Spectroscopic Studies of the Interactions of Coenzymes and Coenzyme Fragments with Pig Heart, Oxidized Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase†

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ABSTRACT: Spectroscopic, ultrafiltration, and kinetic studies have been used to characterize interactions of reduced and oxidized triphosphopyridine nucleotides (TPNH and TPN), 2′-phosphoadenosine 5′-diphosphoribose (Rib-P_2-Ado-P), and adenosine 2′,5′-bisphosphate [Ado(2′,5′)P_2] with TPN-specific isocitrate dehydrogenase. Close similarity of the UV difference spectra and of the protein fluorescence changes accompanying the formation of the binary complexes provides evidence for the binding of these nucleotides to the same site on the enzyme. From the pH dependence of the dissocation constants for TPNH binding to TPN-specific isocitrate dehydrogenase in the absence and in the presence of Mn^{2+}, over the pH range 5.8–7.6, it has been demonstrated that the nucleotide binds to the enzyme in its unprotonated, metal-free form. The involvement of positively charged residues, protonated over the pH range studied, has been postulated. One TPNH binding site per enzyme subunit has been measured by fluorescence and difference absorption titrations. A dramatic effect of ionic strength on binding has been demonstrated: about a 1000-fold decrease in the dissociation constant for TPNH has been observed at pH 7.6 upon decreasing ionic strength from 0.336 (K_d = 1.2 ± 0.2 μM) to 0.036 M (K_d = 0.4 ± 0.1 nM) in the presence and in the absence of 100 mM Na_2SO_4, respectively. Weak competition of sulfate ions for the nucleotide binding site has been observed (K_I = 57 ± 3 mM). The binding of TPN in the presence of 100 mM Na_2SO_4 at pH 7.6 is about 100-fold weaker (K_d = 110 ± 22 μM) than the binding of the reduced coenzyme and is similarly affected by ionic strength. These results demonstrate the importance of electrostatic interactions in the binding of the coenzyme to TPN-specific isocitrate dehydrogenase. The large enhancement of protein fluorescence caused by binding of TPN and Rib-P_2-Ado-P (ΔF_{max} = 50%) and of Ado(2′,5′)P_2 (ΔF_{max} = 41%) has been ascribed to a local conformational change of the enzyme. An apparent stoichiometry of 0.5 nucleotide binding site per peptide chain was determined for TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2 from fluorescence titrations, in contrast to one binding site per enzyme subunit determined from UV difference spectral titration and ultrafiltration experiments. Thus, the binding of one molecule of the nucleotide per dimeric enzyme molecule is responsible for the total increase in protein fluorescence, while binding to the second subunit does not cause further change. The dissociation constant for the binary TPN–enzyme complex is about 10 times lower than the K_m, and the dissociation constants for the binary complexes of the enzyme with Rib-P_2-Ado-P and Ado(2′,5′)P_2 are 10–50 times lower than the K_I values determined kinetically, suggesting that manganese isocitrate weakens binding of the nucleotides. The fluorescence-detected conformational change induced by Rib-P_2-Ado-P and Ado(2′,5′)P_2 is prevented by the presence of manganese isocitrate. These results indicate that conformational differences exist between the enzyme–nucleotide and enzyme–nucleotide–substrate complexes of isocitrate dehydrogenase.

The structural similarities of the coenzyme binding domains in oxidized diphosphopyridine nucleotide (DPN)[1] dependent dehydrogenases have been extensively characterized [e.g., see Grau (1982)]. Evolutionary relationships have been suggested for these enzymes (Rossmann et al., 1975). In contrast, TPN-binding enzymes exhibit much greater variations in their structures as well as their functions. It has been proposed that several classes of structurally related TPN binding proteins might exist (Adams et al., 1981). Further studies of nucleotide binding sites are important to explore structural relationships among these enzymes, especially for the latter group of proteins.

Pig heart TPN-specific isocitrate dehydrogenase [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42], which requires a divalent metal ion for activity, catalyzes the oxidative decarboxylation of isocitrate to produce α-ketoglutarate, CO_2, and TPNH. Previous investigations of coenzyme binding to mammalian TPN-specific isocitrate dehydrogenase (Reynolds et al., 1978; Carlier & Pantaloni, 1976; Ehrlich & Colman, 1975; Colman, 1983) have been performed over a broad range of ionic strength and buffer composition and therefore comparisons of their nucleotide binding properties are difficult. In the present study we have investigated the effect of sulfate ions on nucleotide binding to isocitrate dehydrogenase. We have also examined the pH dependence of the dissociation constants for TPNH in the presence and in the absence of Mn^{2+} in order to obtain information about the protonation state of the ligand as well as the amino acid residues located within the binding site.

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; Rib-P_2-Ado-P, 2′-phosphoadenosine 5′-diphosphoribose; Ado(2′,5′)P_2, adenosine 2′,5′-bisphosphate; TPN, triphosphopyridine nucleotide (oxidized form); TPNH, triphosphopyridine nucleotide (reduced form); DPN, diphosphopyridine nucleotide (oxidized form); EDTA, ethylenediaminetetraacetic acid.

