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Conformations of the Coenzymes and the Allosteric Activator, ADP, Bound to NAD^+-Dependent Isocitrate Dehydrogenase from Pig Heart†

Robert S. Ehrlich and Roberta F. Colman*
Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716
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ABSTRACT: NAD^+-dependent isocitrate dehydrogenase from pig heart is an allosteric enzyme that is activated by ADP and is inhibited by NADPH in the presence of NADH. Transferred nuclear Overhauser effect measurements, made at a range of times to ensure that observed effects are due to direct dipole–dipole transfer and not to spin diffusion, were used to determine the conformations of pyridine nucleotide coenzymes and of the allosteric effector ADP. For NAD^+, significant effects were observed on the N2 proton (on the nicotinamide ring) when the N1′ proton (on the nicotinamide ribose) was saturated and on the N6 proton when the N2′ proton was saturated, indicating that the conformation of the nicotinamide–ribose moiety is anti. The anti conformation is expected because of the stereospecificity of NAD^+-dependent isocitrate dehydrogenase and is the same as for $NADP^+$-dependent isocitrate dehydrogenase. For the adenosine moiety of NAD^+, the predominant nuclear Overhauser effect on the A8 proton is found when the A2′ proton is saturated. This result implies that the adenine–ribose bond is anti with respect to the ribose. Previous kinetic and binding studies of ADP activation have shown an influence of divalent metal ions. The conformation of bound ADP, in the presence of Mg^{2+} and/or Ca^{2+}, is found to be anti about the adenine–ribose bond. The 3′H–8H distance increases when Ca^{2+} is added to the Mg–ADP–enzyme complex. Changes in the 4′H–1′H distance upon addition of isocitrate are indicative of interactions between the ADP activator site and the isocitrate site. ^{31}P NMR of ADP in the presence of enzyme and Mg^{2+} demonstrates a resonance attributable to the bound β-phosphate. This resonance, observed only in the presence of Mg^{2+}, titrates with a lower pK than free ADP, but no changes are seen with different combinations of divalent metals. The conformational analysis and ^{31}P NMR results suggest that the allosteric effects of ADP in the presence of divalent metals are propagated through changes in the enzyme conformation which are manifested at the isocitrate site.

Mammalian heart tissue contains two forms of the enzyme isocitrate dehydrogenase. Both NAD^+-dependent isocitrate dehydrogenase [isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] and $NADP^+$-dependent isocitrate dehydrogenase (EC 1.1.1.42) are located in the mitochondria, and, in contrast to other tissues, a cytoplasmic form is absent or present at low concentrations in heart (Plaut & Gabriel, 1983). An understanding of how substrates bind to these enzymes is sought as part of the elucidation of the roles of both enzymes in heart metabolism. The stereochemistry of the isocitrate dehydrogenase reaction is the same for both enzymes (Nakamato & Vennesland, 1960; Colman, 1983) with hydride transfer to the *pro-R* position of reduced nicotinamide. The configuration of the coenzyme bound to the $NADP^+$-de-

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pendent enzyme has been investigated by proton NMR (Ehrlich & Colman, 1985), and the nicotinamide moiety was found to be anti to the nicotinamide ribose while the adenosine moiety has the ring in a syn configuration. In the present study, the conformation of NAD^+ bound to the NAD^+-dependent enzyme is investigated by using the transferred nuclear Overhauser effect (Albrand et al., 1979; Clore & Gronenborn, 1982).

In addition to NAD^+ which binds at the catalytic site, NAD^+-dependent isocitrate dehydrogenase binds several nucleotides that are activators or inhibitors: NADH, NADPH, ATP, and ADP. ADP activates the enzyme by decreasing the K_m for isocitrate. Both ADP and ATP activation and inhibition have been observed under different conditions (Gabriel et al., 1985). Kinetic and binding studies indicate that, while Mg^{2+} is sufficient to tighten the binding of ADP to the enzyme (Ehrlich & Colman, 1981), other divalent metals such as Ca^{2+} may be necessary to produce the kinetic effect (Denton et al., 1978; Aogaichi et al., 1980). The conformation of bound ADP has now been probed by measuring the transferred nuclear Overhauser effects between protons of ADP in the presence and absence of combinations of Mg^{2+}, Ca^{2+}, and isocitrate. A small change in the ribose conformation of bound ADP is produced by the inclusion of Ca^{2+}. A preliminary version of a portion of this work has been presented (Ehrlich & Colman, 1988).

An additional probe of bound nucleotides is ^{31}P NMR. The ionization state of phosphates can be deduced from the pH dependence of the phosphorus resonance. This approach has been used to probe the 2′-phosphate of $NADP^+$ bound to dihydrofolate reductase (Hyde et al., 1980) and $NADP^+$-dependent isocitrate dehydrogenase (Mas & Colman, 1984). In this paper, we describe the ^{31}P NMR spectra of the β-phosphates of free ADP and ADP bound to NAD^+-dependent isocitrate dehydrogenase.

Experimental Procedures

Materials. NAD^+-dependent isocitrate dehydrogenase was purified from pig hearts following the method of Ramachandran and Colman (1977). The purification of enzyme was scaled up by chromatography of about 30 g of crude ammonium sulfate precipitate on 4 × 60 cm DEAE-Sephacel (Pharmacia) or DEAE-cellulose (Whatman DE-52) columns followed by a cellulose phosphate column. The cellulose phosphate column was eluted at 20–40 mL/h with a linear gradient of 500 mL in the mixing chamber with a reservoir of 500 mL starting from Tris–0.01 M citrate (pH 6.5) to Tris–0.4 M citrate for a 2 × 15 cm column. This procedure yielded about 40 mg of purified protein of specific activity >20 units/mg. Purity was assessed by SDS–gel electrophoresis (Shen et al., 1974). The enzyme was 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. Enzyme concentrations were 10–20 mg/mL before the addition of nucleotide. Enzyme 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.

3-(*N*-Morpholino)propanesulfonic acid (MOPS),[1] MES, ADP, NAD^+, NADH, NADPH, and DL-isocitrate were obtained from Sigma Chemical Co. D_2O (99.8% D) was obtained from Aldrich Chemical Co. Ligands were dissolved in the appropriate buffers prepared in D_2O.

