between A1′ and A8 and the absence of significant NOE's (r > 3.2 Å) between A2, A3′, A5′, and A8 indicate that $NADP^+$ bound to isocitrate dehydrogenase has a predominantly syn conformation about the adenine–ribose glycoside bond.

Binding of NADPH to Isocitrate Dehydrogenase. When the concentration of NADPH is less than the concentration of enzyme, no resonances from NADPH are observed, indicating that the NADPH is bound but the signals are broadened. Unlike $NADP^+$, the line widths of excess free NADPH in the presence of isocitrate dehydrogenase remain narrow, and no shifts can be observed. Hence, either the chemcial shift is almost identical in free NADPH and enyzme-bound NADPH, or exchange is slow and the observed lines are lifetime-broadened lines of free nucleotide. In a sample containing 2.5 mM NADPH and 0.41 mM enzyme, no additional peaks due to bound nucleotide were found. An attempt was made to locate resonances of the bound nucleotide by saturation transfer (Forsén & Hoffman, 1963; Hyde et al., 1980). Irradiation at the position of a resonance of the bound nucleotide should decrease the amplitude of the corresponding resonance of the free nucleotide. Selective irradiation for 0.4–0.5 s was applied between 7.0 and 9.5 ppm, and no effects attributable to saturation transfer were observed. A reduction in A2 due to irradiation at 7.55 ppm was observed but was present even for analogues in fast exchange (see below) and hence cannot arise from saturation transfer at the bound A2 position. It is possible the resonances of bound nucleotide are too broad to saturate or too close to the free positions.

To increase the exchange rate, spectra were measured at 35 °C. From the line widths, the dissociation rate of NADPH is estimated to be less than 7 s^{-1}. Even though exchange is clearly slow on the chemical shift scale, NOE's should be observable provided exchange is sufficiently rapid compared to relaxation rates. Figure 6A shows difference spectra generated when selective irradiation is applied to particular resonance of NADPH. Effects (albeit of different magnitudes) are observable between A8 and N2, A8 and A1′, A2 and N2, and A2 and N6. In order to ascertain which effects may be ascribed to cross-relaxation rather than spin–diffusion, measurements were again made as a function of irradiation time (Figure 6B).

The decreased amplitude of N2 following irradiation of A8 is clearly characterized by a lag, indicative of spin–diffusion.

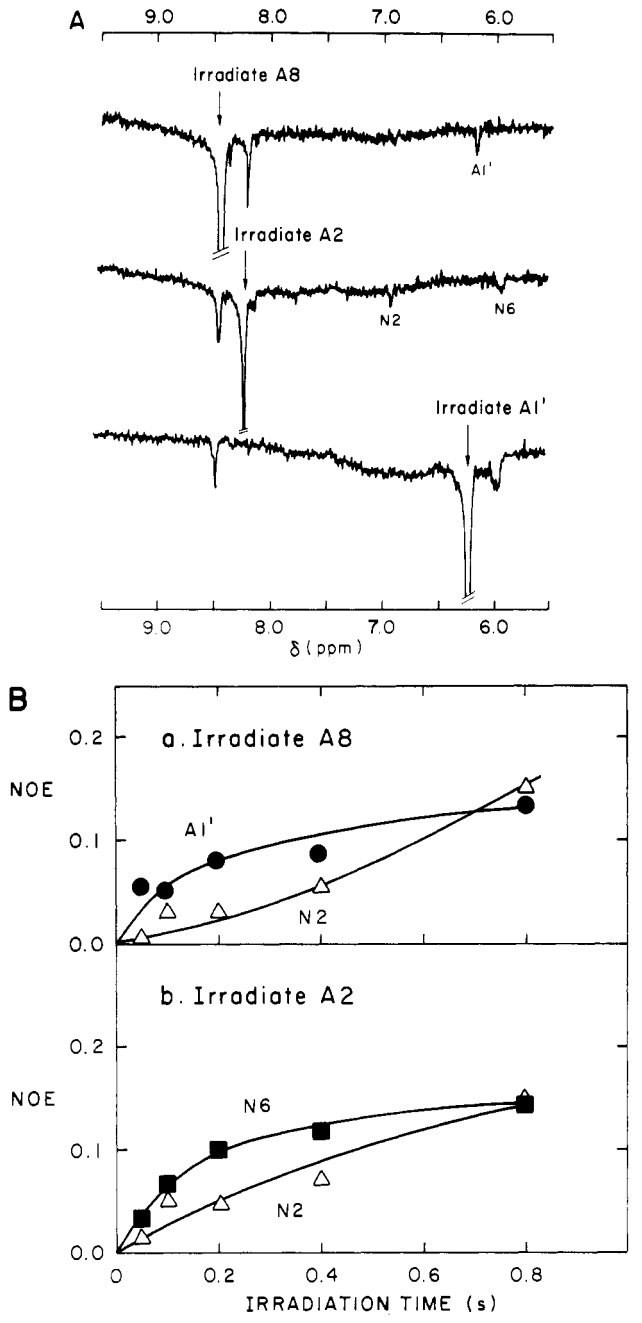

FIGURE 6: Transferred NOE's in NADPH. Irradiation time was 0.2 s. (A) The spectra are difference spectra between those with irradiation as indicated and a control irradiated at −1.5 ppm. (B) The NOE as a function of irradiation time is shown for irradiation of A8 and A2. Measurements of NOE's have estimated errors of ±0.02.