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The results of our phosphorus-31 NMR studies of the nucleotide complexes with pig heart TPN-specific isocitrate dehydrogenase indicated a similar environment of the 2′-phosphate group in these complexes and suggested a common binding site for the 2′-phosphate moiety of TPN, TPNH, and 2′-phosphoadenosine 5′-diphosphoribose (Mas & Colman, 1984). In the present study we have used difference absorption spectroscopy as a complementary method, sensitive to the perturbations of nucleotide and protein chromophores, in order to further characterize similarities and/or differences in the binding of the nucleotide coenzymes to isocitrate dehydrogenase. Differences in the number of nucleotide binding sites in mammalian isocitrate dehydrogenases have been reported (Ehrlich & Colman, 1975; Carlier & Pantaloni, 1976; Reynolds et al., 1978). In this paper, the stoichiometry and binding affinity of TPN, 2′-phosphoadenosine 5′-diphosphoribose (Rib-P_2-Ado-P), and adenosine 2′,5′-bisphosphate [Ado(2′,5′)P_2] for pig heart TPN-specific isocitrate dehydrogenase were investigated by using protein fluorescence titration, UV difference absorption, and ultrafiltration techniques. The effect of the substrate on the binding of the nucleotides has been investigated by comparison of the dissociation constants determined from kinetic measurements with those obtained directly from fluorescence titrations in the absence of manganese isocitrate. CD spectra of the nucleotide complexes in the absence and in the presence of the substrate have been measured to evaluate the extent of conformational changes of the enzyme.

Experimental Procedures

Materials. TPN-specific isocitrate dehydrogenase was isolated from pig hearts as previously described (Bacon et al., 1981). The specific activity of the enzyme used in this study was in the range 35–42 units/mg. Enzymatic activity was measured in the spectrophotometric assay (Colman, 1968) at 340 nm in a total volume of 1 mL containing 0.03 M triethanolamine chloride buffer, pH 7.4, 100 μM TPN, 4 mM DL-isocitrate, and 2 mM $MnSO_4$ at 25 °C. Enzyme concentrations are expressed in terms of molar concentration of the enzyme subunits by assuming a molecular weight of 58 000 for a peptide chain (Colman et al., 1970). Protein concentrations were determined spectrophotometrically at 280 nm by assuming $E_{280}^{1\%} = 10.8$ (Johanson & Colman, 1981). The enzyme samples were dialyzed extensively against an appropriate buffer solution and centrifuged prior to spectroscopic measurements.

Coenzymes (sodium salts), triethanolamine hydrochloride, MOPS and MES (free acids), Tris (base), and DL-isocitrate were purchased from Sigma Chemical Co. Na_2SO_4, NaCl, glycerol, and EDTA (disodium salt) were from Fisher Chemical Co., and $MnSO_4$ was from Mallinckrodt. The pH of buffer solutions was adjusted with NaOH (MOPS and triethanolamine buffers) or with HCl (Tris).

Difference Absorption Spectral Measurements. Absorption difference spectra were measured at 25 °C on a Cary 219 double-beam spectrophotometer (Varian). Tandem quartz cuvettes with a total path length of 1 cm (Savant) were used. One milliliter of a protein solution and 1 mL of ligand solution were placed in separate compartments of both sample and reference cuvettes, and a base line was recorded and subsequently subtracted from the difference spectrum which was measured after a thorough mixing of the contents of the sample cuvette. At least two scans were recorded for each sample. The final base line was recorded after mixing of the contents of the reference cuvette. The spectra for which a zero final base line was not obtained were discarded. Each experiment was repeated at least 3 times. Difference spectra were measured by using a 0.1- or 0.05-Å scale expansion, at 2.5-nm spectral bandwidth. Titration experiments were performed by mixing 1-mL portions of the enzyme solution at a constant concentration with 1 mL of a nucleotide solution of varying concentration or, alternatively, by titrating a protein solution in the sample cuvette and a buffer solution in the reference cuvette with small aliquots of a nucleotide solution. Identical volumes of buffer were added to the second compartment of each cuvette. Corrections for the dilution in the latter case did not exceed 2%. Although only the former procedure allows a continuous control of a base line throughout the titration experiment, the results of both methods were comparable.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a Perkin-Elmer MPF-44B spectrofluorometer operated in the ratio mode and equipped with an X–Y recorder (Hitachi, Model 057), using 1-cm pathlength quartz cells at 25 °C. Fluorescence titrations with TPNH were routinely made at the excitation wavelength at 340 nm and a bandwidth of 5 nm and at the emission wavelength of 440 nm and a bandwidth of 10 nm. In a typical experiment, two parallel samples containing enzyme solution or buffer alone were titrated with identical increments of TPNH. The enzyme concentration was maintained constant by titrating with a TPNH solution containing the same concentration of the enzyme as that of the titrated sample. Whenever a titration was performed with TPNH dissolved in a buffer solution, the final dilution did not exceed 2–3% of the initial volume. The fluorescence of bound TPNH was determined by titrating a low concentration of the nucleotide (≤1 μM) with a concentrated enzyme solution containing the same nucleotide concentration as that of the titrated sample. Final concentrations of the enzyme subunits were in the range of 15–20 μM in these experiments. Each titration experiment was repeated at least twice. The pH of the solutions was measured on a Radiometer pH meter equipped with a combined glass electrode.

The concentrations of protonated and unprotonated, free and metal-bound nucleotides were calculated by using a computer program described by Cohen & Colman (1972), assuming Mn^{2+}–TPNH association constants of 590 M^{-1} for the protonated and of 2560 M^{-1} for the unprotonated nucleotide (Colman, 1972) and the $MnSO_4$ association constant of 139 M^{-1} (Sillen and Martell, 1964).

Protein fluorescence titrations were performed at the excitation wavelength of 300 nm at a spectral bandwidth of 5 nm, and the fluorescence emission intensity was monitored at 350 nm at a spectral bandwidth of 10 nm for about 1 min. Corrections for the inner filter effect were unnecessary at the excitation wavelength and at the concentrations of nucleotide solutions used in this study. Protein solutions were placed in 3-mL quartz cuvettes and titrated with 3–5-μL aliquots of concentrated nucleotide solutions at 25 °C. The solutions were stirred with a magnetic micro stirring bar after addition of ligand for about 30 s. Corrections were made for dilutions (not greater than 2.5% of an initial volume of 2–2.5 mL). Each titration experiment was performed at least twice. Fluorescence titration data were analyzed according to Scatchard (1949).