NMR Measurements. ^1H NNR 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. Samples in 5-mm tubes were not spun in order to avoid 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 radiofrequency 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 1–1.5 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 control irradiation at either 5.0 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 that of the free induction of the off-resonance control. NOE's, expressed as a decimal fraction, are the ratio of the intensity of a resonance in the difference peak to the intensity of the resonance obtained from the Fourier transform of the control. In the presence of enzyme, all NOE's are negative, but the sign has been omitted. Identical experiments were also performed with nucleotide samples in the absence of enzyme. Under the conditions used, no nuclear Overhauser effects were observed in these samples, but in some experiments, decoupler pulses had appreciable power at regions distal from the central frequency, resulting in saturation of resonances other than the selected one. The decrease in intensity of these resonances cannot be attributed to a nuclear Overhauser effect. The presence of these instrumental effects could be determined by the use of compounds (in the absence of enzyme) in which the affected resonances are distal protons or by the sensitivity of the effect to changes in spectrometer settings.

Clore and Gronenborn (1983) have shown that the observed NOE arising from irradiation either of the resonance of a proton on the free compound or of an averaged resonance from free and enzyme-bound compounds (in the case of fast exchange on the cross-relaxation scale) is given by

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

where σ_b and σ_f are the cross-relaxation rates for bound and free molecules, respectively, and P_b and P_f are the fractions of bound and free molecules, respectively. For a sample of fixed composition, the cross-relaxation rates may be determined from the slope of the observed experimental data 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 by using the equation:

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

The protons in the nicotinamide ring of NAD^+ are fixed with r(N5,N6) = r(N4,N5) = 2.48 Å. The distance between the 1′- and 2′-ribose protons is 2.9 ± 0.2 Å based upon the crystal structure of adenosine (Lai & Marsh, 1972). Distances be-

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NOE, nuclear Overhauser effect; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

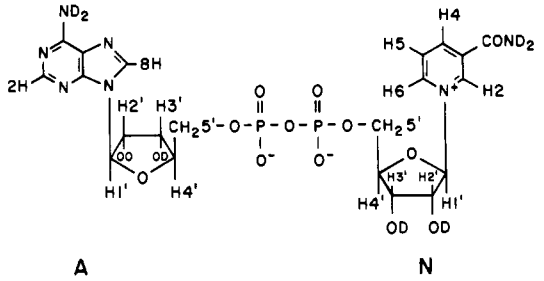

FIGURE 1: Notation for NAD^+ resonances. NAD^+ is shown schematically with both adenosine (A) and nicotinamide (N) moieties in anti configurations.

tween these ribose protons of 2.71 and 2.81 Å, respectively, have been found for $NADP^+$ bound to $NADP^+$-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1985) and NAD^+ bound to yeast alcohol dehydrogenase (Clore & Gronenborn, 1983). Clore and Gronenborn (1983) have suggested that more accurate distances may be obtained by using the observed NOE's instead of cross-relaxation rates in eq 1. Distances were also calculated in this way at a number of fixed irradiation times and the results averaged. These measurements as a function of time allow determination of the contribution of spin diffusion; an appreciable contribution leads to a lag in the NOE development with time.

^{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. Buffers used are the same as for ^1H experiments with the addition of 10% glycerol. Chemical shifts, with downfield taken as positive, were determined by reference to phosphoric acid either in a separate tube or in a concentric 5-mm tube. Samples contained 10% D_2O for the field-frequency lock. Samples were titrated by small additions of 0.1 M NaOH or 1 M MES.

Results

Binding of Coenzymes to NAD^+-Dependent Isocitrate Dehydrogenase. When NAD^+ is added to isocitrate dehydrogenase, resonances with chemical shifts identical with those of free coenzyme are observed. The amplitudes as a function of concentration are consistent with the observed resonances arising from free NAD^+ in equilibrium with enzyme, with a dissociation constant of 40 μM. The measured dissociation constant is 55 μM (Ehrlich & Colman, 1981). No resonances from bound NAD^+ are observed, and attempts to locate these by saturation transfer were unsuccessful. The line widths of all resonances increase as the fractions of bound NAD^+ increase (i.e., as the ratio of total NAD^+ to enzyme decreases). Line broadening is also observed as the temperature is increased from 294 to 308 K. These phenomena are suggestive of exchange-limited relaxation (Lanir & Navon, 1971). By use of the relation $\pi\Delta\nu = k_{off}$ for broadening due only to exchange, the excess line widths for N2, N4, N6, and A8 resonances (protons designated in Figure 1) in the presence of enzyme give a dissociation rate of 44 ± 8 s^{-1}.

For both NADH and NADPH, a single resonance is observed for each proton at the positions of the corresponding resonances in the free compounds. The line widths do not vary with coenzyme/enzyme ratios, and the exchange rates cannot be determined. At concentrations of coenzyme less than half of the molar concentration of enzyme subunits, no resonances from the coenzyme are observed. The amplitudes of the resonances as a function of coenzyme concentration are consistent with resonances from free coenzyme in slow exchange with enzyme-bound coenzyme with no resonances observed for the latter.

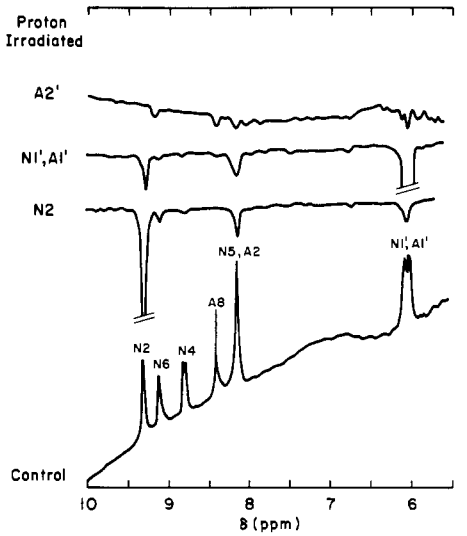

FIGURE 2: NMR spectrum of NAD^+ and difference spectra arising from specific irradiation. The sample contained 0.16 mM enzyme and 2.2 mM NAD^+ in Na–0.05 M MOPS (pH 7.0). The control ^1H NMR spectrum was obtained with radiofrequency irradiation at −1.5 ppm. Spectra result from 512 accumulations into 8K memory at 250.13 MHz. For the difference spectra, the peaks indicated were irradiated for 0.2 s, and the free induction decay of the control spectrum was substrated from the free induction decay of the irradiated spectrum before Fourier transformation.

