Phosphorus-31 Nuclear Magnetic Resonance Studies of the Binding of Nucleotides to $NADP^+$-Specific Isocitrate Dehydrogenase†

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ABSTRACT: The interaction of the 2′-phosphate-containing nucleotides ($NADP^+$, NADPH, 2′-phosphoadenosine 5′-diphosphoribose, and adenosine 2′,5′-bisphosphate) with $NADP^+$-specific isocitrate dehydrogenase was studied by using ^{31}P NMR spectroscopy. The separate resonances corresponding to free and bound nucleotides, characteristic for slow exchange of nuclei on the NMR time scale, were observed in the spectra of the enzyme (obtained in the presence of excess ligand) with $NADP^+$ and NADPH in the absence and presence of Mg^{2+} and with 2′-phosphoadenosine 5′-diphosphoribose in the absence of metal or in the presence of the substrate magnesium isocitrate. The position of the ^{31}P resonance of the bound 2′-phosphate group in these spectra is invariant (δ = 6) in the pH range 5–8, indicating that the pK of this group is much lower in the complexes with the enzyme than that (pK = 6.13) in the free nucleotides. The additional downfield shift of this resonance by 1.8 ppm beyond that (δ = 4.22) of the dianionic form of the 2′-phosphate in free nucleotides suggests interaction with a positively charged group(s) and/or distortion of P–O–P angles as the result of binding to the enzyme. A single resonance of 2′-phosphate was observed in the spectrum of the enzyme complex with 2′-phosphoadenosine 5′-diphosphoribose in the presence of Mg^{2+}, with the chemical shift dependent on the nucleotide to enzyme ratio, characteristic for the fast exchange situation. Addition of metal does not perturb the environment of the 2′-phosphate in the complexes of $NADP^+$ and NADPH with isocitrate dehydrogenase. No detectable enzyme–adenosine 2′,5′-bisphosphate complex was observed under conditions of the NMR experiments. It is postulated that the 2′-phosphate group of $NADP^+$, NADPH, and 2′-phosphoadenosine 5′-diphosphoribose binds in a similar environment, presumably to the same site on the enzyme. It has also been determined that, in contrast to bacterial isocitrate dehydrogenase, both NAD^+- and $NADP^+$-specific enzymes isolated from pig heart do not contain covalently bound phosphate.

Pig heart $NADP^+$-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] catalyzes the oxidative decarboxylation of isocitrate and requires the presence of divalent metal ions such as Mn^{2+} and Mg^{2+}. It has been reported that the enzyme possesses one NADPH binding site and two $NADP^+$ binding sites per peptide chain (Ehrlich & Colman, 1975). Binding of the oxidized form of the coenzyme is 1 order of magnitude weaker than that of NADPH [K_d($NADP^+$) = 49 μM, K_d(NADPH) = 3.5 μM (Ehrlich & Colman, 1975)]. It has been suggested on the basis of a 10-fold difference between the Michaelis constant [K_m = 4.6 μM (Colman, 1968)] and the dissociation constant for $NADP^+$ determined by direct binding in the absence of the substrate, as well as from affinity-labeling studies with the dialdehyde analogue of $NADP^+$ (Mas & Colman, 1983), that the presence of the substrate manganous isocitrate strengthens the binding of the oxidized coenzyme. The binding of NAD^+ is several orders of magnitude weaker than that of nucleotides with a 2′-phosphate group on the adenosine ribose. The K_i for NAD^+ (11.6 mM) was obtained from competitive inhibition with respect to $NADP^+$ at pH 7.4 (Ehrlich & Colman, 1978), while much lower inhibition constants were obtained for 2′-phosphoadenosine 5′-diphosphoribose (Rib-P_2-Ado-P),[1] K_i = 6.8 μM, and adenosine 2′,5′-bisphosphate [Ado(2′,5′)P_2], K_i = 74 μM. The results of these studies strongly imply the importance of the 2′-phosphate group in determining the specificity of binding of nucleotides to isocitrate dehydrogenase.

^{31}P NMR has been used to study interaction of several enzymes with phosphate-containing ligands [for review see Cohn & Rao (1979), Cohn & Reed (1982), and Sykes (1983)]. This technique, which allows selective observation of the ^{31}P resonances of phosphate moieties of free and bound ligands, has been used in this study in order to characterize the environment and ionization state of phosphate groups of nucleotides in binary and ternary complexes with isocitrate dehydrogenase.

Experimental Procedures

Materials. $NADP^+$-specific isocitrate dehydrogenase was isolated from pig hearts according to the method of Bacon et al. (1981). The enzyme was concentrated to about 20–30 mg/mL in an Amicon ultrafiltration cell with PM10 membrane and stored in aliquots at −85 °C. Prior to NMR experiments the enzyme was thawed, dialyzed against appropriate buffer, and centrifuged to remove any insoluble material. Specific activities of the enzyme preparations used in this study were in the range of 35–43 units/mg of protein, as determined by a standard spectrophotometric isocitrate dehydrogenase assay at 25 °C (Colman, 1968). In order to stabilize enzyme activity, 10% glycerol and 0.1 M Na_2SO_4 were included in all buffer solutions. The activity was stable at 25 °C for at least 12 h, depending on the pH of the buffer solution. The enzyme concentrations were determined spectrophotometrically at 280 nm by using $E^{1\%}_{280}$ = 10.8 (Johanson & Colman, 1981); calculations were based on a molecular weight of 58 000 for the isocitrate dehydrogenase monomer (Colman, 1972).

MOPS, MES, nucleotides, DL-isocitrate, and phosphate standard solution were purchased from Sigma Chemical Co. All solutions were prepared with distilled, deionized water.

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[1] Abbreviations: NMR, nuclear magnetic resonance; Ado(2′,5′)P_2, adenosine 2′,5′-bisphosphate; Rib-P_2-Ado-P, 2′-phosphoadenosine 5′-diphosphoribose; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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^{31}P NMR. ^{31}P NMR spectra were measured at 25 °C with a Bruker WM-250 MHz spectrometer at 101.27 MHz with quadrature phase detection and broad band proton decoupling (1 W). Spectra were obtained with a spectral width of 10 kHz, memory size of 16K (or 8K), 10-μs pulse width, and 0.82-s (or 0.41-s) acquisition time. Before Fourier transformation, the free induction decay signal was multiplied by an exponential function, resulting in a line broadening of 1 Hz for the spectra of free nucleotides and of 6–10 Hz for the enzyme spectra. The conditions of individual NMR experiments are given in the legends to the figures. Samples of 1.7–2.0 mL were contained in 10-mm diameter NMR tubes (Wilmad). Protein samples were not spun during data accumulation. The NMR samples contained 10% D_2O (Norell, Inc.) as an internal field frequency lock and 0.5 mM (or 0.25 mM) EDTA (Mallinckrodt) to eliminate line broadening due to paramagnetic impurities. All chemical shifts were determined relative to an external standard of 85% H_3PO_4 (Fisher Scientific Co.) contained in a concentric capillary tube. Chemical shifts downfield from the H_3PO_4 resonance are given a positive sign. The pH of the solutions are direct readings (uncorrected for the deuterium isotope effect on the glass electrode) on a Radiometer pH meter standardized with normal H_2O buffers.