Ultrafiltration Experiments. The protein and nucleotide solutions of known concentrations (about 2 orders of magnitude higher than the respective dissociaton constants) were mixed in a 1:1 (v/v) ratio and placed in an Amicon ultrafiltration cell, Model 10PA, containing a Diaflo PM-10 membrane (Amicon). About 1 mL of the filtrate was collected following an initial equilibration of the solution at room temperature

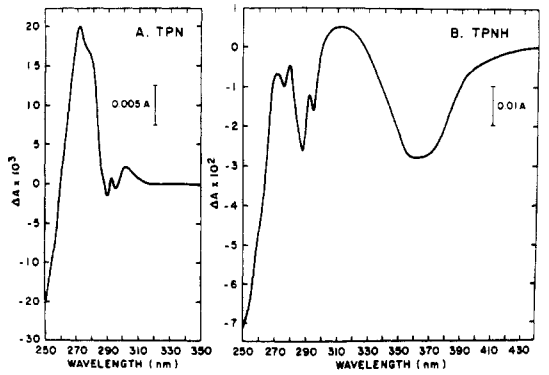

FIGURE 1: Difference absorption spectra of isocitrate dehydrogenase (31 μM) with nucleotides in 50 mM MOPS buffer, pH 7.6, containing 0.1 M Na_2SO_4, 10% glycerol, and 0.5 mM EDTA at 25 °C. (A) 75.8 μM TPN; (B) 100 μM TPNH.

for 5–10 min. The initial concentration and the concentration of free nucleotide in the filtrate were measured spectrophotometrically at 259 nm by using an extinction coefficient of 18 000 M^{-1} cm^{-1} for TPN and 15 400 M^{-1} cm^{-1} for Rib-P_2-Ado-P. The concentration of bound nucleotide was calculated from the difference between the total and free ligand concentrations.

Circular Dichroism. CD spectra were measured at room temperature on a Durrum Jasco Model J-10 recording spectropolarimeter (Jasco, Inc., Easton, MD), calibrated with *d*-10-camphorsulfonic acid (Cassim & Young, 1969). Samples were placed in cylindrical quartz windowed (Precision Cells, Inc., Hicksville, NJ) with a path length of 1 mm. The mean residue ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$), was calculated from the following expression:

$$[\theta] = \frac{100\theta}{Cln} \quad (1)$$

where θ is measured ellipticity in degrees, C is the subunit concentration in moles per liter, l is the path length in centimeters, and n is the number of amino acid residues per subunit of isocitrate dehydrogenase [523 residues per M_r 58 000 of a monomer (Johanson & Colman, 1981)].

Kinetic Measurements. The spectrophotometric assays were performed at 25 °C on a Cary 219 recording spectrophotometer (Varian) by monitoring at 340 nm the appearance of TPNH produced in the isocitrate dehydrogenase reaction (Colman, 1968). The initial rate measurements were performed in a 1-mL solution containing 50 mM triethanolamine chloride buffer, pH 7.6, 10% glycerol, 2 mM $MnCl_2$, 4 mM DL-isocitrate, and 3–80 μM TPN in the absence and in the presence of an inhibitor. The K_m values were determined from the Lineweaver–Burk plots, and the K_I constants for Rib-P_2-Ado-P and for Ado(2′,5′)P_2 were determined from the plots of the apparent K_m vs. inhibitor concentration (Segal, 1975), at three concentrations of each inhibitor.

RESULTS

Absorption Difference Spectra of the Binary Complexes of Isocitrate Dehydrogenase with TPN and TPNH in the Presence of 100 mM Sulfate. Difference absorption spectroscopy is a method sensitive to the changes in the environment of protein and/or ligand chromophores and capable of providing quantitative information about stoichiometry and affinity of ligand binding in favorable cases. This technique has been used in the present study in order to evaluate binding parameters for the complexes of pig heart isocitrate dehydrogenase with nucleotides. The absorption difference spectra of 31 μM enzyme subunits in 50 mM MOPS buffer, pH 7.6, containing 0.1 M $NaSO_4$, 10% glycerol, and 0.5 mM EDTA, at 25 °C, in the presence of an excess of ligands are shown in panels A and B of Figure 1 for TPN and TPNH complexes, respectively. Identical conditions were used in our previous ^{31}P NMR studies of these complexes (Mas & Colman, 1984). Common features can be observed in both spectra in the wavelength range 250–290 nm, with a characteristic minimum near 290 nm, a large negative band at about 250 nm due to a hypochromicity of the adenine absorption band (Leonard et al., 1967; Subramanian et al., 1981), and positive absorption bands in the region of 270–280 nm. In addition, a hypochromic blue shift of the 340-nm absorption band of the reduced nicotinamide chromophore (Fisher et al., 1969) is observed in the difference spectrum of the enzyme–TPNH complex (Figure 1B), with a minimum at about 360 nm and a broad maximum at about 310 nm. The spectral difference between these extrema, $\Delta A_{310\text{-}360nm}$, was used as a quantitative measure of a complex formation between the enzyme and the reduced coenzyme. The spectral titration of 31 μM enzyme subunits with increasing concentrations of TPNH is shown in Figure 2. The stoichiometry of binding was calculated from the equivalence point at the intersection of the two linear portions of the titration curve, corresponding to 31 μM TPNH and indicating one binding site per enzyme subunit. [A lower value reported in the literature, 0.7 mol of TPNH/mol of peptide chain (Ehrlich & Colman, 1975), was probably due to the nonstoichiometric conditions at the much lower enzyme concentration used for the determination of the dissociation constant and number of ligand binding sites from the same experiment.] Parallel changes in other regions of the TPNH–enzyme spectrum were observed during the titration. Titrations monitored at $\Delta A_{288\text{-}292nm}$ and $\Delta A_{280\text{-}288nm}$ yielded the same stoichiometry (not shown). For the determination of a dissociation constant of approximately 1 μM (as estimated from the nonlinear segment of the titration curve in Figure 2) the concentration of protein subunits should be of the same order of magnitude ([P] $\leq K_d$). The molar extinction coef-