Nuclear Overhauser Effects and the Conformation of NAD^+ Bound to Isocitrate Dehydrogenase. The spectrum of NAD^+ in the presence of pig heart NAD^+-dependent isocitrate dehydrogenase is shown in Figure 2. The positions of the proton resonances are identical with those of free NAD^+ but are broadened due to exchange between the free and bound states.

The transferred nuclear Overhauser effect was measured by subtracting the control spectrum from a spectrum irradiated at the position of the specific nucleotide resonances identified, as shown in Figure 1. Several of these difference spectra are shown in Figure 2. Nuclear Overhauser effects on ligands bound to large molecules are always negative (Clore & Gronenborn, 1982), and the result of irradiating one resonance is a decrease in the intensity of resonances arising from protons close to the irradiated proton. When the 2 proton on the nicotinamide ring (N2) is irradiated, decreases in the intensity of the N5 and N1′ resonances are observed. Resonances in the ribose region of both the nicotinamide and adenine moieties of NAD^+ overlap, and their positions are determined by decoupling experiments and reference to assignments in the literature (Oppenheimer, 1982). Irradiation at the A1′–N1′ resonances decreases the amplitudes of the N5, A2 resonance, in addition to the decrease in the N2 resonance. Irradiation at the position of the A2′ resonance causes a decrease in intensity of the A8 resonance. The effects observed in Figure 2 on tne N5, A2, or N6 resonances do not arise from the nuclear Overhauser effect, as shown by experiments described below.

The specificity and relative magnitude of the Overhauser effects arising from irradiation in the ribose region are indicated by the data presented in Figure 3. The irradiation position is varied in steps through the region, and the relative intensities of ring resonances are observed. The largest decrease in the intensity of N6 is seen to arise from irradiation at the position of N2′ (Figure 3A). The largest decrease in the intensity of A8 arises from irradiation of A2′ (Figure 3B).

While the protons whose irradiation gives rise to the largest NOE's may be presumed to be closest to the observed proton,

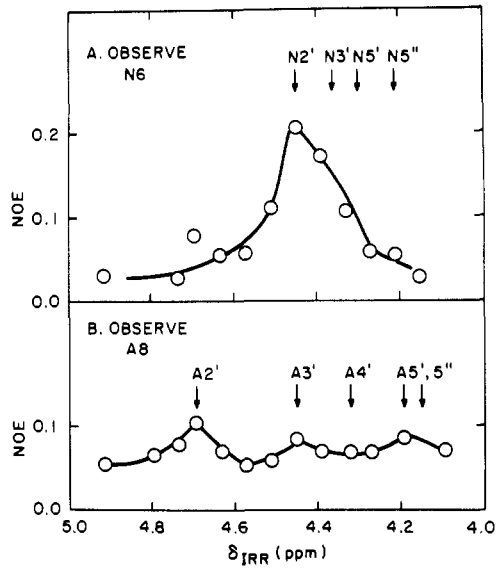

FIGURE 3: Dependence of the nuclear Overhauser effect upon irradiation position in the ribose region. The sample composition was similar to that in Figure 2. Irradiation time was 0.3 s. (A) N6 was observed. (B) A8 was observed. The positions of ribose proton resonances are indicated based upon literature values (Oppenheimer, 1982).

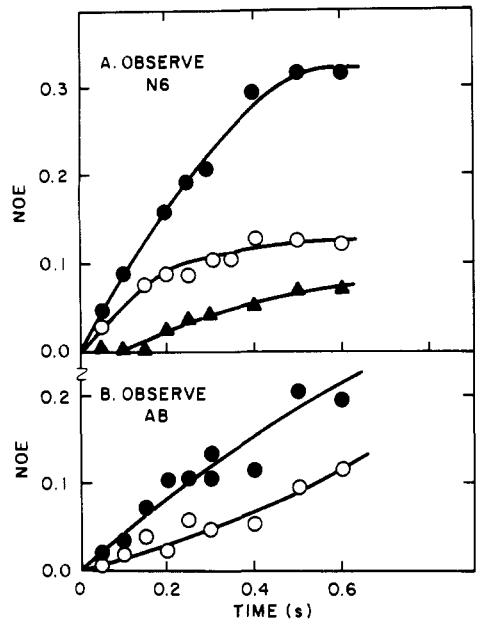

FIGURE 4: Dependence of the nuclear Overhauser effect on irradiation time. The nuclear Overhauser effects were calculated from the decrease in intensity of the observed resonance divided by its intensity in the control. (A) The N6 resonance was observed, and N2′ (●), N5 (○), or N1′ (▲) was irradiated. (B) The A8 resonances was observed and A2′ (●) or A1′ (○) irradiated. Sample compositions are similar to those in Figure 2.

more detailed measurements as a function of irradiation time must be carried out since observed effects may include contributions from spin diffusion as well as dipole–dipole interactions through space. Some representative measurements as a function of time of irradiation are shown in Figure 4. The relative decrease in amplitude of the N6 resonance upon irradiation of N2′ is found to increase with irradiation time (Figure 4A). The effect upon irradiation of N5 is shown for comparison since N5 and N6 are known to be a fixed distance apart in the nicotinamide ring. A decrease in the amplitude of N6 arises from irradiation of N1′ only for long irradiation times, and the major contribution to the effect is attributable to spin diffusion.