Phosphorus Determination. Determination of the phosphorus content in isocitrate dehydrogenase was performed essentially according to the method of Hess & Derr (1975). Three 100-μL samples each containing 6.3 nmol of the enzyme (dialyzed extensively against 50 mM MOPS buffer, pH 7.5, containing 10% glycerol, and 0.1 M Na_2SO_4) and duplicate samples containing phosphate standard (0–8 nmol) and 100 μL of the above buffer were digested with 20 μL of 10 N H_2SO_4, in tubes capped with aluminum foil, at 190 °C for 2 h. Several additions of 30% H_2O_2 (Fisher Scientific Co.) were made to the cooled tubes, followed by a 30-min incubation at 190 °C in open tubes in order to bleach the samples; 20 μL of 10 N H_2SO_4 and 800 μL of a fresh mixture of malachite green (Sigma Chemical Co.) and ammonium molybdate (Mallinckrodt) solution at a 3/1 ratio were added to the protein samples and to the standards. The absorbance was measured after 5 min at 660 nm.

Analysis of Data. The NMR pH titration curves were analyzed by using a nonlinear least-squares curve fitting computer program based on Marquardt's algorithm (Marquardt, 1963). Concentrations of free NADPH and NADPH–magnesium complex were calculated from the total concentrations by using the method described by Cohen & Colman (1972). The following association constants were used for calculations: 179 M^{-1} for unprotonated NADPH–Mg^{2+} complex (Kuchel et al., 1980), 42 M^{-1} for protonated NADPH–Mg^{2+} [about a 4-fold decrease in the association constant for the protonated form was assumed by analogy to the corresponding constants for $NADP^+$ (Cohen & Colman, 1972)], and 139 M^{-1} for $MgSO_4$ (Sillén & Martell, 1964).

Results

Effect of pH and Mg^{2+} on the ^{31}P NMR Spectra of NADPH, $NADP^+$, and Rib-P_2-Ado-P. The effects of pH and the presence of Mg^{2+} on ^{31}P resonances of nucleotides were investigated for free nucleotides in solution to allow for further comparisons with the spectra of these nucleotides in their complexes with the enzyme. In the absence of metal the spectrum of NADPH at pH 7.83 (Figure 1A) exhibits two ^{31}P resonances. The downfield resonance corresponds to 2′-phosphate. Despite nonequivalence of both phosphates of the pyrophosphate moiety, by coincidence their resonances occur at the same or very close frequency (Feeney et al., 1975) and

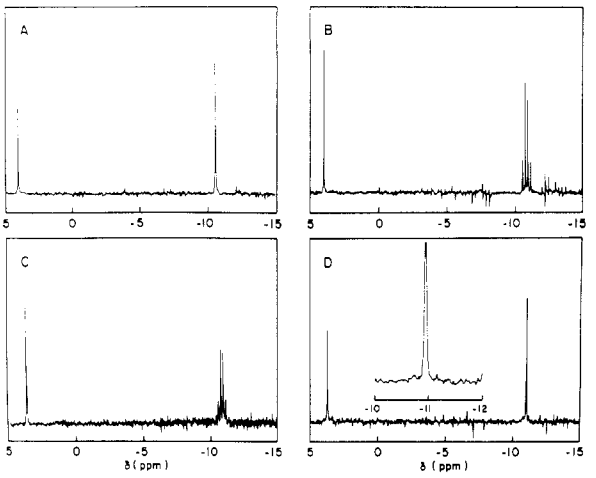

FIGURE 1: ^{31}P NMR proton-decoupled spectra (at 101.27 MHz) of free nucleotides in 50 mM MOPS, 10% glycerol, 0.25 mM EDTA, and 10% D_2O at 25 °C. (A) 1.72 mM NADPH, pH 7.83; (B) 1.85 mM $NADP^+$, pH 7.84; (C) 1.72 mM NADPH, 4 mM $MgSO_4$, pH 7.83; (D) 1.85 mM $NADP^+$, 4 mM $MgSO_4$, pH 7.82. NMR parameters: 1500 scans, spectral width 10 kHz, pulse width 10 μs, memory size 16K, acquisition time 0.82 s, and exponential line broadening 1 Hz.

cannot be resolved at 101.27 MHz (at digital resolution 1.2 Hz/point). In contrast, the spectrum of $NADP^+$ (Figure 1B) at a similar pH (7.84) exhibits a quartet of lines in the pyrophosphate region, characteristic of an AB system of spins (Hyde et al., 1980). Resonance of the ribose 2′-phosphate of $NADP^+$ occurs at a chemical shift very close to that of the reduced coenzyme (compare parts A and B of Figure 1). A pattern of two groups of resonances (singlet and quartet) similar to that of $NADP^+$ has been observed in the spectrum of Rib-P_2-Ado-P, both in the absence and in the presence of metal (spectra not shown).

In the presence of excess metal (4 mM Mg^{2+} and 2 mM nucleotide) the spectrum of NADPH shows a quartet in the pyrophosphate region (Figure 1C) and resembles the spectrum of $NADP^+$ in the absence of metal (Figure 1B). Addition of metal to $NADP^+$ results in a change in the intensity ratio of the lines of the pyrophosphate quartet (Figure 1D) in a manner dependent on the pH of the solution. As the pH increases, the intensity of two side lines of the quartet decreases. At pH 7.82 these lines are barely detectable (inset to Figure 1D), and the pyrophosphate region of the spectrum appears as a doublet of closely spaced lines (inset to Figure 1D). This behavior indicates that as the pH increases, the chemical shifts of the phosphates become closer. The differences in the pyrophosphate region of NADPH and $NADP^+$ spectra cannot be attributed to an electrostatic interaction of the positive charge of the nicotinamide ring of $NADP^+$ with the pyrophosphate moiety, in view of the fact that Rib-P_2-Ado-P, which does not have a nicotinamide ring, also exhibits a quartet of lines. Thus, other structural factors must contribute to the observed spectral changes.

Titration behavior of phosphate moieties of free nucleotides ($NADP^+$, NADPH, and Rib-P_2-Ado-P) in solution in the presence or absence of Mg^{2+} was studied by ^{31}P NMR spectroscopy. ^{31}P NMR titration curves for the 2′-phosphate resonance were analyzed by using a nonlinear least-squares curve fitting procedure based on Marquardt's algorithm (Marquardt, 1963), assuming a simple proton dissociation model.

$$XH \rightleftharpoons X^- + H^+; \qquad K = \frac{[X^-][H^+]}{[XH]} \qquad (1)$$

Table I: Comparison of the ^{31}P NMR Spectral Parameters of Free Nucleotides[a]

nucleotide	2′-phosphate[b]				pyrophosphate[c]		
	pK	δ_p	δ_u	Δ (ppm)	δ_1	δ_2	$^2J_{P-O-P}$ (Hz)
NADPH	6.52 ± 0.03	0.45 ± 0.05	4.22 ± 0.03	3.77	−10.60 ± 0.03	*d*	*d*
$NADP^+$	6.40 ± 0.03	0.36 ± 0.05	4.15 ± 0.04	3.79	−10.68 ± 0.02	−10.99 ± 0.02	20.60 ± 0.12
Rib-P_2-Ado-P	6.36 ± 0.02	0.34 ± 0.03	4.13 ± 0.03	3.77	−10.38 ± 0.03	−10.70 ± 0.04	20.65 ± 0.18
NADPH + Mg^{2+}	6.17 ± 0.03	0.38 ± 0.08	3.78 ± 0.03	3.40	−10.71 ± 0.02	−10.98 ± 0.07	19.28 ± 0.28
$NADP^+$ + Mg^{2+}	6.14 ± 0.02	0.33 ± 0.03	3.80 ± 0.02	3.47	−10.85 ± 0.05	−11.00 ± 0.01	20.14 ± 0.25
Rib-P_2-Ado-P + Mg^{2+}	5.95 ± 0.03	0.26 ± 0.05	3.69 ± 0.04	3.43	−10.57 ± 0.02	−11.00 ± 0.08	19.75 ± 0.28