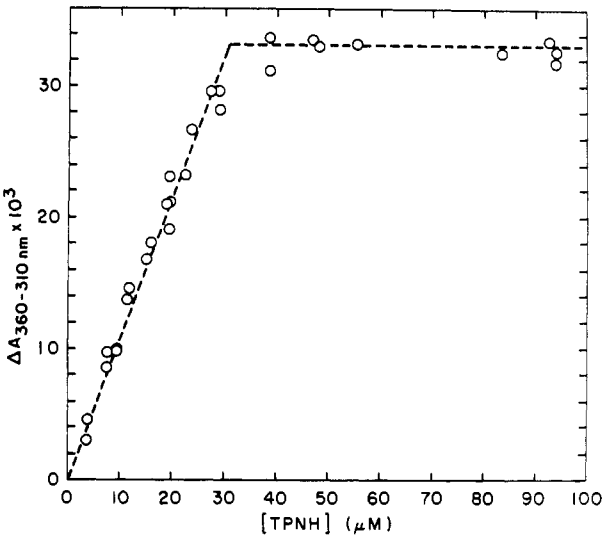

FIGURE 2: Spectrophotometric titration of isocitrate dehydrogenase (31 μM) with TPNH. The enzyme in 50 mM MOPS buffer, pH 7.6, containing 0.1 M Na_2SO_4, 10% glycerol, and 0.5 mM EDTA, was titrated with successive additions of TPNH at 25 °C as described under Experimental Procedures. The absolute value of the spectral difference between a minimum at 360 nm and a maximum at 310 nm ($\Delta A_{310\text{-}360nm}$) was monitored during the titration (see spectrum in Figure 1B). The molar extinction coefficient, $\Delta\epsilon_{310\text{-}360nm}$, of 1.26 mM^{-1} cm^{-1} was calculated on the basis of the molecular weight of 58 000 per peptide chain. The stoichiometry of 1 mol of TPNH/mol of subunit is obtained from extrapolation of the two linear portions of the titration curve representing four separate experiments.

ficient, $\Delta\epsilon_{310-360nm}$, of 1.26 mM^{-1} cm^{-1} was calculated for the spectral change $\Delta A_{310-360nm}$. The maximum spectral change corresponding to complete saturation of 1 μM enzyme subunits with TPNH can be calculated as approximately equal to 0.001 absorbance unit, i.e., 30-fold less than the magnitude of the spectral change observed under the condition of stoichiometric binding (Figure 2); this method is therefore unsuitable for the purpose of accurate determination of the dissociation constant for TPNH under these conditions.

A positive spectral change at 270 nm in the spectrum of the TPN–enzyme complex (Figure 1A) was used to monitor binding of the oxidized coenzyme. Titration of 31 μM enzyme subunits with TPN up to 90 μM ligand did not lead to saturation (not shown). The maximum absorbance of the solution in each cuvette was maintained at about 2 absorbance units at the constant spectral bandwidth of 2.5 nm used for recording difference spectra, which limits further increase in TPN concentration and thereby prevents the direct determination of the end point. Furthermore, the lack of linearity in the initial portion of the titration curve did not allow for the calculation of the end point from the molar extinction coefficient. These titration characteristics indicated that the binding of TPN was much weaker than that of TPNH under identical conditions. The dissociation constants for TPN and for TPNH were determined instead by using a fluorescence technique as described below.

Absorption Difference Spectra of the Binary Complexes of Isocitrate Dehydrogenase with TPNH, TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2 in the Absence of Sulfate. In order to compare spectral properties of the binary complexes of nucleotides with isocitrate dehydrogenase, we have measured the difference absorption spectra of the binary complexes in 50 mM MOPS and in 50 mM triethanolamine buffers (pH 7.6), containing 10% glycerol and 0.5 mM EDTA. All experiments described in this section were performed at relatively low ionic strength (I = 0.05–0.08 M). These spectra were also compared with those obtained in 50 mM MOPS (pH 7.6) containing 10% glycerol and 0.5 mM EDTA, in the presence of 100 mM sulfate (Figure 1A,B). The difference spectra of the binary complexes with Ado(2′,5′)P_2, Rib-P_2-Ado-P, and with TPN in MOPS buffer in the absence of sulfate are shown in Figure 3A. The spectral perturbations observed for all three complexes are remarkably similar. A minimum at about 250 nm and positive bands occurring in a 270–280-nm spectral region closely resemble spectral features observed by Subramanian et al. (1981) in the difference spectra of adenine nucleotides produced by low pH, salts, and by dioxane. By analogy, they can be largely attributed to the perturbations of the electronic transitions of the purine ring which might result from the interaction with a cationic or a combination of a cationic and nonpolar residues on the enzyme (Subramanian et al., 1981). A characteristic minimum at about 290 nm might be ascribed to the perturbation of an indole chromophore(s) entering a more polar environment (Ananthanarayanan & Bigelow, 1969). Isocitrate dehydrogenase contains 9 tryptophans per monomer, in addition to 24 phenylalanyl and 14 tyrosyl residues (Johanson & Colman, 1981). The location of these aromatic amino acids with respect to the nucleotide binding sites is unknown at present. Perturbation of protein chromophores could also contribute to some extent to the spectral changes observed in the 270–280-nm region. Whatever the origin of the spectra bands observed in the difference spectra of the binary complexes, it is evident that the interactions of TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2 with the enzyme in MOPS buffer give rise to nearly identical spectral changes