For (A8, A1′), (A2, N6), and (A2, N2), a more rapid rate of increase in the NOE at short times is seen. This is evidence that NADPH, like $NADP^+$, has a predominantly syn conformation for the adenine–ribose bond. A new feature obtained from the NOE pattern for NADPH is the close proximity of the nicotinamide and adenine rings. This can only occur if NADPH bound to isocitrate dehydrogenase exists in a folded conformation. The conformation of the nicotinamide–ribose bond could not be ascertained. Irradiation of N1′ coincides with irradiation of N5, and irradiation at this position (4.9 ppm) produces an NOE on the N6 resonance. If the conformation were syn, irradiation at N3′ should affect N2. No effect on either N2 or N6 was seen upon irradiation in the vicinity of N3′.

Binding of Rib-P_2-Ado-P to Isocitrate Dehydrogenase. As with $NADP^+$, the resonances of Rib-P2-Ado-P broaden in the presence of enzyme. For the A2 resonance, exchange is intermediate in that the peak position shifts as the amount bound increases. Variation of shift with nucleotide concentration is consistent with K_D <0.01 mM. Extrapolation to completely bound analogue gives a downfield shift of 0.19 ppm comparable to the 0.16 ppm estimated for $NADP^+$. The A1′ resonance shows an upfield shift of about 0.10 ppm. Using these values and the excess width of A2 and A1′ in eq 4 yields $k_{off} \geq 480 \pm 120\ s^{-1}$.

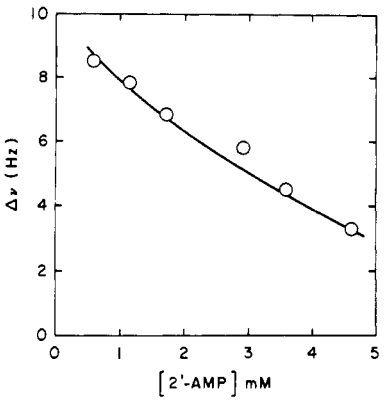

FIGURE 7: Shift in the A2 resonance of 2′-AMP as a function of concentration with 0.4 mM NADP-dependent isocitrate dehydrogenase. The line is a fit to the data with $\Delta\nu_b$ = 118 Hz and a dissociation constant of 2.9 mM.

Irradiation of the A8 resonance led to reduction in the amplitude of the A1′ resonance. This reduction was measured at various irradiation times to give a cross-relaxation rate of 14.4 s^{-1}. A similar experiment in which A2′ was irradiated and A1′ observed gave a cross-relaxation rate of 4.6 s^{-1}. Taking the distance between A1′ and A2′ to be 2.9 Å, the distance between A1′ and A8 is calculated to be 2.4 Å. Again, the adenosine glycoside bond is in a syn conformation.

Binding of Ado(2′,5′)P_2 and 2′-AMP. Binding of Ado-(2′,5′)P_2 produces a downfield shift of A2 (0.23 ppm) and a small upfield shift of A1′ (0.06 ppm). Line broadening is reduced compared with that of Rib-P_2-Ado-P. An approximate value for k_{off} of $3.0 \times 10^3\ s^{-1}$ is calculated with K_D = 0.15 mM. Nuclear Overhauser effects are obtained between A1′ and A8 (NOE = 0.08 for t = 0.3 s with Ado(2′,5′)P_2/ICDH = 12.5), and again, the adenosine is in the syn conformation.

With 2′-AMP, binding could not be detected in the presence of 0.15 M Na_2SO_4. Na_2SO_4 has been found to increase the dissociation constants for NADPH, $NADP^+$, and Rib-P_2-Ado-P (Mas & Colman, 1985). The Na_2SO_4 concentration was reduced to 0.025 M, and binding could then be observed as described below.

Binding of Nucleotides in the Presence of 0.025 M Na_2SO_4. At 0.025 M Na_2SO_4, NMR spectra of 2′-AMP in the presence of isocitrate dehydrogenase indicate conditions of fast exchange: line widths remain narrow, but the position of the A2 resonance changes with nucleotide/protein ratio. The shift can be fit to the expression

$$\delta_{obsd} = P_b(\delta_b - \delta_f) + \delta_f \quad (7)$$

where δ_{obsd}, δ_b, and δ_f are the chemical shifts of the observed, enzyme bound, and free nucleotide and P_b is calculated from an assumed dissociation constant. Equation 7 is used to calculate $\delta_b - \delta_f$ for each 2′-AMP concentration. K_D is varied until the variation in $\delta_b - \delta_f$ is a minimum. The data (Figure 7) are best fit with K_D = 2.9 mM and a shift $\delta_b - \delta_f$ corresponding to 118 ± 20 Hz (0.48 ppm). This latter value is larger than was seen for other nucleotides at 0.15 M Na_2SO_4.