[a] Proton-decoupled ^{31}P NMR spectra were obtained under conditions described in the legend to Figure 1 in the pH range 5–8 with MOPS buffer (or MES below pH 6.5). Positive chemical shifts are downfield from 85% H_3PO_4. Digital resolution of 1.2 Hz/point introduces maximum uncertainty of 2.4 Hz in each coupling constant and 0.02 ppm in each chemical shift. [b] The pK values and chemical shifts of protonated (δ_p) and unprotonated (δ_u) 2′-phosphate were calculated by nonlinear least-squares curve fitting (Marquardt, 1963) to eq 4. [c] Average values of chemical shifts and coupling constants obtained over the pH range of 5.0–8.0. [d] The resonances of two pyrophosphate nuclei were too close to be resolved.

The observed chemical shift (δ_{obsd}) can be expressed as a weighted average of the chemical shifts of unprotonated and protonated form (δ_u and δ_p, respectively).

$$\delta_{obsd} = \frac{[XH]}{[XH] + [X^-]}\delta_p + \frac{[X^-]}{[XH] + [X^-]}\delta_u \quad (2)$$

$$pH = pK + \log\frac{\delta_{obsd} - \delta_p}{\delta_u - \delta_{obsd}} \quad (3)$$

From the above equations, the relationship between observed chemical shift and pK of the titratable group can be derived:

$$\delta_{obsd} = \frac{(\delta_u \times 10^{(pH-pK)}) + \delta_p}{1 + 10^{(pH-pK)}} \quad (4)$$

The pK values and end points of the titration curves obtained by computer curve fitting of the experimental data to eq 4 are presented in Table I. The 2′-phosphate resonance shifts downfield from the fully protonated to the unprotonated form by about 3.8 ppm for $NADP^+$, NADPH, and Rib-P_2-Ado-P in the absence of metal and by about 3.4 ppm in the presence of magnesium ion (Table I). A total shift of 3.90 ppm was observed for NADPH in the presence of metal by Feeney et al. (1975). The pK values for $NADP^+$, NADPH, and Rib-P_2-Ado-P in the absence of metal differ from the values obtained in the presence of metal only by about 0.3 pH unit (Table I). Therefore, the 2′-phosphate resonance cannot be used as a diagnostic tool to determine whether a nucleotide exists as a metal complex. A more pronounced effect of metal ion was observed on the pyrophosphate region of the spectra, as discussed above. Quantitative differences in chemical shifts of 5′-phosphates are shown in Table I together with $^2J_{P-O-P}$ coupling constants. An average coupling constant of the pyrophosphate group ($^2J_{P-O-P}$) of approximately 20 Hz was obtained over the pH range 5–8 for $NADP^+$, NADPH, and Rib-P_2-Ado-P in the presence and absence of magnesium ion (Table I). The pyrophosphate coupling constant of 18–20 Hz for free $NADP^+$ and for $NADP^+$ and its analogues bound to dihydrofolate reductase has been reported by Hyde et al. (1980). A similar value (20.8 Hz) was also found by Feeney et al. (1975) for NADPH bound to dihydrofolate reductase.

The ^{31}P NMR spectrum of the dialdehyde analogue of $NADP^+$, used as an affinity label for the nucleotide binding sites of isocitrate dehydrogenase in our previous study (Mas & Colman, 1983), has been compared to that of $NADP^+$ at pH 7.9. Virtually identical spectra were obtained for both compounds, indicating that structural changes introduced by periodate oxidation do not cause significant structural changes in phosphate moieties of the coenzyme.

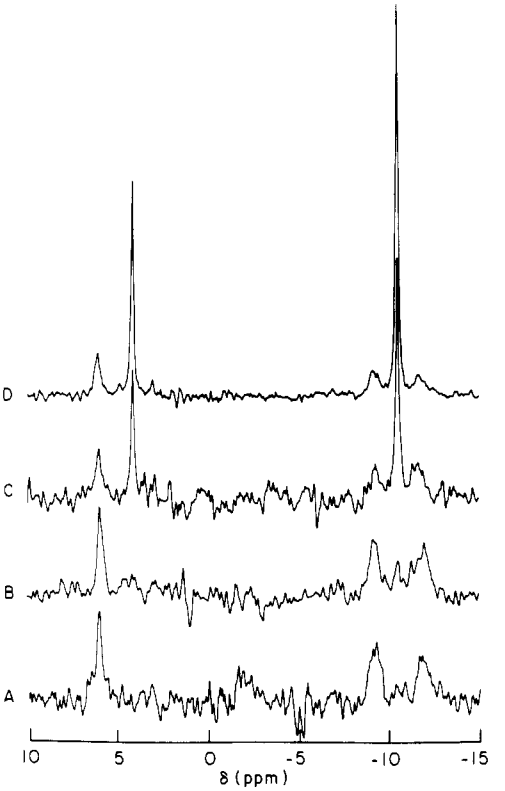

FIGURE 2: ^{31}P NMR proton-decoupled spectra (at 101.27 MHz) of NADPH in the presence of isocitrate dehydrogenase at 25 °C as a function of NADPH/enzyme ratios. The samples contained 0.3 mM enzyme in 50 mM MOPS (pH 7.53), 10% glycerol, 0.1 M Na_2SO_4, 0.5 mM EDTA, 10% D_2O, and NADPH concentration expressed as moles of nucleotide per mole of enzyme: (A) 0.5; (B) 1.0; (C) 2.0; (D) 3.0. NMR parameters: 14 000 scans, spectral width 10 kHz, pulse width 10 μs, memory size 16K, acquisition time 0.82 s, and exponential line broadening 6 Hz.

^{31}P NMR Spectra of Nucleotides Bound to Isocitrate Dehydrogenase. (1) NADPH. Figure 2 shows spectra of the binary complex of the enzyme with NADPH at pH 7.5 at various coenzyme/enzyme ratios. In the presence of the excess of enzyme (ligand/enzyme ratio = 0.5; Figure 2, spectrum A) three broad resonances are observed: a downfield resonance corresponding to the 2′-phosphate of bound nucleotide (δ = 5.97) and two signals in the pyrophosphate region of the spectrum, centered at about −9.2 and −11.7 ppm. These two broad resonances are apparently two sets of doublets of a quartet, unresolved at the accessible signal to noise ratio. As shown above (Figure 1B–D), four narrow resonances ($\Delta\nu_{1/2} \leq 4$ Hz, uncorrected for 1-Hz line broadening due to exponential multiplication of the free induction decay) of a quartet