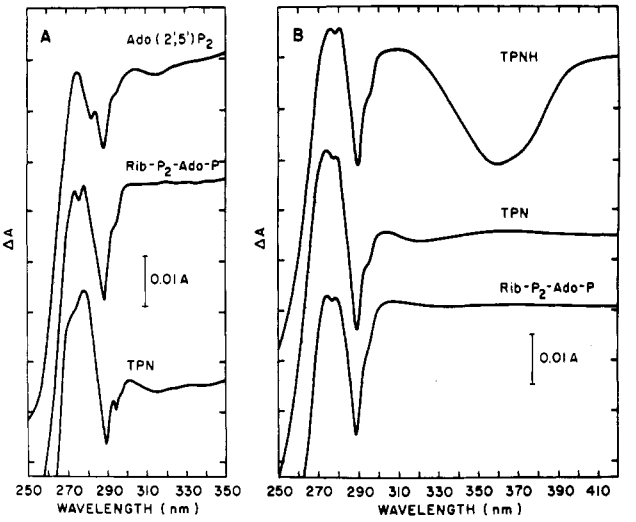

FIGURE 3: Difference absorption spectra of the binary complexes of isocitrate dehydrogenase with coenzymes and coenzyme fragments. The enzyme (1 mL) and the nucleotide (1 mL) solutions were placed in separate compartments of the sample and the reference cuvettes, of a total path length of 1 cm. After a base line was recorded, the contents of the sample cuvette were mixed, and the spectrum was recorded with an automatic base-line subtraction. (A) Spectra in 50 mM MOPS buffer (pH 7.6), containing 10% glycerol and 0.5 mM EDTA, at 25 °C. Final concentrations were (top to bottom) 18.9 μM enzyme subunits plus 39.1 μM Ado(2′,5′)P_2, 18.9 μM enzyme subunits plus 40.7 μM Rib-P_2-Ado-P, and 18.4 μM enzyme subunits plus 42.6 μM TPN. The saturation of nucleotide binding sites were 94.3%, 99.3%, and 98.8%, resp., assuming one site per subunit and the dissociation constants for the nucleotides calculated in the text. (B) Spectra in 50 mM triethanolamine chloride buffer (pH 7.6), containing 10% glycerol and 0.5 mM EDTA, at 25 °C. Final concentrations were (top to bottom) 18 μM enzyme subunits plus 38.1 μM TPNH, 18 μM enzyme subunits plus 39.2 μM TPN, and 18 μM enzyme subunits plus 38.8 μM Rib-P_2-Ado-P. These concentrations correspond to 100%, 98.7%, and 99.3% saturation of nucleotide binding sites with TPNH, TPN, and Rib-R_2-Ado-P, respectively, assuming one nucleotide binding site per subunit, K_d = 0.5 nM for TPNH, and the dissociation constants for other nucleotides as calculated in the text.

(Figure 3A). This similarity also applies to the spectra of the binary complexes of the enzyme with TPN, Rib-P_2-Ado-P, and TPNH obtained in triethanolamine buffer (Figure 3B). Moreover, a comparison of the spectra of the enzyme complexes with TPN and with TPNH measured in the absence of sulfate (Figure 3A,B) with the corresponding spectra obtained in the presence of 100 mM sulfate (shown in Figure 1A,B) implies interaction with the same site on the enzyme, regardless of the buffer conditions used. The molar extinction coefficient of 1.25 mM^{-1} cm^{-1} was calculated for the spectral change $\Delta A_{310-360nm}$ for the TPNH–enzyme complex in the absence of sulfate in triethanolamine buffer (Figure 3B), in excellent agreement with the molar extinction coefficient of 1.26 mM^{-1} cm^{-1} obtained in the presence of sulfate. The essentially identical perturbations observed in the 250–290-nm region in the spectra of the binary complexes with TPNH, TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2 strongly suggest similar interactions of the adenosine ribose moiety with the same binding site on the enzyme. There are no additional spectral features in the difference spectrum of the binary complex with TPN, as compared to that with Rib-P_2-Ado-P, which could be ascribed to the perturbations resulting from the interaction of the oxidized nicotinamide ring with the enzyme. On the other hand, a direct interaction of the reduced nicotinamide is evident from the perturbation of the 340-nm absorption band of TPNH (Figure 3B), resulting in a large negative band with a minimum at about 360 nm. The hypochromic blue shift of

this band might be attributed to the interaction of the reduced nicotinamide ring with an environment of lower refractive index or, in part, to the unfolding of TPNH (Fisher et al., 1969).

Effect of pH and Mn^{2+} on the Dissociation Constants for TPNH in the Presence of 100 mM Sulfate. The binding of TPNH to TPN-specific isocitrate dehydrogenase in the pH range 5.8–7.6, in 50 mM MOPS buffer (MES below pH 6.5) containing 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA or in the same buffer containing 0.08 M Na_2SO_4, 10% glycerol, and 0.02 M $MnSO_4$, was studied by monitoring the fluorescence emission intensity at 440 nm (excitation at 340 nm). The subunit concentrations were in the range 1–4 μM. The results of a typical fluorescence titration experiment are illustrated in Figure 4A, at pH 5.8 in the absence of manganous ions. The observed fluorescence (F) at any point of a titration curve is the sum of the fluorescence of bound and free TPNH:

$$F = F_{free}[L]_{free} + F_{bound}[L]_{bound} \quad (2)$$

where F_{free} and F_{bound} are the molar fluorescence of the free and bound ligand, respectively. From eq 2, by substitution of $[L]_{free} = [L]_{total} - [L]_{bound}$ and rearrangement:

$$F/F_{free} = [L]_{total} + Q[L]_{bound} \quad (3)$$

where Q is the fluorescence enhancement, defined as

$$Q = F_{bound}/F_{free} - 1 \quad (4)$$

From the expression for the protein–ligand dissociation constant

$$K_d = \frac{[P]_{free}[L]_{free}}{[L]_{bound}} \quad (5)$$

$$[L]_{bound} = \frac{[L]_{total}[P]_{free}}{K_d + [P]_{free}} \quad (6)$$

and eq 3

$$\frac{F}{F_0} - 1 = \frac{Q[P]_{free}}{K_d + [P]_{free}} \quad (7)$$

where $F_0 = F_{free}[L]_{total}$. F and F_0 represent the observed fluorescence in the presence and absence of the enzyme, respectively, as in Figure 4A. Taking the reciprocal of eq 7, one obtains

$$\frac{1}{F/F_0 - 1} = \frac{K_d}{Q}\frac{1}{[P]_{free}} + \frac{1}{Q} \quad (8)$$