Table II: Dissociation Rates for Nucleotides from Isocitrate Dehydrogenase

addition		k_{off} (s^{-1})[a]				
Na_2SO_4 (M)	Mg-IC[b]	NADPH[c]	$NADP^+$	Rib-P_2-Ado-P	Ado(2′,5′)P_2	2′-AMP
0.025	−	<7	36 ± 8	220 ± 30	1600	*d*
0.15	−	<7	115 ± 30	480 ± 120	3000	*d*
0.025	+	110 ± 40	*e*	28 ± 7	300 ± 60	*d*
0.15	+	61 ± 11	*e*	54 ± 10	500 ± 200	*d*

[a] Determined from the line widths of various amounts of nucleotide in the presence of enzyme. [b] 5 mM $MgSO_4$ and 4 mM DL-isocitrate. [c] Determined at 35 °C in the absence of DL-isocitrate. [d] Cannot be determined because of fast exchange. [e] Cannot be determined because of enzymatic conversion to NADPH.

Titrations of enzyme were performed with Ado(2′,5′)P_2, Rib-P_2-Ado-P, $NADP^+$, and NADPH in the presence of 0.025 M Na_2SO_4.

The shift determined for Ado(2′,5′)P_2, 0.28 ppm, is in close agreement with the value 0.19 ppm obtained at 0.15 M Na_2SO_4. The other nucleotides exhibited slow exchange, and the shifts could not be determined. Dissociation rates were determined and are compared with those obtained with 0.15 M Na_2SO_4 in Table II. Only small changes in the dissociation rate occur when the Na_2SO_4 concentration is changed. The nuclear Overhauser effects are unchanged by lowering the Na_2SO_4 concentration from 0.15 to 0.025 M. Adenosine glycoside bonds are syn in all cases. NOE's are observed between N2 and N1′ and between N3′ and N6 of $NADP^+$, indicating an anti conformation for the nicotinamide–ribose bond.

Effect of Magnesium Isocitrate upon Nucleotide Binding. Comparison of dissociation constants with Michaelis constants indicates that, in the presence of sulfate, the substrate magnesium isocitrate enhances the binding of nucleotides with the exception of NADPH, for which the affinity is reduced in the presence of substrate (Ehrlich & Colman, 1975, 1978). The ^1H NMR spectra of nucleotides bound to isocitrate dehydrogenase in the presence of magnesium isocitrate were compared to spectra obtained in the absence of substrate.

For NADPH, the most striking difference upon adding substrate is the transition from very slow exchange to intermediate exchange with broadening and shifting of the A2 resonance. From analysis of line widths as a function of NADPH/enzyme ratio, a dissociation rate $\geq 110 \pm 40$ s^{-1} in the presence of 0.15 M Na_2SO_4 was obtained. Downfield shifts of A2, N2, and N6 are 0.16, 0.11, and 0.08 ppm, respectively. A small upfield shift of 0.04 ppm is found for the A1′ resonance.

In contrast, the binding of Rib-P_2-Ado-P and Ado(2′,5′)P_2 is characterized by slower exchange rates in the presence of magnesium isocitrate as presented in Table II. For Rib-P_2-Ado-P, slow exchange prevents determination of chemical shifts. For Ado(2′,5′)P_2, an upfield shift of 0.13 ppm for A2 and a downfield shift of 0.04 ppm for A1′ are observed. For 2′-AMP, shifts of 0.22 ppm (A2) and $\leq$0.04 ppm (A1′) are found. Comparison with data obtained in the absence of substrate indicates that the chemical shifts of A2 and A1′ are reduced somewhat by magnesium isocitrate.

The changes in chemical shifts could arise from changes in enzyme conformation or in nucleotide conformation. To see if nucleotide conformation is changed, we made nuclear Overhauser measurements between the same protons as in the absence of substrate. For NADPH, the observed effects are similar to those obtained in the absence of substrate (Figure 6A). Cross-relaxation rates obtained by measuring NOE's as a function of irradiation rate are given in Table III, along with the calculated distances between protons. Cross-relaxation between N1′ and N2 indicates an anti conformation for the nicotinamide–ribose bond, which in the presence of substrate could be confirmed by observation of an NOE between N3′ and N6. An NOE between A1′ and A8 was observed. NOE's of the same magnitude are observed in the rest of the ribose region (4–4.7 ppm), but these may be due to irradiation of the protein since the effects on A8 and A2 are the same. Thus, it is most likely that the adenosine glycoside bond conformation is syn. An NOE between the A2 and N6 protons was observed. NOE's of the same magnitude are observed in the rest of the ribose region (4–4.7 ppm), but these may be due to irradiation of the protein since the effects on A8 and A2 are the same. Thus, it is most likely that the adenosine glycoside bond conformation is syn. An NOE between the A2 and N6 protons was observed. This demonstrates proximity of the adenine and nicotinamide rings in a folded conformation of NADPH bound to isocitrate dehydrogenase.