in the spectra of free nucleotides, for which ^{31}P–^{31}P spin–spin coupling constant $\simeq$ 20 Hz (Table I), are well resolved. Line broadening of the resonances of bound ligand can be due to several factors. Slow rotation of a macromolecule causes ^{31}P resonances to broaden considerably [the molecular weight of isocitrate dehydrogenase is 58 000 per peptide chain, and the enzyme exists under many conditions as a dimer (Kelly & Plaut, 1981; J. M. Bailey and R. F. Colman, unpublished data)]. An exchange contribution to the line width can be also expected for a system of nuclei exchanging between free and bound states. Line broadening of 6 Hz was introduced in the enzyme spectra in order to improve the signal to noise ratio at the expense of resolution. The appearance of a quartet in the spectrum of bound NADPH, in contrast to a single resonance of ^{31}P nuclei of pyrophosphate moiety in the spectrum of free nucleotide in the absence of metal ion (Figure 1, spectrum A), indicates that upon binding to the enzyme, the two phosphoryl groups experience different environments and/or undergo some structural changes. One of the ^{31}P resonances of the pyrophosphate moiety exhibits a downfield shift with respect to the single resonance of the free ligand, whereas the other resonance is shifted upfield. The ^{31}P–^{31}P spin–spin coupling constant cannot be directly obtained for the spectrum of bound nucleotide due to lack of resolution of the individual lines of a quartet. The line width at half-height of each signal observed for the two side lines of the quartet is equal to 75 Hz. It can be estimated that the distance between the two unresolved resonances (^{31}P–^{31}P coupling constant) which give rise to this broad signal cannot be much different from the coupling constant for free nucleotide ($^2J_{P\text{-}O\text{-}P} \simeq 20$ Hz, from Table I). A coupling constant of 20.8 Hz was reported for NADPH bound to dihydrofolate reductase (Feeney et al., 1975) and of 18–20 Hz for $NADP^+$ and its analogues (Hyde et al., 1980). Increase of the ligand to enzyme ratio (0.5, 1.0, 2.0, and 3.0 in Figure 2A–D, respectively) leads to the appearance of separate resonances of free nucleotide in addition to signals of bound ligand. The large downfield shift ($\Delta\delta$ = 1.9 at pH 7.5) of the 2′-phosphate resonance of bound ligand with respect to that of free nucleotide indicated a change in the environment of this group as the result of its interaction with the enzyme. The pH dependence of this shift will be discussed later. The shape of a spectrum of ligand (L) interacting with an enzyme (E) depends on the rate of chemical exchange (Pople et al., 1959):

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \tag{5}$$

The lifetimes of the free ligand (τ_F) and bound ligand (τ_B) are given by

$$1/\tau_F = k_1[E]; \qquad 1/\tau_B = k_{-1} \tag{6}$$

The occurrence of two signals, corresponding to free and bound ligand, is characteristic of slow exchange of nuclei, on the NMR time scale, between free and bound states. This condition occurs when the lifetime of exchanging nuclei

$$\tau_{EX} \geq \frac{1}{2\pi\Delta\nu} \tag{7}$$

where

$$\tau_{EX} = \frac{\tau_F\tau_B}{\tau_F + \tau_B} \text{ and } \Delta\nu = |\nu_B - \nu_F| \tag{8}$$

From the difference of the chemical shifts ($\delta_F - \delta_B$ = 1.8 ppm at 101.27 MHz) observed for the unprotonated 2′-phosphate resonance of free and bound NADPH (Figure 5), the lower limit for the lifetime of the exchanging nuclei can be estimated. For $\Delta\nu$ = 182 Hz, $\tau_{EX} \geq 0.87$ ms. The line width of the resonance of the nuclei in the bound state depends on the spin–spin relaxation time, T_2, and on the lifetime (Hyde et al., 1980):

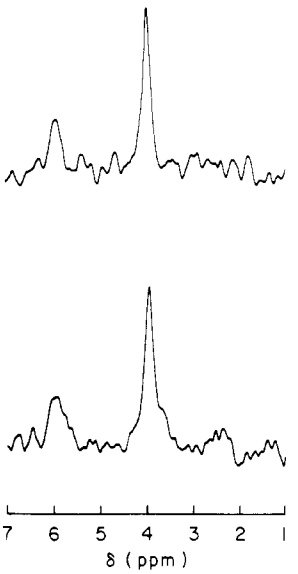

FIGURE 3: 2′-Phosphate region of the ^{31}P NMR proton-decoupled spectra of 0.54 mM NADPH in the presence of 0.27 mM isocitrate dehydrogenase in 50 mM MOPS, 10% glycerol, 0.5 mM EDTA, 0.1 M Na_2SO_4, and 10% D_2O at 25 °C. Top: in the absence of metal, pH 7.55. Bottom: in the presence of 2 mM $MgSO_4$, pH 7.51. NMR parameters: operating frequency 101.27 MHz, 8000 scans, spectral width 10 kHz, pulse width 10 μs, memory size 8K, acquisition time 0.41 s, and exponential line broadening 10 Hz.

$$\Delta\nu_{1/2,B}^{obsd} = \frac{1}{\pi T_{2,B}^{obsd}} \tag{9}$$

$$\frac{1}{T_{2,B}^{obsd}} = \frac{1}{T_{2,B}^{*}} + \frac{1}{\tau_B} \tag{10}$$

$$\pi\Delta\nu_{1/2,B}^{obsd} = \frac{1}{T_{2,B}^{*}} + \frac{1}{\tau_B}; \qquad \pi\Delta\nu_{1/2,B}^{obsd} = \frac{1}{T_{2,B}^{1}} + k_{-1} \tag{11}$$

Similarly for the free ligand

$$\pi\Delta\nu_{1/2,F}^{obsd} = \frac{1}{T_{2,F}^{*}} + \frac{1}{\tau_F}; \qquad \pi\Delta\nu_{1/2,F}^{obsd} = \frac{1}{T_{2,F}^{*}} + \frac{P_B k_{-1}}{P_F} \tag{12}$$

where P_F and P_B are mole fractions of ligand in free and bound states. From eq 11 and 12 it can be seen that the line width of the signal of ligand in the bound state is independent of ligand concentration, while that of free ligand will decrease with increasing concentration. From the constant line width of the 2′-phosphate resonance in the bound state, estimated from Figure 2 as $\Delta\nu_{1/2,B}$(2′-P) = 28 Hz, the observed spin–spin relaxation time, $T_{2,B}^{obsd}$, is equal to 0.011 s.

The spectrum of the ternary complex of the enzyme with NADPH and Mg^{2+} at pH 7.55 is indistinguishable from that in the absence of metal. Figure 3 presents the 2′-phosphate regions of the spectra at a ligand to enzyme ratio = 2/1 in the absence and presence of 2 mM $MgSO_4$ (top and bottom, respectively).