Table I: Interproton Distances between Protons on Coenzymes Bound to Isocitrate Dehydrogenase[a]

irradiate	observe	NAD+, r (Å)	NADH, r (Å)
	(A) Nicotinamide Moiety		
N1′	N2	2.22 (0.08)	2.25 (0.07)
N2′	N2	>3.9	*b*
N3′	N2	>3.8	*b*
N1′	N6	>3.5	*c*
N2′	N6	2.27 (0.08)	2.84 (0.07)
N3′	N6	2.24 (0.11)	*b*
N2′	N1′	2.65 (0.13)	*b*
	(B) Adenosine Moiety		
A1′	A8	>3.1	>3.5
A2′	A8	2.48 (0.08)	2.94 (0.05)
A3′	A8	2.69 (0.14)	3.03 (0.07)
A5′,A5″	A8	2.94 (0.15)	3.03 (0.07)
A2′	A1′	2.45 (0.06)	2.86 (0.12)
A4′	A1′	2.55 (0.07)	*b*

[a] All distances were calculated from ratios of observed NOE's as described in the text, taking the N4–N5 and N5–N6 distances as 2.48 Å. The numbers in parentheses are obtained from the standard deviations of at least three experimental measurements. [b] Not determined. [c] This distance could not be unequivocally determined because of the overlap of the N5 and N1′ resonances. The observed NOE's obtained upon irradiating the combined N5–N1′ resonance were attributed to N1′–N2 and N5–N6 interactions, assuming that for NADH, as for NAD+, the NOE's between N1′ and N6 and between N5 and N2 are negligible.

Observation of A8 as a function of irradiation time demonstrates that a dipole–dipole interaction is operant for the effect arising from irradiation of A2′. The effect arising from irradiation of A1′ shows a lag, and the distance from A1′ to A8 is thus greater than the distance from A2′ and A8. Similar time-dependent measurements show that the apparent nuclear Overhauser effects observed in Figure 2 between N2 and N5, between N1′ or A1′ and N5 or A2, and between A2′ and N6 arise time from spin diffusion or instrumental effects. In all these latter cases, the effect either shows a lag due to spin diffusion or includes a constant component arising from irregularities in the decoupler. Instrumental effects may also be indicated since irradiation at a symmetric position with respect to the observed resonance (at which no proton NMR peak occurs) causes similar effects.

Distances between protons in NAD+ were obtained as described under Experimental Procedures using both the initial time dependence of the nuclear Overhauser effects and ratios of effects at fixed times less than 0.25 s. The distances between N5 and N6 or between N5 and N4 were taken as 2.48 Å, and the average nuclear Overhauser effect for these protons was used in eq 2 where the cross-relaxation rates are determined from either initial slopes or ratios of the nuclear Overhauser effects at fixed times. The calculated distances given in Table I represent averages from measurements of nuclear Overhauser effects for at least three irradiation times and two separate samples. Distances obtained by using initial slopes are within 0.05 Å of these averages. Extrapolation to zero irradiation time as suggested by Majumdar and Hosur (1989) gave values within 0.05 Å of these averages and indicated clearly pairs of protons for which the observed decreases in intensity upon irradiating one of the protons are determined largely by spin diffusion. Entries in Table I for which no values are given represent pairs of protons either for which no effects are observed or for which effects arise from spin diffusion and for which the distances are greater than 4 Å. The distances for all ribose protons from A2 are greater than 3.6 Å since the nuclear Overhauser effects are not greater than the errors in measurement.

The results in Table I, column 3, show that, for NAD+, the

N1′ proton is closest to the N2 proton and distant from the N6 proton. The N2′ proton is close to the N6 proton but distant from the N2 proton. This combination of distances is diagnostic of an anti conformation of the nicotinamide–ribose bond. The proximity of the A2′ proton to the A8 proton combined with the limit on the distance between the A1′ proton and the A8 proton suggest an anti conformation for the adenine ribose. The distances between ribose protons 1′ and 2′ are constrained in all possible ribose puckers to the range 2.7–2.9 Å (Lai & Marsh, 1972; Neidle et al., 1976). This constraint appears to hold for the nicotinamide ribose, but the distance calculated for A2′–A1′ is too short. This discrepancy could result from different correlation times for the adenine ribose and nicotinamide ribose, implying different degrees of freedom of the bound moieties. Such differences would affect the absolute distances but not the relative distances from which conclusions about conformation are drawn.

Nuclear Overhauser Effect Measurements for NADH and NADPH. Transferred nuclear Overhauser effects were observed on protons of free NADH as a function of irradiation time. Distances between protons (Table I, column 4) were calculated from the initial slopes of the time-dependent decreases in observed resonances and from the ratios of NOE's at 0.2-, 0.3-, and 0.5-s irradiation. The conformation about the glycosidic bond is anti for the adenosine moiety and anti for the nicotinamide ribose moiety. Comparison between NAD^+ and NADH of the calculated distances for N2′ and N6 suggests a difference in sugar pucker in the nicotinamide ribose, but the inability to separate the other sugar resonances makes this conclusion tentative. The relative distances between adenosine ribose protons and the A8 proton are greater for NADH than for NAD^+, suggesting a slight difference in sugar pucker. The distance from A2′ and A8 is 2.94 Å, within the expected range for adenosine. No nuclear Overhauser enhancements attributable to interactions between adenine and nicotinamide rings were observed, which is consistent with the bound forms of the coenzymes being in extended conformations.

No nuclear Overhauser effects were observed when resonances of NADPH were irradiated and other resonances observed. This is probably caused by an exchange rate that is too slow for appreciable cross-relaxation to occur because NADPH is known to bind tightly to one site for every two subunits of NAD^+-dependent isocitrate dehydrogenase (Harvey et al., 1972; Ehrlich & Colman, 1982). The absence of observed peaks due to free NADPH for molar ratios of coenzyme/enzyme subunit less than 0.5 is consistent with this tight binding.

Binding and Activation of Isocitrate Dehydrogenase by ADP plus Divalent Metals. Previous binding studies have shown that ADP binding to NAD^+-dependent isocitrate dehydrogenase is enhanced by divalent metals with Mn^{2+} being more effective than Mg^{2+} in decreasing the dissociation constant (Ehrlich & Colman, 1981). Kinetic studies have indicated that Ca^{2+} is required for the allosteric reduction in the Michaelis constant for isocitrate by ADP (Denton et al., 1978; Aogaichi et al., 1980), but Ca^{2+} did not serve to promote the binding of ADP or alter the binding of ADP in the presence of Mg^{2+} (Ehrlich & Colman, 1981). The activity of isocitrate dehydrogenase was measured at pH 7.2 by using an assay mixture containing 0.2 mM DL-isocitrate, 1 mM Mg^{2+}, and 0.75 mM EGTA. Only a small increase (1.7-fold) in velocity was obtained upon the addition of 1 mM ADP. When Ca^{2+} was added in concentrations greater than the concentration of EGTA, the velocity increased about 30-fold. (Identical results were obtained at 0.3 and 1.5 mM EGTA.) A lesser but significant enhancement in velocity was obtained by the addition of Cd^{2+}.