Table III: Cross-Relaxation between Protons of NADPH in the Presence of Magnesium Isocitrate

irradiated resonance	obsd resonance	cross-relaxation rate, σ_B (s^{-1})	r (Å)[a]
N5	N6	4.0 ± 1.3	2.48[b]
N1′	N2	5.4 ± 1.4	2.36 ± 0.21
N3′	N6	4.8 ± 0.7	2.41 ± 0.14
N6	A2	4.7 ± 1.7	2.55 ± 0.21
A2′	A1′	1.8 ± 0.4	2.83 ± 0.19
A1′	A8	6.0 ± 1.6	2.32 ± 0.16

[a] Calculated assuming r(N5, N6) = 2.48 Å. [b] Assumed.

For Rib-P_2-Ado-P, NOE's between A1′ and A8 were observed, indicating a syn conformation as in the absence of substrate. Cross-relaxation rates were 12.7 s^{-1} for (A1′, A8) and 4.3 s^{-1} for (A2′, A1′), yielding a distance of 2.42 Å between A1′ and A8. These measurements indicate that there are no appreciable differences between the conformations of nucleotides bound to isocitrate dehydrogenase in the presence and absence of magnesium isocitrate.

^1H NMR Spectrum of Isocitrate Dehydrogenase. Except in the aromatic region, the ^1H NMR spectrum of isocitrate dehydrogenase is relatively featureless. A broad peak centered at 0.9 ppm arises from the contribution of many methyl groups. The failure to observe individual peaks arises from the relatively slow rotation of the isocitrate dehydrogenase dimer (M_r 116 000) causing broadening. Figure 8A shows the aromatic region at several different pHs. Peaks at 7.04 ppm and 7.3 ppm remain largely unchanged as the pH is lowered from 7.5 to 5.4 and may arise from several tyrosines or tryptophans.

Three narrow peaks appear at even lower field, and these peaks change with pH. Judging from the area, the peak labeled 3 probably arises from more than one residue. On the basis of the pH behavior, the residues giving rise to these peaks are histidines. The chemical shifts of peaks in the region above 7.6 ppm are plotted in Figure 8B. Where individual peaks may be observed, the data are fit to a standard titration curve. This is extrapolated to lower pH, yielding the assignments given in Figure 8A. The values obtained for p*K*s and chemical shifts are given in Table IV. Figure 8B includes additional points