(2) *$NADP^+$*. In analogy to the spectra of binary complexes of the enzyme with NADPH described above, the corresponding spectra (not shown) with $NADP^+$ at pH 7.5 measured at various ligand to enzyme ratios (0.8 to 3 molar excess of ligand with respect to enzyme concentration) exhibit separate resonances for free and bound ligand, typical for the slow exchange between ^{31}P nuclei in both states. Both regions of the spectra (2′-phosphate and 5′-pyrophosphate region) exhibit

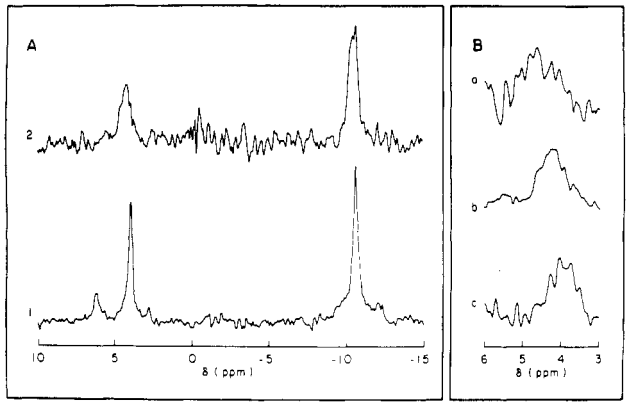

FIGURE 4: ^{31}P NMR proton-decoupled spectra (at 101.27 MHz) of Rib-P_2-Ado-P in the presence of isocitrate dehydrogenase in 50 mM MOPS, 10% glycerol, 0.1 M Na_2SO_4, 0.5 mM EDTA, and 10% D_2O at 25 °C. (A) Spectrum 1, 0.29 mM enzyme, 0.57 mM nucleotide, pH 7.36, 53 800 scans (identical spectrum was obtained in the presence of 1 mM DL-isocitrate and 2 mM $MgSO_4$); spectrum 2, 0.23 mM enzyme, 0.46 mM nucleotide, 2 mM $MgSO_4$, pH 7.3, 44 300 scans. (B) 0.23 mM enzyme, 2 mM $MgSO_4$, pH 7.3; nucleotide to enzyme ratios, (a) 1.0, (b) 2.0, and (c) 3.0; number of scans, 24 800, 44 300, and 38 850, respectively. Spectrum b is an expanded region of spectrum 2 in (A). NMR parameters identical with those in the legend to Figure 3.

close similarity to those observed in the spectra of binary complexes with NADPH shown in Figure 2. Although the fraction of bound ligand appears lower for $NADP^+$ complexes, as indicated by lower intensity of bound resonances under comparable conditions, the chemical shifts of bound 2′-phosphates are identical (about 6 ppm) for both binary complexes. The line width of this relatively sharp resonance, $\Delta\nu_{1/2,B}$(2′-P) is equal to 31 Hz for $NADP^+$ as compared to 28 Hz for NADPH complex. The similar line widths of these resonances may indicate a similar degree of immobilization of the 2′-phosphate in both complexes. Two broad peaks are observed in the pyrophosphate region of the spectrum, one located downfield and the other upfield with respect to the position of the free pyrophosphate signal. Addition of Mg^{2+} alone or in the presence of isocitrate does not cause any significant changes in the spectrum.

(*3*) *Rib-P_2-Ado-P.* A binary complex of the enzyme and the nucleotide analogue Rib-P_2-Ado-P at pH 7.3 at a ligand to enzyme ratio of 2/1 yields a ^{31}P NMR spectrum (Figure 4A, spectrum 1) similar to the analogous spectra of the binary complexes with $NADP^+$ and NADPH. All these spectra are typical of the slow exchange condition and exhibit separate resonances of bound and free ligand. The positions of the resonances of bound 2′-phosphates are identical (within experimental error) in all three spectra ($\delta \simeq 6$) and are independent of the ligand to enzyme ratio. There is also close similarity within the pyrophosphate region of these spectra, which exhibits two broad signals suggesting a quartet of unresolved lines. The quartet structure of free pyrophosphate resonances is not evident in spectrum 1 of Figure 4A due to additional line broadening (10 Hz) introduced to enhance signal to noise level, as well as to partial overlap with broad, low-intensity resonances of bound nucleotide.

In contrast to all spectra of nucleotide complexes with the enzyme described above, only a single 2′-phosphate resonance is observed in the spectrum of the tertiary complex in the presence of a 2-fold molar excess of Rib-P_2-Ado-P and 2 mM Mg^{2+} (Figure 4A, spectrum 2). This suggests that, in the presence of metal ion, exchange between the free and bound states of Rib-P_2-Ado-P is fast on the NMR time scale, whereas slow exchange was observed for ternary complexes of the enzyme with Mg^{2+} and NADPH or $NADP^+$. In the case of fast exchange between the bound and free states [$\tau_{EX} \leq 1/(2\pi\Delta\nu)$], only one signal of ^{31}P nucleus is expected whose chemical shift is a weighted average of those of free and bound ligand and depends on the ligand concentration. The 2′-phosphate regions of the spectra at different ligand to enzyme ratios are shown in Figure 4B, spectra a, b, and c (ratios 1, 2, and 3, respectively). Spectrum b in Figure 4B presents the expanded region of spectrum 2 in Figure 4A. It is apparent that the position of the 2′-phosphate resonance shifts upfield as the concentration of ligand increases, approaching the chemical shift position of free ligand. It is not possible in this case to distinguish between fast and intermediate exchange. It has been pointed out that a full line-shape analysis can distinguish between these two conditions (Feeney et al., 1979). In the intermediate exchange case, the line width of the resonance reaches a maximum at some intermediate ligand concentration, in contrast to the case of fast exchange, in which the line width decreases monotonically with increasing ligand concentration. Large errors can be expected if the equilibrium constant is calculated from the line width by assuming fast exchange if the case is actually an intermediate exchange situation (Feeney et al., 1979). Although a poor signal to noise ratio does not allow for meaningful line-shape analysis of these spectra, fast (or intermediate) exchange observed for the ternary complex with Rib-P_2-Ado-P may indicate weaker binding of this ligand in the presence of metal, in contrast to ternary complexes with $NADP^+$ and NADPH.

A virtually identical spectrum with that shown in Figure 4 (spectrum 1) is obtained for Rib-P_2-Ado-P–enzyme complex (at 2/1 molar ratio) in the presence of the substrate, Mg^{2+}–isocitrate, indicating a slow exchange situation.

(*4*) *Ado(2′,5′)P_2.* Free adenosine 2′,5′-bisphosphate in a solution containing 50 mM MOPS, 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA at 25 °C and pH 7.5 gives ^{31}P NMR spectrum (not shown) with two equal intensity resonances at $\delta_1 = 4.10$ and $\delta_2 = 3.98$. No evidence for binding of Ado(2′,5′)P_2 was obtained from the spectrum measured in the presence of 0.42 mM enzyme at ligand/enzyme ratios of 0.5 to 3.6, in the absence of other ligands, as well as in the presence of Mg^{2+} or Mg^{2+} and isocitrate.