The enhancement, Q, can be obtained from the intercept of the linear plot of $1/(F/F_0 - 1)$ vs. $1/[P]_{total}$, according to eq 8, with the assumption that $[P]_{free} - [P]_{total}$ at sufficiently high excess of enzyme with respect to the enzyme–ligand dissociation constant. The results of a typical determination of the fluorescence enhancement are shown in Figure 4B. An average enhancement of $Q = 6$, corresponding to a 7-fold increase of the fluorescence intensity of bound with respect to that of free ligand, was obtained over the pH range studied.

Once the value of Q is known, the concentration of bound ligand can be calculated from a fluorescence titration experiment such as that shown in Figure 4A from the relationship

$$[L]_{bound} = [L]_{total}\frac{F/F_0 - 1}{Q - 1} \quad (9)$$

The dissociation constants were obtained from Scatchard plots according to

$$\frac{r}{[L]_{free}} = \frac{n}{K_d} - \frac{r}{K_d} \quad (10)$$

where r is the ratio of the concentration of bound ligand (calculated from eq 9) to the total protein concentration and n represents the maximum number of binding sites per enzyme subunit. A typical Scatchard plot is shown in Figure 4C for the experiment conducted at pH 5.8.

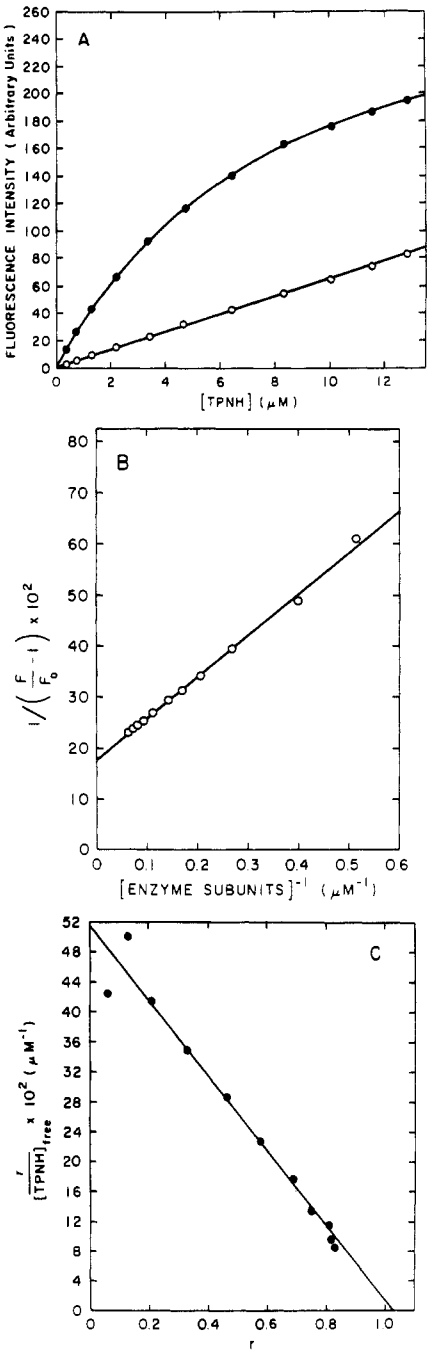

FIGURE 4: Fluorescence determination of the TPNH–isocitrate dehydrogenase dissociation constant in 50 mM MES buffer, pH 5.8, containing 0.1 M Na_2SO_4, 10% glycerol, and 0.5 mM EDTA at 25 °C. (A) Fluorescence titration of 3.7 μM enzyme subunits (●) and buffer alone (○) with TPNH. (B) Determination of the fluorescence enhancement, $Q = F_{bound}/F_{free} - 1$, for TPNH by fluorescence titration of 1.2 μM total TPNH with isocitrate dehydrogenase subunits. The enhancement $Q = 6$ was obtained from the data plotted according to eq 8. The line is a least-squares fit ($R^2 = 99.9\%$). F and F_0 represent fluorescence intensity in the presence and absence of the enzyme, respectively. (C) Scatchard plot (eq 10) of the titration data in (A). The line is a least-squares fit with $K_d = 2.0$ μM, $n = 1.03$, and $R^2 = 97\%$. Fluorescence emission was monitored at 440 nm (bandwidth = 10 nm) by using an excitation wavelength of 340 nm (bandwidth = 5 nm).