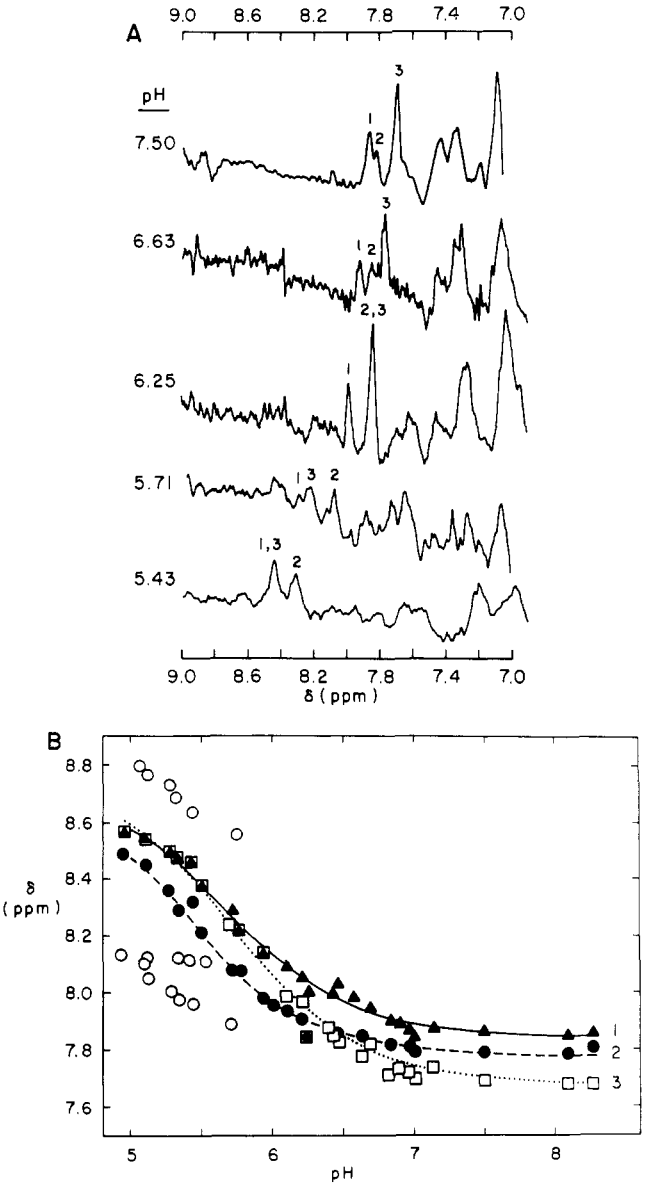

FIGURE 8: ^{1}H spectra of isocitrate dehydrogenase. (A) The downfield region of the spectra at five different pHs. Spectra are convolution spectra with line broadenings of 2 and 10 Hz and an additive constant of 1.0. A total 2000 or more scans was taken with protein concentrations in the range 0.25–0.45 mM. The narrow peaks that move with changes in pH have been labeled. Assignments have been made by determining which peaks followed a standard titration curve. (B) pH dependence of all peaks observed between 7.6 and 9.0 ppm. The lines are fits of peaks 1 (—), 2 (– –), and 3 (···) to titration curves with values given in Table IV.

Table IV: p*K*s and Chemical Shifts of Histidines[a]

peak	p*K*	δ_{prot}	δ_{unprot}
1	5.68 ± 0.04	8.74 ± 0.04	7.84 ± 0.01
2	5.35 ± 0.16	8.79 ± 0.26	7.79 ± 0.01
3	5.75 ± 0.05	8.76 ± 0.04	7.68 ± 0.01

[a] Values are obtained from nonlinear least-squares of data in Figure 8B.

corresponding to peaks appearing at low pH, which cannot be assigned p*K*s. These could be histidines or, in the case of the one that does not shift with pH, a very slowly exchanging peptide amide proton (Clark et al., 1982; Markley, 1975).

Perturbation of the ^{1}H Spectrum by Nucleotides. Figure 9 shows the spectrum of the histidines in the presence and absence of $NADP^+$ and NADPH at pH 7.5. Peaks labeled 1 and 3 do not move when nucleotides are added. Peak 2 shifts

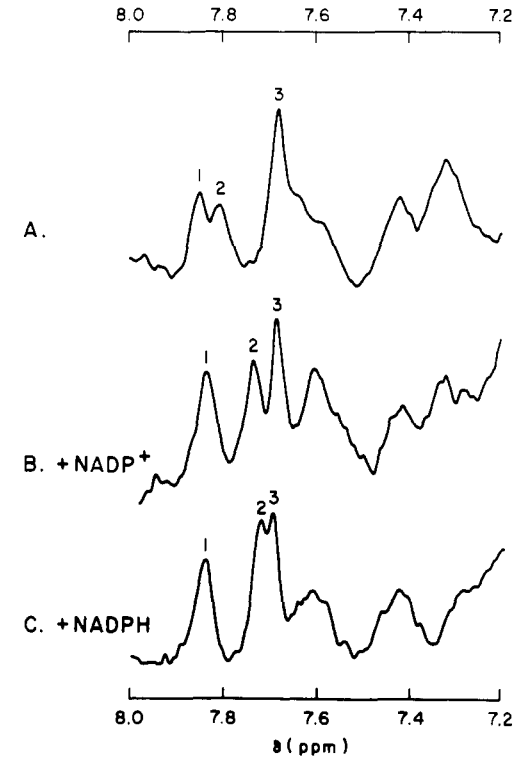

FIGURE 9: ^{1}H spectra of isocitrate dehydrogenase in the region 7.2–8.0 ppm with and without nucleotides. All were recorded at pH 7.50.

upfield by 0.07 ppm in the presence of $NADP^+$ or NADPH. The chemical shifts in the presence or absence of nucleotide of all three peaks are unchanged by addition of 5 mM $MgSO_4$ and 4 mM DL-isocitrate. Comparison of spectra at several different pHs between 5.4 and 7.6 indicate that magnesium isocitrate produces little or no change in the p*K*s of any of the observed histidines. In the presence of nucleotides, peak 2 could not be followed throughout the pH range covered in Figure 8, but results in the range 6.3–8.1 indicate there is little change in the p*K* and the major effect is the 0.07 ppm upfield shift.

Saturating concentrations of Rib-P_2-Ado-P, Ado(2′,5′)P_2, or 2′-AMP fail to produce any changes in the observed ^{1}H NMR spectrum. Thus, the histidine giving rise to peak 2 is most likely in the vicinity of the nicotinamide ring and not near the adenine ring or the phosphates.