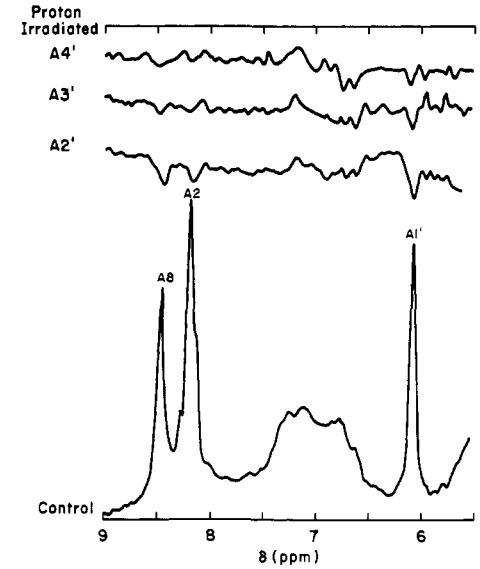

FIGURE 5: Nuclear Overhauser effects on ADP in the presence of isocitrate dehydrogenase. The samples contain 0.2 mM isocitrate dehydrogenase, 5.0 mM Mg^{2+}, and 1.5 mM ADP in Na–0.05 M MES (pH 6.1). Irradiation at the indicated positions was for 0.1 s. The control was irradiated at 5 ppm, and difference spectra were obtained as in Figure 2.

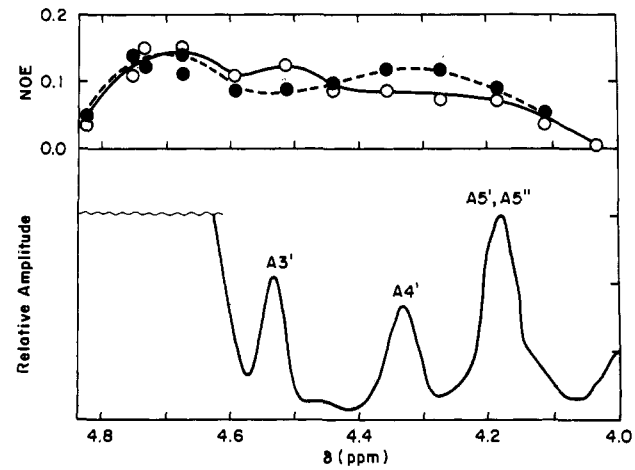

FIGURE 6: Dependence of the nuclear Overhauser effect upon irradiation position in the ribose region. Sample conditions were the same as for the spectra shown in Figure 4 except that the irradiation time was 0.3 s. The bottom panel shows the proton NMR spectrum in the ribose region. The A2′ resonance is obscured by the HDO peak. The upper panel shows the nuclear Overhauser effect for the A1′ resonance (●) and A8 resonance (○).

Nuclear Overhauser Effects and Conformation of ADP Bound to Isocitrate Dehydrogenase. Figure 5 shows the downfield region of the proton NMR spectrum of ADP at pH 6.1 in the presence of isocitrate dehydrogenase and Mg^{2+}. Difference spectra obtained upon subtracting the control spectra from spectra obtained with irradiation in the region of several ribose protons are shown. The largest decrease in the amplitude of A8 is obtained when the A2′ proton is irradiated. This irradiation also affects the amplitude of the A1′ resonance. The specificity of the effects is demonstrated in Figure 6 where the decreases in the amplitudes of the A8 and A1′ resonances are plotted as a function of irradiation position through the ribose proton region. For A8, the effects are maximal at the positions of the A2′ (obscured by HDO) and

Table II: Interproton Distances in Enzyme-Bound ADP[a]

	r (Å) (standard deviation) for additions				
proton pair	none	5 mM Mg^{2+}	5 mM Mg^{2+}, 0.5 mM Ca^{2+}	5 mM Mg^{2+}, 0.5 mM Ca^{2+}, 2 mM DL-isocitrate	5 mM Mg^{2+}, 2 mM DL-isocitrate
A4′–A1′	3.54 (0.27)	3.20 (0.08)	3.24 (0.14)	3.77 (0.36)	3.07 (0.21)
A1′–A8	>3.8	>3.6	>3.8	3.64 (0.14)	>3.8
A2′–A8	3.23 (0.19)	2.78 (0.09)	2.83 (0.12)	2.89 (0.09)	2.88 (0.07)
A3′–A8	3.45 (0.29)	3.12 (0.15)	3.58 (0.10)	3.58 (0.17)	3.46 (0.18)
A5′–A8	3.51 (0.30)	3.22 (0.30)	3.89 (0.19)	3.65 (0.22)	3.27 (0.24)

[a] Distances were measured from ratios of nuclear Overhauser enhancements as detailed in the text taking the A2′–A1′ distance as 2.9 Å. All measurements were made on samples containing 0.2 mM isocitrate dehydrogenase, 1–1.5 mM ADP, and additional components as indicated. Paramagnetic impurities were complexed by addition of 0.3 mM EGTA to Na-MES buffer (pH 6.1) prepared in D_2O.

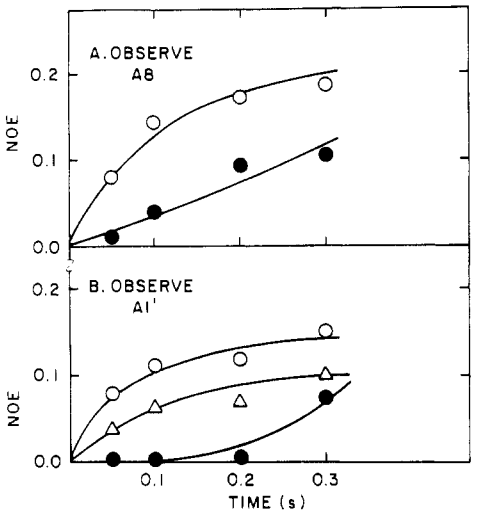

FIGURE 7: Time dependence of nuclear Overhauser effects of ADP. (A) The A8 proton was observed and the A2′ (○) or A1′ (●) proton irradiated. (B) The A1′ proton was observed and the A2′ (○), A4′ (△), or A8 (●) proton irradiated. Amplitude decreases were obtained by transformation of the control and irradiated free induction decays.