Studies of the variation of the TPNH dissociation constant with pH were undertaken in order to obtain information about

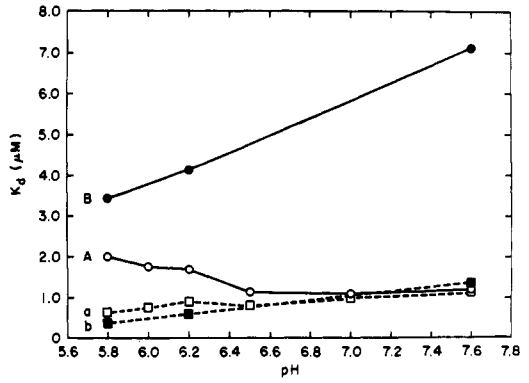

FIGURE 5: pH dependence of the dissociation constants for the binding of TPNH to isocitrate dehydrogenase in the absence (○) (A) and in the presence (●) of 20 nM Mn^{2+} (B). The dissociation constants in lines A and B were also calculated in terms of unprotonated, metal-free TPNH (□, a), and (■, b), respectively. The dissociation constants were determined by fluorescence titrations of 1–6 μM enzyme subunits with TPNH as illustrated in Figure 4. Titration were performed in 50 mM MOPS (MES below pH 6.5) containing 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA (A and a) or in 50 mM MOPS (MES), 10% glycerol, 0.08 Na_2SO_4, and 0.02 M $MnSO_4$ (B and b), at 25 °C.

the ionization state of TPNH as well as about the nature of amino acid residues located in the nucleotide binding site. The apparent dissociation constants for TPNH determined in the absence of manganous ion is approximately constant and equal to about 1 μM in the pH range 6.5–7.6 and increases gradually with decreasing pH to reach the value of 2 μM at pH 5.8 (Figure 5, line A). The titration data can be recalculated in terms of the concentration of completely ionized nucleotide with the assumption that only this species can bind to the enzyme. For these calculations, a computer program described by Cohen & Colman (1972) was used, assuming the pK of 6.13 for the 2′-phosphate group of the adenosine ribose. This pK was determined under identical conditions from ^{31}P NMR studies (Mas & Colman, 1984). A virtually constant value of the K_d for fully ionized TPNH was obtained throughout the entire pH range (Figure 5, line a). An alternative assumption, that only the species with a protonated 2′-phosphate group binds to the enzyme, leads to a 34-fold increase in K_d as the pH is decreased from 7.6 to 5.8 (K_d is equal to 0.04 μM and 1.36 μM, respectively). Thus, the increase observed at lower pH in the dissociation constant, expressed in terms of total nucleotide, can be almost entirely accounted for as the result of the protonation of the 2′-phosphate group. It has been postulated on the basis of the results of our ^{31}P NMR studies (Mas & Colman, 1984) that it is the dianionic form of the 2′-phosphate that binds to the enzyme in its complexes with the nucleotides. When 20 mM $MnSO_4$ is included in the equilibrium mixture, the measured dissociation constants are appreciably higher at every pH tested, and furthermore, the apparent K_d increases with increasing pH, as shown in Figure 5 (line B). However, the K_d values calculated in terms of unprotonated, metal-free species are almost constant over the entire pH range (Figure 5, line b) and are remarkably similar to those determined in the absence of Mn^{2+} (Figure 5, line a). Thus, binding of the unprotonated, metal-free species can largely account for the pH dependence of the dissociation constants both in the absence and in the presence of manganous ions. A slight decrease in the K_d observed (Figure 5, lines a and b) may indicate involvement of a group(s) on the enzyme, the protonation of which lowers the K_d of the nucleotide. The pK of such an enzymic group would have to be below the pH range evaluated in this study; measurements below pH 5.8 were not possible due to decreased stability both of TPNH and

Table I: Effect of Sulfate on TPNH Binding at pH 5.8 and 7.6 at 25 °C

sulfate concn (mM)	K_d (μM)[a] pH 5.8	pH 7.6
100	2.0	1.2
25	0.2	0.1

[a] The dissociation constants were determined in 50 mM MES (pH 5.8) or MOPS (pH 7.6) buffer, containing 10% glycerol, 0.5 mM EDTA, and indicated concentrations of sulfate. Fluorescence titrations were conducted at 0.1–1 μM enzyme subunits as described in the legend to Figure 4.

Table II: Dependence of the Dissociation Constants for TPNH on Sulfate Concentration at Constant Ionic Strength

line	additions	ionic strength (M)	K_d[a] (μM)
1	0.100 M Na_2SO_4	0.336	1.20 ± 0.2
2	0.025 M Na_2SO_4 0.225 M NaCl	0.336	0.66 ± 0.1
3	0.300 M NaCl	0.336	0.45 ± 0.3
4		0.036	0.0004 ± 0.0001

[a] The fluorescence determinations of the dissociation constants were conducted in 50 mM MOPS buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA, at 25 °C as described in the legend to Figure 4, at concentrations of enzyme subunits of about 1 μM. The dissociation constant determined in the absence of added salts was measured indirectly, as described in footnote *a* of Table IV.

of the enzyme at lower pH. Since our pH dependence studies have been conducted in the presence of 100 mM sulfate, it can be argued that SO_4^{2-} ions might specifically interact with a positively charged enzymic group and thereby mask its effect on the dissociation constant. This possibility has been addressed in the next section.

Effect of Sulfate Ions on Binding of TPNH to Isocitrate Dehydrogenase. The effect of sulfate ions on the dissociation constants for TPNH was studied at pH 5.8 and 7.6. The comparison of the dissociation constants obtained from fluorescence titrations at low and at high pH, in the presence of 100 and 25 mM sulfate ions, is presented in Table I. A 4-fold decrease in sulfate concentration (from 100 to 25 mM) causes a 10–12-fold decrease in the dissociation constant at pH 5.8 as well as at pH 7.6. The similar effect of sulfate observed at low and at high pH does not rule out a preferential interaction of sulfate ions with a group on the enzyme (e.g., arginine) which is protonated over the entire pH range of 5.8–7.6. It can be concluded, however, that the pH dependence of the dissociation constant for the binding of TPNH to isocitrate dehydrogenase, discussed in the preceding section, does not involve pH dependence of the binding of SO_4^{2-}. The binding of TPNH in the absence of sulfate was too tight to allow for the direct determination of binding constants, both at low and at high pH. At protein concentrations as low as 30 nM, a stoichiometric binding was observed in both cases.