NOE between Enzyme Protons and Nucleotide Protons. Even though individual residues of isocitrate dehydrogenase other then the histidines shown in Figure 10 are not discernable, it may be possible to identify groups close to the bound nucleotides by means of transferred nuclear Overhauser effects between protons on these groups and protons on the nucleotides. In the presence of $NADP^+$, irradiation was applied between 0 ppm and 8 ppm, and the amplitudes of the resonances of free $NADP^+$ were measured. The effects observed from 7 to 8 ppm and 0 to 2 ppm are shown in Figure 10. In the region 7–8 ppm (Figure 10A), a pronounced effect on A2 is observed at 7.53 ppm. This coulds arise from a broad histidine resonance, a tryptophan, or a phenylalanine. For N6 (Figure 10B) and other nicotinamide protons (not shown), the observed reductions in amplitude show no pronounced maximum. In contrast, A2 (Figure 10B) and to an even greater extent A8 (Figure 10C) show maxima in the vicinity of 1 ppm. Methyl groups of several hydrophobic side chains have peaks in this region. A small peak observed only for A8 at 1.8–1.9 ppm could be due to methylene protons of arginine, lysine, or proline (Roberts & Jardetsky, 1970).

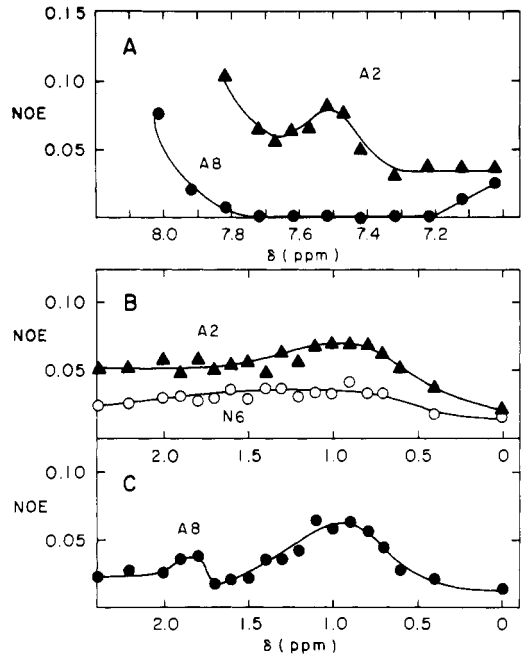

FIGURE 10: Nuclear Overhauser effects on $NADP^+$ arising from irradiation of isocitrate dehydrogenase protons. The sample contains 5.3 mM $NADP^+$ and 0.47 mM enzyme at pH 7.50. Irradiation time was 0.3 s. Differences between peak heights obtained with irradiation at the indicated chemical shift, δ, and the peak height of a control irradiated at −1.5 ppm are divided by the peak height of the control and expressed as NOE. (A) δ 7–8 ppm; (B and C) δ 0–2.5 ppm.

To determine whether the observed interactions between enzyme and nucleotide are specific for the nucleotide, the above experiments were repeated with other nucleotides. For NADPH, peaks at 0.9 ppm are obtained for both A2 and A8. In addition, N2 shows a definite effect arising from irradiation in this vicinity. No peaks are observed when the amplitude of N6 or A1′ is monitored. The peak seen at 1.8–1.9 ppm for A8 of $NADP^+$ is not seen for NADPH. For both nucleotides, a shoulder extending to around 1.5 ppm is seen. The peak in the irradiation spectrum for A2 at 7.5 ppm is present for NADPH as well as $NADP^+$.

When the nicotinamide ring is absent, the irradiation spectra in the region 0 to 2 ppm are less well defined (more like that of A2 in Figure 10B). For all the nucleotides examined in this paper, the peak at 7.5–7.55 ppm (affecting A2) is present. An attempt to further identify the residue(s) giving rise to this striking effect was made by repeating the experiments at lower pH. For Rib-P_2-Ado-P and Ado(2′,5′)P_2, the peak effects were obtained at 7.55 and 7.60 ppm, respectively, at pH 6.1. At pH 5.2, the binding is weakened, and only a shoulder is observed on the peak arising from direct irradiation of A2, but the maximum cannot be higher than 7.7 ppm. Thus, if the residue giving rise to this effect is a histidine, it is not an "ordinary" histidine in terms of width, pK, or variation of chemical shift with pH (Markley, 1975). A buried or partially buried histidine cannot be ruled out.

Discussion

Distance measurements between protons of $NADP^+$ bound to $NADP^+$-dependent isocitrate dehydrogenase (Table I) demonstrate that the conformation of the nicotinamide–ribose bond is anti. This is verified by the distances (~2.3 Å) between N1′ and N2 and between N3′ and N6 compared with distances of greater than 3.5 Å between other combinations of ribose protons and ring protons. Thus, isocitrate dehydrogenase conforms with the generalization obtained both from nuclear Overhauser effect measurements (Levy et al., 1983) and from crystallographic measurements (Moras et al., 1975) that dehydrogenases in which hydrogen transfer involves the *pro-R* (A) side of the nicotinamide ring bind the nicotinamide ring in an anti conformation. A primary NOE between N1′ and N2 is also observed for NADPH bound to isocitrate dehydrogenase, suggesting that NADPH in equilibrium with free NADPH is also in the anti conformation. An NOE between N3′ and N6 was not observed, but this may be due to the slow exchange rate. When substrate, magnesium isocitrate, is added, the NADPH proton distances (Table III) fit an anti configuration for the nicotinamide ribose.