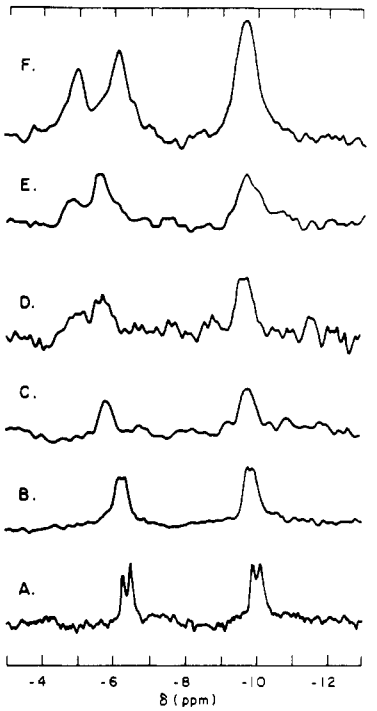

FIGURE 8: ^{31}P spectra of ADP in the presence of isocitrate dehydrogenase. Spectra were obtained at 101 MHz in 8K using a 45° pulse and a 0.5-s repetition rate. Chemical shifts are referenced to external phosphoric acid. An exponential line broadening of 4 Hz was used for ADP alone and 10 Hz for all other spectra. Samples contain 0.6–1 mM ADP in 0.05 M Na–MOPS buffer containing 10% D_2O. Additional components and conditions are as follows: (A) no additions, 6165 scans, pH 6.82; (B) 0.24 mM enzyme subunits, 20000 scans, pH 6.9; (C) 5 mM Mg^{2+}, 7139 scans, pH 6.95; (D) 2 mM Mg^{2+}, 0.24 mM enzyme, 29350 scans, pH 7.0; (F) sample as in (E), 40000 scans, pH 6.45.

A3′ resonances. A smaller Overhauser effect at the position of the 5′,5″ resonances is suggested by the data. For A1′, the expected interaction with the nearby A2′ proton is observed as well as an interaction with the A4′ proton. In Figure 7, the relative decreases in amplitude are plotted as a function of irradiation time for several pairs of protons. The NOE's observed for A8 upon irradiation of A1′ show a lag indicating the presence of spin diffusion. This is more clearly indicated by the lag when A1′ is observed and A8 irradiated (Figure 7B). Likewise, the decreases observed in the amplitude of A2 (Figure 5) can be shown to arise from spin diffusion plus a non time-dependent effect rather than a dipole–dipole interaction indicating that all ribose protons are more than 3.6 Å from A2.

Using 2.9 Å for the distance between A1′ and A2′, we calculated distances for other pairs of protons from NOE's at various irradiation times (Table II). For all combinations of divalent metals and isocitrate, the A1′ and A8 protons are relatively distant, indicating an anti conformation for the adenine–ribose bond. In the absence of divalent metals, the distances between ribose protons and A8 are all greater than 3 Å. This may indicate that the measurements result from a mixture of conformations of the bound ribose when ADP is weakly bound to the enzyme (Ehrlich & Colman, 1982). When Mg^{2+} (column 3) is added, the distance between A2′ and A8 protons decreases significantly ($p < 0.025$) consistent with an anti conformation about the adenine–ribose bond. The further addition of Ca^{2+} (column 4) results in increases ($p < 0.025$) in the distances of the A8 protons from the A3′ and A5′,A5″ protons. Isocitrate does not change these distances but increases the distance between the A1′ and A4′ protons in the complex containing both metal ions (column 5). When Mg^{2+} and isocitrate are present but Ca^{2+} is absent (column 6), all distances except the A3′–A8 distance are similar to the distances obtained for the same complex in the absence of isocitrate. All the experiments shown in Figures 4–6 and summarized in Table II were conducted at pH 6.1 where ADP is tightly bound (Ehrlich & Colman, 1982), but the distances obtained at pH 7 are the same, within the standard deviations of the results obtained at pH 6.1.

^{31}P NMR of ADP in the Presence of Isocitrate Dehydrogenase. While small changes in the ribose conformation of enzyme-bound ADP were observed in the presence of different activating metals, significant changes could arise at the diphosphate moiety which appears to be necessary for binding specificity (Plaut et al., 1979). The ^{31}P NMR spectrum of ADP is shown in Figure 8A. Resonances at −9.9 and −6.2 ppm arise respectively from the α- and β-phosphates of ADP.

Table III: Titration of the Chemical Shift of the β-Phosphate of ADP[a]

addition						
enzyme	Mg^{2+}	Ca^{2+}	isocitrate	pK	δ_u	δ_p
0	0	0	0	6.24 (0.06)	−5.44 (0.10)	−9.25 (0.11)
+	0	0	0	6.42 (0.08)	−5.37 (0.17)	−9.15 (0.17)
0	+	0	0	6.07 (0.10)	−5.50 (0.17)	−9.76 (0.24)
0	+	+	0	6.02 (0.18)	−5.41 (0.09)	−9.60 (0.25)
+	+	0	0	6.18 (0.08)	−5.05 (0.09)	−9.18 (0.25)
				4.8 (0.3)[b]	−4.63 (0.06)[b]	[c]
+	+	+	0	6.19 (0.20)	−5.12 (0.09)	−9.4 (1.3)
				4.81 (0.28)[b]	−4.49 (0.70)[b]	[c]
+	+	+	+	5.98 (0.20)	−5.10 (0.20)	−9.64 (0.77)
				5.31 (0.79)[b]	−4.55 (0.08)[b]	[c]

[a] Chemical shifts for protonated (δ_p) and unprotonated (δ_u) β-phosphate resonances of ADP and apparent pK's were determined from nonlinear least-squares fits to data shown in Figure 9. Where indicated, 0.2–0.24 mM NAD^+-dependent isocitrate dehydrogenase, 2 mM Mg^{2+}, 0.5 mM Ca^{2+}, and 2 mM isocitrate were included. Numbers in parentheses are standard deviations. [b] Values obtained from fits to the downfield resonance attributed to bound ADP (upper curve in Figure 9B). [c] Values (−8 to −14) for protonated ADP have large errors due to the inability to obtain chemical shifts at low pH.