^{31}P NMR pH Titration of 2′-Phosphate Resonances in Binary Complexes of Nucleotides with Isocitrate Dehydrogenase. Chemical shifts of ^{31}P resonances of phosphate groups are very sensitive to the state of protonation (Cohn & Rao, 1979). In order to characterize their environment and compare the ionization state of 2′-phosphate groups in complexes with the enzyme, we measured spectra of the nucleotides ($NADP^+$, NADPH, and Rib-P_2-Ado-P) in the presence of isocitrate dehydrogenase over a range of pH (5.5–8.0) in 50 mM MOPS (or MES), 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA at 25 °C. All spectra were measured at a ligand to enzyme ratio of 2/1. At all pH values studied spectra characteristic for the slow-exchange condition were obtained. The positions of free and bound 2′-phosphate resonances are plotted as a function of pH as shown in Figure 5. The average pK value for free nucleotides obtained by computer curve fitting is slightly lower than values obtained in the absence of 0.1 M Na_2SO_4, which was included to stabilize enzyme activity during several hours of data accumulation. A value of pK = 6.13 was obtained from the data in Figure 5 (open symbols), whereas a pK of 6.4–6.5 was obtained for free nucleotides in the absence of Na_2SO_4 (Table I). For the free coenzymes, $NADP^+$ and NADPH, pK's of 6.1 and 6.4, respectively, were reported by Feeney et al. (1975). The

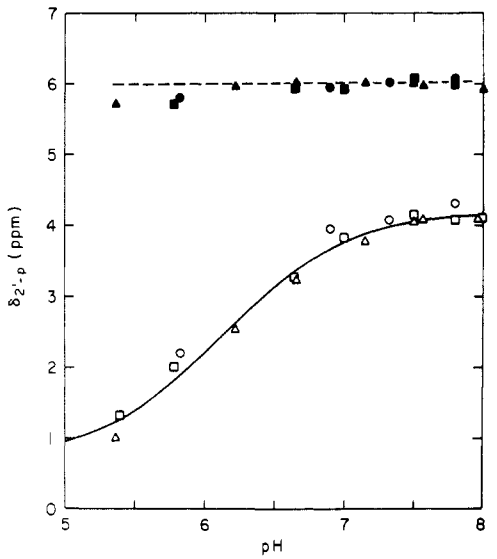

FIGURE 5: pH dependence of the ^{31}P chemical shift of the 2′-phosphate resonance of NADPH (△, ▲), $NADP^+$ (□, ■), and Rib-P_2-Ado-P (○, ●). The open symbols refer to the free nucleotides and the solid symbols to the nucleotides bound to isocitrate dehydrogenase. The samples contained 0.2–0.3 mM enzyme and a 2-fold molar excess of a nucleotide in 50 mM MOPS (or MES below pH 6.5), 10% glycerol, 0.1 M Na_2SO_4, 0.5 mM EDTA, and 10% D_2O at 25 °C; 8000–40 000 scans were collected at 101.27 MHz with broad band proton decoupling and NMR parameters the same as those in the legend to Figure 2. The pH values are uncorrected for the effect of 10% D_2O present in the samples. The solid line represents the best fit to eq 4 with pK = 6.13 ± 0.08, δ_u = 4.20 ± 0.06, and δ_p = 0.71 ± 0.18. The broken line does not have any theoretical significance.

chemical shifts of the bound 2′-phosphate resonances are identical and constant for the complexes with $NADP^+$, NADPH, and Rib-P_2-Ado-P within the available pH range (closed symbols in Figure 5). The resonance of bound 2′-phosphate occurs at δ = 6 and is shifted by 1.8 ppm with respect to that of the dianionic form of free nucleotide (δ = 4.2).

Determination of Phosphate in Isocitrate Dehydrogenase. Phosphorylation of isocitrate dehydrogenases isolated from bacterial sources has been reported (Garnak & Reeves, 1979a,b; Wang & Koshland, 1982). A phosphoserine residue has been identified in the *E. coli* enzyme (Garnak & Reeves, 1979b), and phosphorylated and nonphosphorylated forms of the enzyme were separated by using chromatofocusing (Wegener et al., 1983). Phosphorylation which has been shown to be ATP and Mg^{2+} dependent and cAMP independent (Homa & Reeves, 1983; Wegener et al., 1983) results in a less active form of isocitrate dehydrogenase. It has been proposed for the *S. typhimurium* enzyme (Wang & Koshland, 1982) that reversible phosphorylation of isocitrate dehydrogenase plays a major role in the control of the Krebs and glyoxylate pathways.

No evidence has been obtained from the ^{31}P NMR spectra for the existence of phosphorylation site(s) of the pig heart enzyme. Chemical shifts of the resonances of phosphorylated residues in different proteins have been shown to be either dependent or independent of pH [for review see Cohn & Rao (1979)] within the pH range of NMR experiments described in this paper (pH 5–8). Since chemical shifts of the ^{31}P resonances of phosphorylated amino acid residues fall in the range of the chemical shifts of the nucleotide resonances, [e.g., the chemical shift of a model compound, serine phosphate, is 0.4 and 4 ppm for the mono- and dianion, respectively (Cohn & Rao, 1979)], it seemed possible that a resonance of a hypothetical phosphorylated residue in pig heart $NADP^+$-dependent isocitrate dehydrogenase could have been obscured by the resonances of the nucleotides. Alternatively, the broad resonance of covalently bound phosphate(s) could have been undetectable under the conditions of NMR experiments. To test these possibilities, we have used the colorimetric method (Hess & Derr, 1975) described under Experimental Procedures to determine the phosphate content in $NADP^+$-specific isocitrate dehydrogenase. No more than 0.11 equiv of phosphate has been found per peptide chain (M_r 58 000) of the enzyme. It has also been determined by the same method that NAD^+-specific isocitrate dehydrogenase contains less than 0.05 equiv of phosphate per 40 000 subunit molecular weight (M. M. King and R. F. Colman, unpublished data). It can therefore be concluded that pig heart isocitrate dehydrogenases isolated according to previously described procedures (Bacon et al., 1981; King & Colman, 1983) do not contain covalently bound phosphate.

Discussion

Comparison of X-ray crystallographic data of four NAD^+-linked dehydrogenases revealed topological similarity of the nucleotide binding domains with characteristic supersecondary structure composed of β-sheets and α-helices (Rossmann et al., 1975). The "Rossmann fold" has been found in other nucleotide binding enzymes [for review see Grau (1982)]. It has been demonstrated, however, that the secondary structure of the NADPH-binding domain of *L. casei* dihydrofolate reductase differs from the nucleotide fold in NAD^+-dependent dehydrogenases (Matthews et al., 1978). It has been argued by Matthews et al. (1979) that superficial resemblance of secondary structures of dinucleotide binding domains in enzymes is probably due to requirements for efficient protein folding (Chothia & Levitt, 1979; Richardson, 1977). Crystallographic studies have demonstrated that dinucleotides bind to enzymes in an extended form, in contrast to their structure in solution, which consists of equilibrium mixtures of open and folded conformations, with the latter predominating (Grau, 1982). In all known structures dinucleotides bind at the C-terminal end of β-sheets with the phosphate groups at the N-terminal end of α-helices (Hol et al., 1978).