The distance measurements for $NADP^+$ (Table I) and NADPH (Table III) and NOE's for Rib-P_2-Ado-P, Ado-(2′,5′)P_2, and 2′-AMP all suggest that the adenine–ribose bond is syn. The only NOE measurable is between A1′ and A8. While amplitude changes in A8 (data not shown) are noted upon irradiating through the rest of the ribose region (4.0–4.7 ppm), the changes show no definite peaks in this region, and changes of similar magnitude are observed in the amplitude of A2. These nonspecific effects could more likely arise from α protons of the enzyme (Roberts & Jardetzky, 1970). A syn conformation is unusual for adenine nucleotides bound to proteins. The dehydrogenases examined by Levy et al. (1983) and Clore & Gronenborn (1982), protein kinase (Rosevear et al., 1983), dihydrofolate reductase (Feeney et al., 1983), sorbitol dehydrogenase (Gronenborn et al., 1984a), and glucose-6-phosphate dehydrogenase (Gronenborn et al., 1984b) all bind the nucleotides in the anti adenine–ribose conformation. On the other hand, Lappi et al. (1980) have suggested that a number of dehydrogenases can bind modified nucleotides that are predominantly syn and that lactate dehydrogenase even favors these analogues. Gronenborn & Clore (1982) have used NOE measurements to show that the *Escherichia coli* adenosine cyclic 3′,5′-phosphate receptor protein binds adenosine cyclic 3′,5′-phosphate in the syn conformation. X-ray crystallography has indicated that glyceraldehyde-3-phosphate dehydrogenase from lobster binds the adenosine portion of NAD^+ in a syn conformation to two of its four asymmetrically arranged subunits (Moras et al., 1975), but this has been disputed (Biesecker, et al., 1977). In addition, solution studies using the nuclear Overhauser effect have shown that, while 5′-AMP exists predominantly in the anti conformation, 2′-AMP exists predominantly in the syn conformation (Guéron et al., 1973). Thus, it is not implausible that an enzyme with strong specificity for nucleotides with a 2′-phosphate binds them in a syn conformation.

For NADPH, the nuclear Overhauser effect measurements suggest that the nicotinamide and adenine rings are in close proximity. While a number of techniques have been used to demonstrate base interaction through stacking of NAD(P)H (Oppenheimer, 1982), crystallographic evidence (Grau, 1982) indicates that binding of NAD(P)H to many dehydrogenases occurs in an extended form. Phosphorylase *b* binds NADH at two regulatory sites in two different folded forms, one with the rings close to parallel and the other with the rings almost perpendicular (Stura et al., 1983). NOE measurements on sorbitol dehydrogenase have shown that NAD^+ binds in a form in which the nucleotide is folded but the rings are not stacked, having an angle between the planes of 120° (Gronenborn et al., 1984a). A coenzyme with the nicotinamide ring replaced by 3-iodopyridine crystallizes with liver alcohol dehydrogenase in a folded form (Samama et al., 1981), which has been postulated to represent a step in the binding of NAD^+. The folded form of NADPH observed in our measurements may

also be a conformer obtained on the binding pathway. Rapid fluorescence measurements have revealed at least two steps in the release of NADPH from isocitrate dehydrogenase (Fatania et al., 1982). The faster step has a rate of 3–16 s^{-1} depending upon buffer and is consistent with the dissociation rate measured here (Table II) while the slower step has a rate <0.9 s^{-1} and would not be perceived in NMR spectra of free nucleotide. The fluorescence of both bound forms is enhanced. This enhancement could arise from disruption of the parallel ring stacking, which produces quenching in aqueous solutions by means of energy transfer between the adenine and nicotinamide rings (Scott et al., 1970). The association of NADPH and dihydrofolate reductase is also characterized by two steps (Dunn et al., 1978). NMR evidence shows three different slowly interconverting conformational states in bound $NADP^+$ (Birdsall et al., 1982). Analogously, the folded conformer postulated for NADPH bound to isocitrate dehydrogenase may be only one of two or more slowly interconverting forms. The TNOE effects could arise from one of these which is in more rapid exchange with free nucleotide. Mas & Colman (1984) found only one peak for the 2′-phosphate in the ^{31}P spectrum; hence, the forms probably differ chiefly in the environment of the nicotinamide ring.

An additional possibility that cannot be ruled out is that the interaction between nicotinamide and adenine rings is intermolecular. Free NAD^+ crystallizes in a folded conformation with intermolecular ring stacking (Parthasarathy & Fridey, 1984). If intermolecular interaction between nucleotides bound to isocitrate dehydrogenase occurred, one might expect to see effects on the binding curve. No evidence of any variation of binding constant or fluorescence occurs with NADPH titration (Ehrlich & Colman, 1975; Mas & Colman, 1985). On the other hand, enhancement of enzyme fluorescence follows binding of 1 mol of $NADP^+$/mol of dimer, but no further enhancement is produced upon addition of the second mole (Reynolds et al., 1978; Mas & Colman, 1985). A burst of formation of NADPH equivalent to 1 mol/mol of dimer has been observed under certain conditions (Dalziel et al., 1978).