Each phosphate resonance is split by spin–spin interaction with its neighbor. When enzyme is present and metals are absent (0.3 mM EGTA added), the resonances appear at the same positions with slight broadening (Figure 8B). The spectrum with added Ca^{2+} (not shown) is identical. With the addition of Mg^{2+} to ADP in the absence of enzyme, the β-phosphate resonance is shifted slightly downfield (Figure 8C). In the presence of enzyme, Mg^{2+} addition leads to the appearance of a new resonance at −4.5 ppm (Figure 8D) without appreciably changing the chemical shifts of the other resonances. That this resonance arises from enzyme-bound ADP is demonstrated by an increase in the relative height of the upfield resonance as the total ADP concentration is increased (data not shown). This second resonance is present in samples that contain both divalent metals (Figure 8E). Addition of isocitrate to the samples for which spectra are shown in Figure 8D,E does not change the chemical shift. Lowering the pH of a sample containing Mg^{2+} to 6.45 causes a change in the chemical shift of the upfield resonance arising from the β-phosphate but causes little change in the resonance at −4.5 ppm (Figure 8F).

Titration of the phosphate resonances of ADP over the pH range 5–8 shows that only small changes in the chemical shift of the α-phosphate occur (~0.3 ppm). The β-phosphate resonance undergoes a large upfield shift upon protonation as shown in Figure 9A. In the absence of Mg^{2+}, the titration of this resonance [Figure 9A (○)] is the same [Figure 9A (●)] as it is in the presence of isocitrate dehydrogenase, suggesting that this phosphate is freely accessible to solvent when ADP is weakly bound to isocitrate dehydrogenase. Addition of Ca^{2+} does not enhance the binding of ADP (Ehrlich & Colman, 1981) and produces only small changes in the chemical shifts of the β-phosphate without the appearance of a new peak [Figure 9A (▲)]. The pK's and chemical shifts of protonated and unprotonated forms of ADP are summarized in Table III.

The titration of the β-phosphate resonance of ADP complexed to Mg^{2+} is similar to that of the free compound [Figure 9B (○)]. In the presence of enzyme, the upfield resonance shows the same titration pattern (●), and this is not altered by the addition of Ca^{2+} (▲) and/or isocitrate [Figure 9B, lower curve (▼)]. The data, summarized in Table III, show that the pK's for this resonance in the presence of enzyme are the same as for free ADP although a small change in chemical shift is consistently present.

The chemical shift of the downfield resonance, attributed to enzyme-bound ADP, changes little in the region from pH 7.7–6.4 but moves upfield at lower pH, as indicated by the representative spectra in Figure 8. Data at pH below 5.9 could not be obtained because of precipitation of the enzyme and broadening of the β-phosphate resonance associated with bound ADP. A fit to the shifts of this resonance gives a pK for this resonance of 5.2 when the data obtained in the presence of all metals are combined. The inability to obtain data over a wider pH range does not allow us to rule out the possibility

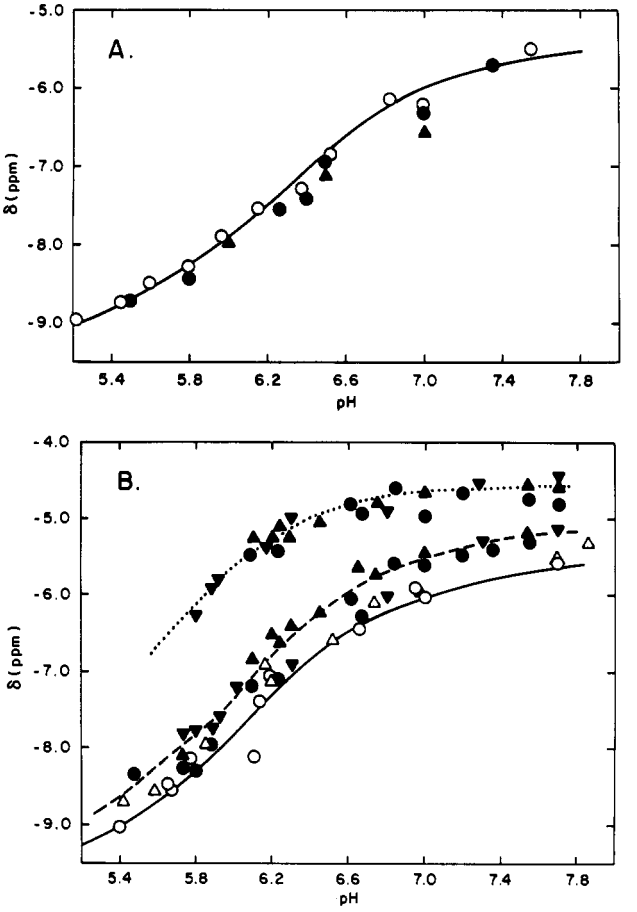

FIGURE 9: Dependence of ADP chemical shifts on pH. The chemical shift of the β-phosphate resonance is plotted as a function of pH for samples (A) containing 0.3 mM EGTA (○, ●) or 0.3 mM EGTA and 0.5 mM Ca^{2+} (▲) and (B) for 2 mM Mg^{2+} and 0.3 mM EGTA (○, ●) and additional components 0.5 mM Ca^{2-} (△, ▲) or 0.5 mM Ca^{2+} and 2 mM DL-isocitrate (▼). Solid symbols are for samples containing 0.2–0.24 mM enzyme, while open symbols are for samples lacking enzyme. The solid lines are nonlinear least-squares fits to the data in the absence of enzyme. The dashed and dotted lines are fits to the chemical shifts of the upfield and downfield resonances, respectively, in the presence of enzyme. Parameters for these fits are given in Table III.

that the changes at lower pH arise from a complex pattern of titrations of several groups. The environment of enzyme-bound ADP is clearly altered at the β-phosphate when ADP is bound in the presence of Mg^{2+}, but no changes can be demonstrated when Ca^{2+} and/or isocitrate is present.