Since neither the three-dimensional structure nor the amino acid sequence is known for mammalian $NADP^+$-dependent isocitrate dehydrogenase, the structural insights regarding specific ligand bindings sites at present depend on the results of indirect methods and analogies with other dehydrogenases for which more data have been accumulated. The results of the ^{31}P NMR studies presented in this paper demonstrate that the environment of the 2′-phosphate group of adenine ribose is identical in binary complexes of isocitrate dehydrogenase with $NADP^+$, NADPH, and Rib-P_2-Ado-P. This conclusion is based on the identity of the ^{31}P NMR spectra of these complexes within the pH range studied. The total downfield shift of 3.8 ppm has been observed for the 2′-phosphate upon its complete ionization in free nucleotides in solution (Table I). The downfield direction of the chemical shift of the ^{31}P resonance in the binary complexes of these nucleotides with isocitrate dehydrogenase (Figure 5) suggests that their 2′-phosphate groups exist in the dianionic form. The constant value of the chemical shift within the pH range 5.5–8.0 can be explained by a decrease in pK, by at least a few pH units, presumably as the result of interaction with a positively charged amino acid residue(s) within the nucleotide binding site. Comparison of the chemical shift of the dianionic form of 2′-phosphate of free nucleotides in solution, δ = 4.22, to that found in their complexes with isocitrate dehydrogenase, δ =

6, indicates, however, that other factors must contribute to the observed change in chemical shift. Although ^{31}P NMR has been widely applied to characterize enzymes which bind phosphate-containing ligands (Cohn & Rao, 1979; Cohn & Reed, 1982; Sykes, 1983), no reliable theory exists to correlate observed chemical shifts and coupling constants with molecular structure. The importance of electronegativity difference in the P–X bond and changes in the π-electron overlap and in the σ-bond angles have been listed as the most significant factors governing ^{31}P chemical shifts (van Wazer & Letcher, 1967). From the empirical correlation between ^{31}P chemical shifts and O–P–O angles in a variety of model compounds, it has been determined that a reduction of the ester bond angle by 1 deg results in a downfield shift of 2–3 ppm (Gorenstein, 1981).

There is striking similarity between our ^{31}P NMR data for isocitrate dehydrogenase and that obtained for *L. casei* dihydrofolate reductase (Feeney et al., 1975; Birdsall et al., 1977, 1982; Hyde et al., 1980). Dihydrofolate reductase is the only NADP(H) binding enzyme for which both ^{31}P NMR data and a detailed X-ray structure for the ternary complex with the reduced coenzyme are available (Matthews et al., 1978, 1979; Bolin et al., 1982; Filman et al., 1982). The interpretation of the NMR spectra for the *L. casei* dihydrofolate reductase based on its X-ray structure has been published (Matthews, 1979). Considering the close similarity of ^{31}P NMR spectra for isocitrate dehydrogenase and dihydrofolate reductase and the wealth of structural data available for the latter enzyme, it is tempting to speculate about analogies between the nucleotide binding domains of the two enzymes. The discimination between NAD^+ and $NADP^+$ is mainly the result of multiple specific interactions of the adenine ribose with several amino acid residues within its binding cleft. It has been found that for NAD^+-specific dehydrogenases there is an invariant, negatively charged group (an aspartate residue) which is involved in hydrogen bonding to the 2′-hydroxyl of ribose (Grau, 1982). This residue is replaced by an arginine in NADPH-dependent dihydrofolate reductase (Matthews et al., 1978). It is known from crystallographic data for that enzyme that the oxygens of the 2′-phosphate are involved in hydrogen bonds and salt bridges to four protein side chains: arginine-43, threonine-63, histidine-64, and glutamine-65 (Filman et al., 1982). Additional stabilization of negatively charged phosphate results from polarity of the electric field near the amino end of the helix (Hol et al., 1978). This unique array of amino acid residues surrounding the 2′-phosphate of the adenosine ribose must be in some way responsible for an usual downfield shift of the ^{31}P resonance of 1.7 ppm beyond that of the dianionic form of free NADPH in solution (Feeney et al., 1975). It is not clear whether a combination of electrostatic and hydrogen-bonding interactions, a distortion of the O–P–O bond angles, or both cause the observed change. The magnitude of this extra shift is very similar to that we observed for the isocitrate dehydrogenase–NADPH complex, for which Δ = 1.8 ppm; a similar shift is observed for the complexes with $NADP^+$ and Rib-P_2-Ado-P. The pH-independent position of the ^{31}P resonance of bound 2′-phosphate is also characteristic for both enzymes. Whether specific amino acid residues within the 2′-phosphate binding site of isocitrate dehydrogenase are the same as those in *L. casei* dihydrofolate reductase cannot be answered until the detailed X-ray structure for this enzyme is known. From the chemical modification and affinity-labeling studies (Ehrlich & Colman, 1978; Mas & Colman, 1983) the presence of histidyl and lysyl residues has been reported within a nucleotide binding site of isocitrate dehydrogenase. It is interesting that the extra chemical shift of the ^{31}P resonance of 2′-phosphate is only 0.2 ppm in the NADPH complex with *E. coli* dihydrofolate reductase in which histidine-64, known to interact with the 2′-phosphate in *L. casei* enzyme, is replaced by a serine (Cayley et al., 1980).

X-ray crystallographic data have been published for the following NADP(H) binding enzymes: *p*-hydroxybenzoate hydroxylase (Wierenga et al., 1979), glutathione reductase (Thieme et al., 1981; Schulz et al., 1982; Pai & Schulz, 1983), ferrodoxin-$NADP^+$ oxidoreductase (Sheriff & Herriott, 1981), and glucose-6-phosphate dehydrogenase (Adams et al., 1983). Interaction of the 2′-phosphoryl group of NADPH with arginine-218, histidine-219, and arginine-224 has been reported for glutathione reductase (Kraut-Siegel et al., 1982). Similarly, involvement of two arginine residues (166 and 269) and a histidine (162) in the binding of the 2′-phosphate of NADPH has been found for *p*-hydroxybenzoate hydroxylase (Wierenga et al., 1983).

The properties exhibited by $NADP^+$-dependent isocitrate dehydrogenase such as discrimination between $NADP^+$ and NAD^+, different affinity with respect to oxidized and reduced forms of the coenzyme, and stronger binding of the coenzyme in the presence of substrate (Ehrlich & Colman, 1978; Ehrlich & Colman, 1975; Mas & Colman, 1983) are not uncommon among dehydrogenases (Grau, 1982). However, examination of the ^{31}P NMR spectra of the binary complexes of NADPH and $NADP^+$ with isocitrate dehydrogenase does not reveal significant differences. In both cases separate resonances are observed for bound and free ligands, indicating slow exchange between bound and free states. A larger proportion of bound vs. free ligand observed for NADPH than for $NADP^+$, as estimated from the areas under ^{31}P resonances (in the spectra obtained for any given nucleotide to enzyme ratio at the same protein concentration), is consistent with stronger binding reported for the reduced form of the coenzyme (Ehrlich & Colman, 1975).

In addition to the close similarity of the 2′-phosphate region of the spectra of binary complexes of isocitrate dehydrogenase with $NADP^+$, NADPH, and Rib-P_2-Ado-P discussed above, their pyrophosphate regions also exhibit similar features. Characteristic shifts of the resonances of two ^{31}P nuclei of the pyrophosphate moiety in the opposite directions with respect to the resonance of free nucleotide, shown in Figure 2, for the NADPH–enzyme complex, are observed for all three binary complexes. This indicates that the environment of the pyrophosphate moiety, as well, must be similar in these complexes and the difference between these two nonequivalent ^{31}P nuclei is enhanced as the result of interaction with the enzyme. We found no evidence from the ^{31}P NMR spectra for the presence of a second (different) binding site for $NADP^+$. If the reported number of sites obtained from binding studies (Ehrlich & Colman, 1975) is correct, the environment of both 2′-phosphate and 5′-pyrophosphate moieties in two $NADP^+$ molecules bound per subunit of isocitrate dehydrogenase must be identical and therefore indistinguishable by ^{31}P NMR.