Limits on the dissociation rates of nucleotides and nucleotide fragments have been obtained from line-width measurements (Table II). The dissociation rate increases as more of the original nucleotide is removed (progression from $NADP^+$ to 2′-AMP). The dissociation rate for NADPH is less than 7 s^{-1} and is in agreement with the faster of the rates measured by stopped flow experiments (Fatania et al., 1982). Addition of 5 mM $MgSO_4$ and 4 mM DL-isocitrate decreases the dissociation rates of Rib-P_2-Ado-P and Ado(2′,5′)P_2. This is consistent with the decreased equilibrium dissociation constant for these nucleotides in the presence of isocitrate and sodium sulfate obtained from protection against histidine modification (Ehrlich & Colman, 1978). At low ionic strength (i.e., in the absence of sodium sulfate), the effects of net charge on the association rate appear to dominate and to cause increases of the equilibrium dissociation constants in the presence of substrate (Mas & Colman, 1985). Magnesium isocitrate increases the dissociation rate of NADPH consistent with an increase in the dissociation constant (Ehrlich & Colman, 1975; Reynolds et al., 1978). Substrate- (or product-) induced release of NADPH is necessary because the steady-state rate (≈34 s^{-1}) is greater than the estimated dissociation rate for NADPH in the absence of ligands (<7 s^{-1}).

Binding of $NADP^+$ and nucleotide fragments to isocitrate dehydrogenase results in downfield shifts of the A2 protons of 0.16–0.47 ppm In the presence of magnesium isocitrate the shifts are in the same direction but smaller (0.13 ppm for Rib-P_2-Ado-P compared with 0.19 ppm). In contrast, the A8 proton shows less than a 0.02 ppm shift. This indicates that the adenine ring is bound in such a way that the side of the adenine ring containing A2 is in intimate contact with the enzyme, while the A8 region interacts weakly with charged or aromatic residues. These conclusions are consistent with the transferred NOE observed between an aromatic group and A2 but not A8 (Figure 10). The A1′ resonance shows a small (0.04–0.1 ppm) upfield shift. For $NADP^+$ alone and NADPH in the presence of substrate, the N6 resonance is shifted downfield by about 0.08 ppm. The N4 resonance shows little shift, indicating that it is probably not interacting with the enzyme as might be expected for direct hydride transfer (Englard & Colowick, 1957).

The NMR spectrum of isocitrate dehydrogenase at pH 7.5 has three narrow resonances between 7.68 and 7.84 ppm. One of those probably arises from two groups. On the basis of their pH dependence, all three resonances may be attributed to histidines. The remainder of the resonances from the 13 histidines in the enzyme (Johanson & Colman, 1981b) must be broad or lie under the peaks of other aromatic groups. The resonance with pK = 5.35 is shifted upfield 0.07 ppm by $NADP^+$ and NADPH. The shift is the same in the presence of magnesium isocitrate. Fragments lacking the nicotinamide ring have no effect on any of the observable histidine resonances. Thus, it is unlikely that the histidines distinguished by NMR measurements include the critical histidine detected by ethoxyformic anhydride modification (Ehrlich & Colman, 1978) since that histidine is protected by all nucleotides containing a 2′-phosphate, including those that lack the nicotinamide. A residue (or residues) on the enzyme that resonates at 7.55 ppm interacts with the adenine ring of $NADP^+$ and of $NADP^+$ fragments as demonstrated by NOE with the A2 proton. This residue cannot be definitely assigned as a histidine since its position varies little in the pH range 5.2–7.5.

Nuclear Overhauser effect measurements between the enzyme and nucleotides indicate proximity of methyl groups to the A8 proton and to a lesser extent to the A2 proton of $NADP^+$ (Figure 10B,C). No interaction with nicotinamide protons are apparent (Figure 10B). The results for NADPH with or without isocitrate are similar to those for $NADP^+$ except that an interaction between methyl group(s) and the N2 proton is apparent. A hydrobic region containing methyl side groups of valine, isoleucine, leucine, and alanine has been found in the nucleotide binding sites of a number of dehydrogenases (Grau, 1982). Direct interaction of the reduced nicotinamide ring of NADPH with isocitrate dehydrogenase has been postulated on the basis of the absorption difference spectrum of the complex in the region of 340 nm (Mas & Colman, 1985). The presence of an NOE for the N2 proton of NADPH but not of $NADP^+$ suggests that the tighter binding of the reduced nucleotide could arise from specific interactions of the reduced nicotinamide ring with the enzyme.

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