Discussion

Distance measurements between protons of NAD^+ in the presence of NAD^+-dependent isocitrate dehydrogenase (Table I) indicate that the conformation of the nicotinamide–ribose glycosidic bond is anti. The proximities of the N2 and N1′ protons and the N6 and N2′ protons are supportive of this conformation, and the conclusion is further verified by the relatively larger distances between N6 and N1′ protons and between N2 and N2′ protons. NAD^+-dependent isocitrate dehydrogenase is thus another example of a dehydrogenase with *pro-R* specificity that binds nucleotides in the anti conformation at the nicotinamide ribose (Levy et al., 1983). In this respect, the NAD^+-dependent isocitrate dehydrogenase is similar to $NADP^+$-dependent isocitrate from the same source, pig heart (Ehrlich & Colman, 1985). Distances that could be measured from transferred NOE effects on NADH are consistent with the same conformations for reduced and oxidized coenzymes.

Similar measurements on the adenosine moiety of NAD^+ (Table I) indicate that its glycosidic bond is also anti. Additionally, the closer proximity of the A2′ and A3′ protons to the A8 proton than of the A1′ proton to the A8 proton supports this conclusion. Moreover, no ribose protons are close to the A2 proton, which is consistent with the anti conformation (Gronenborn & Clore, 1982; Leanz & Hammes, 1986). The anti conformation has been observed for NAD^+ sites on most dehydrogenases (Levy et al., 1983), but a contribution from a bound syn conformer was postulated for $NADP^+$-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1985). The anti adenosine conformation for NADH bound to the NAD^+-isocitrate dehydrogenase is also indicated by NOE measurements (Table I), although different relative distances between the A2′, A3′, A5′, and 5″-ribose protons and A8 may arise from an alteration in ribose pucker. No interaction between adenosine and nicotinamide moieties was identified by NOE measurements. This contrasts with $NADP^+$-dependent isocitrate dehydrogenase for which an interaction observed in bound NADPH can be attributed to a folded form of the bound reduced coenzyme. Extended conformations of bound NADH are consistent with the enhanced fluorescence of the coenzyme which is bound to the NAD^+-specific isocitrate dehydrogenase (Harvey et al., 1972; Ehrlich & Colman, 1982).

Kinetic evidence for ADP activation based upon the observation of an altered Michaelis constant for isocitrate or enhanced velocities at low isocitrate concentrations has indicated that a divalent metal other than Mg^{2+} is required for the activation (Denton et al., 1978; Aogaichi et al., 1980). Activation by Mn^{2+} could not be demonstrated at low (<2.5 μM) concentrations (Denton et al., 1978), but ADP activation has been observed when Mn^{2+} is the only added metal (Cohen & Colman, 1974). In all experiments with Mn^{2+}, the presence of low levels of endogenous Ca^{2+} cannot be ruled out since chelation is not specific for either metal. NOE and chemical shift measurements cannot be made in the presence of paramagnetic Mn^{2+}. Kinetic measurements made in the presence of the chelator EGTA indicate that either Ca^{2+} or Cd^{2+} can support ADP activation.

NOE's attributable to a single conformation of bound ADP are only observed when a divalent metal is present. In the absence of divalent metals, NOE's between ribose protons of ADP and the A8 proton are indicative of a range of ribose puckers since no one proton is close to the adenine ring (Table II, column 2). When Mg^{2+} is present (Table II, columns 3–6), the A2′ proton is close (2.8–2.9 Å) to the A8 proton while the A1′ proton is distal (>3.6 Å) from the A8 proton. For all divalent metal combinations, the adenine–ribose bond is anti as has generally been observed for bound adenine 5′-nucleotides (Fry et al., 1987). When Ca^{2+} is added to enzyme complexes of Mg–ADP, the distances of the A3′ and A5′, 5″ protons from the ring increase (Table II, columns 4 and 5) compared with Mg^{2+} alone. The major change observed when isocitrate is added to form the Mg^{2+}–Ca^{2+}–isocitrate–enzyme complex is an increase in the A4′–A1′ distance (Table II, column 5). This increase is indicative of a change from C3′ endo to C2′ endo ribose pucker (Levitt & Warshel, 1978). Since all distance calculations presume that the correlation times for relative proton motions remain constant, any of these changes could in fact arise from differences in relative correlation times. Nevertheless, the changes in NOE's as reflected in the calculated distances given in Table II demonstrate that small changes are produced by the inclusion of components of the ADP-activating system such as Ca^{2+} and isocitrate.

The presence of the pyrophosphoryl group of ADP is considered important for ADP activation (Plaut et al., 1979) although lesser activation has been observed with nonphysiological levels of 3′,5′-cyclic AMP (Gabriel et al., 1985). The ^{31}P NMR spectrum of ADP in the presence of enzyme was obtained in order to explore the possibility that the effects of divalent metals could alter the environment of the bound phosphates. In the absence of Mg^{2+}, the β-phosphate resonance of ADP in the presence of NAD^+-dependent isocitrate dehydrogenase had a chemical shift only slightly different from that of ADP in the absence of enzyme (Table III, Figures 8 and 9). Thus, the weak ADP binding in the absence of divalent ions probably results from interaction between the enzyme and the adenine ring, with the β-phosphate group remaining exposed to solvent. When Mg^{2+} is added in the presence of enzyme, a new resonance appears that is downfield of the resonance of free ADP (Figure 8D). The chemical shift of this resonance changes less in the pH range 5.9–7.7 (Figure 9B) than that of free ADP, consistent with a reduced pK for this bound group (Figure 8A). This resonance is in slow exchange ($2\pi\Delta\nu \gg k_{off}$) with the ADP resonance of free ADP as indicated by the presence of two resonances when ADP is present in excess; an exchange rate of less than 25 s^{-1} for bound ADP would account for this observation. Shifts in phosphate resonances may arise from changes in O–P–O bond angles (Gorenstein, 1981). Downfield shifts have been observed for ADP bound at the catalytic sites of arginine kinase (Nageswara Rao & Cohn, 1977) and creatine kinase (Nageswara Rao & Cohn, 1981). The titration of the β-phosphate resonance of ADP in the presence of Mg^{2+} and NAD^+-dependent isocitrate dehydrogenase reveals no changes in pK or chemical shift when Ca^{2+} and/or isocitrate is added. Thus, the allosteric effects of ADP mediated by Ca^{2+} cannot be attributed to alterations in the mode of ADP binding at the β-phosphate site, but rather to synergistic changes in the enzyme conformation that modify the isocitrate site.

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