The results of indirect studies have indicated that the presence of the substrate facilitates binding of $NADP^+$ and Rib-P_2-Ado-P to isocitrate dehydrogenase, presumably via a conformational change of the enzyme (Ehrlich & Colman, 1978; Mas & Colman, 1983). In order to test this hypothesis we have studied the effect of the substrate magnesium isocitrate on the ^{31}P NMR spectra of the enzyme complexes with these nucleotides. In both cases, an unchanged spectrum was obtained, characteristic of the slow exchange situation, with the position of the resonances of the 2′-phosphate and 5′-

pyrophosphate moieties essentially identical with those observed for the binary complexes. This result suggests that if there is a conformational change caused by substrate binding, it does not affect the environment of the phosphate moieties of nucleotides bound to the enzyme. Other methods, sensitive to structural changes of the enzyme as well as the environments of adenine and/or nicotinamide fragments of the nucleotides, must be applied to evaluate changes accompanying the formation of nucleotide complexes with the enzyme.

It has been pointed out by Jaffe & Cohn (1978) and by Cohn & Rao (1979) that although a specific metal binding site on a polyphosphate chain cannot be unequivocally determined on the basis of ^{31}P chemical shifts, ^{31}P NMR can still be useful to determine qualitatively changes in metal chelation in phosphate-containing ligands upon binding to an enzyme. The presence of metal ion (Mg^{2+}) has been shown to produce characteristic changes in the pyrophosphate region of the ^{31}P NMR spectra of the free nucleotides but not in that of the 2′-phosphate (Figure 1 and Table I). On the basis of these model studies one might expect to find a quartet of pyrophosphate resonances for the metal–nucleotide complexes (assuming that contributions from the enzyme are insignificant and do not cancel the metal effect). The same result, however, is observed for the pyrophosphate resonance of NADPH in the absence of metal in the NADPH–enzyme complex (Figure 2), in contrast to a single pyrophosphate resonance in the ^{31}P NMR spectrum of free nucleotide (Figure 1A). Thus, occurrence of a quartet in this region of the spectrum merely reflects a distinction in magnetic environment between two ^{31}P nuclei. Subtle differences between chemical shifts of the two 5′-phosphates can be observed for the free nucleotides in solution at different pH values, in the presence and absence of metal (ν_1 and ν_2 in Table I). Unfortunately, this region of the spectrum in the enzyme–nucleotide complexes is rather obscure due to large line widths of the resonances of bound ligands. The chemical shift of the 2′-phosphate resonance, as well as its titration behavior, is not significantly affected by the presence of metal, and therefore, this portion of the molecule cannot provide information about metal chelation.

The only circumstance in which the involvement of metal is reflected in the ^{31}P NMR spectrum of nucleotide complexes is when metal ion changes the mode of binding of the nucleotide. This may occur when the 2′-phosphate moiety experiences a different environment or there is a significant change in the lifetime of the exchanging nuclei as the result of altered strength of binding in the presence of metal ion. The latter situation is observed when the spectrum of Rib-P_2-Ado-P is measured in the presence of the enzyme and magnesium ion (Figure 4A, spectrum 2, and Figure 4B). Change from slow exchange in the absence of Mg^{2+} (Figure 4A, spectrum 1) to a fast exchange situation in the presence of metal ion is unique for Rib-P_2-Ado-P. The presence of Mg^{2+} does not produce any detectable changes in the ^{31}P NMR spectra of the enzyme complexes with $NADP^+$ or NADPH (Figure 3). Apparently, binding of Mg^{2+} to the enzyme does not alter binding of the 2′-phosphate group of these nucleotides. It has previously been suggested that the reduced coenzyme binds in its free form (Ehrlich & Colman, 1975). The addition of metal would decrease the amount of free nucleotide available for binding by only about 2% under the conditions of our experiment,[2] and therefore, a corresponding decrease in the intensity of the bound ^{31}P resonances would not be detectable. The effect of metal on the spectrum of the enzyme complex with Rib-P_2-Ado-P but not $NADP^+$ or NADPH demonstrates that binding of the former nucleotide is easier to perturb than that of the other two. The nicotinamide ring in $NADP^+$ and NADPH apparently serves as an important additional point of attachment.

The results presented in this paper demonstrate that $NADP^+$, NADPH, and Rib-P_2-Ado-P bind in similar environments, probably at the same site on $NADP^+$-specific isocitrate dehydrogenase. The 2′-phosphate of the adenine ribose in these complexes is in the dianionic form, and its pK is considerably lower than that of free nucleotides. The nature of the interaction of this group in complexes with $NADP^+$-specific isocitrate dehydrogenase and *L. casei* dihydrofolate reductase appears to be strikingly similar and may be characteristic for other NADP(H) linked enzymes.

Registry No. NADP, 53-59-8; NADPH, 53-57-6; Rib-P_2-Ado-P, 53595-18-9; Ado(2′,5′)P_2, 3805-37-6; NADP-specific isocitrate dehydrogenase, 9028-48-2.

[2] Calculations of free and metal-bound species were performed as described under Experimental Procedures.

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Biosynthesis of Isoleucine in Methanogenic Bacteria: A ^{13}C NMR Study†

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ABSTRACT: The biosynthesis of isoleucine by seven species of methanogenic bacteria was studied by using ^{13}C NMR, following growth in media containing specifically ^{13}C-labeled compounds. It was found that in these bacteria α-ketobutyrate is not derived from threonine. In all cases, when acetate and CO_2 serve as the total carbon sources, the labeling pattern of isoleucine is consistent with the operation of the pyruvate pathway (condensation of pyruvate with acetyl coenzyme A to form α-ketobutyrate). When propionate or 2-methylbutyrate is added to the medium, alternative pathways contribute to isoleucine biosynthesis. Two additional pathways are postulated: carboxylation of propionate to α-ketobutyrate and conversion of 2-methylbutyrate to isoleucine probably by a carboxylation reaction.

In addition to the standard pathway, in which α-ketobutyrate is derived from threonine, microorganisms have developed several alternative pathways for the synthesis of isoleucine (Figure 1). α-Ketobutyrate can be derived from homoserine (Flavin & Segal, 1964), from glutamate via β-methylaspartate (Phillips et al., 1972), from pyruvate by chain elongation (Vollbrecht, 1974; Charon et al., 1974; Westfall et al., 1983; Kisumi et al., 1977), and from propionate by carboxylation (Sauer et al., 1975; Buchanan, 1969). In some cases, when there is an abundance of volatile fatty acids in the medium, 2-methylbutyrate can be carboxylated directly to isoleucine by some anaerobic bacteria from the rumen (Robinson & Allison, 1969).

Methanogens are members of a recently discovered third kingdom designated the Archaebacteriae (Woese, 1982). Because a number of structural and biochemical features are already known to separate the methanogens from the eubacterial and eukaryotic lines of evolutionary descent, differences in metabolic pathways are likely to occur as well. Metabolic studies have special relevance because methane, a fuel gas, is the major excretion product of metabolism, and a common intermediate may be shared between methanogenic and anabolic pathways (Kenealy & Zeikus, 1982). Metabolic control mechanisms have yet to be explored. In a previous paper (Ekiel et al., 1983), it was concluded from ^{13}C labeling patterns that isoleucine in *Methanospirillum hungatei* (*Msp. hungatei*)[1]

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[1] Abbreviations: *Msp.*, *Methanospirillum*; *Ms.*, *Methanosarcina*; *Mbr.*, *Methanobrevibacter*; *Mc.*, *Methanococcus*; *Mb.*, *Methanobacterium*; CoA, coenzyme A.

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