It is evident from the above results that the presence of sulfate ions dramatically affects interaction of TPNH with the enzyme. In order to ascertain whether this phenomenon is due to the difference in the ionic strength, to the specific interaction of sulfate with the enzyme, or to both, we determined the dissociation constants for TPNH at constant ionic strength (I = 0.336 M) at various concentrations of sulfate ions at pH 7.6. The results are compared in Table II, together with the K_d for TPNH, determined indirectly (see below) in the absence of sulfate at ionic strength I = 0.036 M. A large increase in the dissociation constant, of several orders of magnitude, is observed as the result of increasing the ionic strength of the solution by 0.3 M by the addition of 0.3 M NaCl (compare lines 3 and 4, Table II) or 0.1 M Na_2SO_4 (line

Table III: Determination of Dissociation Constant for TPN from Its Effect on the K_d for TPNH at pH 7.6

[TPN] (μM)	K'(TPNH)[a] (μM)	K_I(TPN) (μM)
116.5	3.0	78
290.0	3.9	129
401.7	4.9	130
511.6	6.8	110
514.0	7.3	101
		110 ● 22[b]

[a] The apparent dissociation constants for TPNH were determined from the fluorescence titrations conducted in 50 mM MOPS buffer, containing 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA, at 25 °C, in the presence of various concentrations of TPN. The dissociation constants, K_I, for TPN were calculated from eq 11 by using the value of 1.2 μM for the dissociation constant for TPNH in the absence of TPN. [b] The average K_I(TPN).

Table IV: Determination of the Dissociation Constant for TPNH in the Absence of Sulfate from the Effect of TPN and Rib-P_2-Ado-P on the Apparent Dissociation Constant

inhibitor	K'(TPNH)[a] (μM)	K^0(TPNH) (nM)
55 μM TPN	0.049	0.3
110 μM TPN	0.12	0.3
441 μM TPN	0.77	0.5
433 μM Rib-P_2-Ado-P	1.55	0.5
		0.4 ± 0.1[b]

[a] The dissociation constants in the presence of inhibitors [K'(TPNH)] were measured fluorometrically in 50 mM MOPS buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA. Fluorescence titrations were performed at 25 °C as illustrated in the legend to Figure 4 at the concentration of enzyme subunits ≤1 μM. The dissociation constant for TPNH in the absence of inhibitors [K^0(TPNH)] was calculated from eq 11, for each inhibitor concentration. The dissociation constants for TPN and Rib-P_2-Ado-P of 0.29 μM and 0.15 μM, respectively, were used for these calculations (as determined from the protein fluorescence titrations). [b] The average K^0(TPNH).

1, Table II). The dissocation constant for TPNH determined at high ionic strength (I = 0.336 M) is about 2.7-fold higher in the presence of 100 mM sulfate than that in the presence of 300 mM chloride. Assuming that sulfate acts as a competitive inhibitor, a K_I of 57 ± 3 mM can be calculated from the apparent dissociation constants determined at 25 mM and at 100 mM Na_2SO_4 (Table II, lines 1 and 2) and the K_d for TPNH measured in the absence of sulfate (Table II, line 3).

Dissociation Constant for TPN at pH 7.6 in the Presence of 100 mM Sulfate. The results of the UV difference spectral titrations indicated that the binding of TPN is weaker than that of the reduced nucleotide at 100 mM sulfate concentration. The dissociation constant for TPN in 50 mM MOPS, pH 7.6, containing 10% glycerol, 0.1 M Na_2SO_4, and 0.5 mM EDTA, was determined indirectly from the fluorescence titrations with TPNH of 1–5 μM enzyme subunits in the presence of several concentrations of TPN by assuming the competition of both ligands for the same binding site. The dissociation constants for TPN (K_I) were calculated from eq 11

$$K' = K^0(1 + [I]/K_I) \qquad (11)$$

by using the value of 1.2 μM for the dissociation constant for TPNH in the absence of TPN (K^0). K' is an apparent dissociation constant determined at a given concentration of TPN (I). From the results of these determinations (Table III), the calculated dissociation constant for TPN was reasonably constant within the range of concentrations of TPN used and gave an average of K_I = 110 ± 22 μM. This value is 2 orders of magnitude higher than the dissociation constants for TPNH (K_d = 1.2 ± 0.2 μM) obtained under identical conditions.

Dissociation Constant for TPNH at pH 7.6 in the Absence of Sulfate. The results of the fluorometric titration of 30 nM enzyme subunits in 50 mM MOPS, pH 7.6, containing 10% glycerol and 0.5 mM EDTA, at 25 °C demonstrated stoichiometric binding of TPNH, indicating extremely tight binding of TPNH in the absence of sulfate at the ionic strength I = 0.036 M. The enhancement factor F_{bound}/F_{free} = 7 was obtained directly from the ratio of the fluorescence intensity of 0.74 μM TPNH free in solution to that of the same amount of TPNH bound to the enzyme (data not shown). The fluorescence enhancement Q = 6.00 ± 0.05 agreed with the enhancement values determined previously in the presence of sulfate. The sensitivity limits of fluorometric measurements did not allow for a direct determination of K^0 for TPNH. The dissociation constant was therefore determined indirectly, in a similar manner to that described above for TPN, by using eq 11. The fluorescence titrations of low enzyme concentrations (≤1 μM) with TPNH were performed in the presence of the inhibitors TPN and 2′-phosphoadenosine 5′-diphosphoribose (Rib-P_2-Ado-P), and the apparent dissociation constants, K', were determined by Scatchard analysis. In this case, however, the K_I(TPN) of 0.29 μM and K_I(Rib-P_2-Ado-P) of 0.15 μM, determined in the absence of sulfate in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA (as described below) were assumed for the calculation of K^0 for TPNH at several concentrations of the inhibitors. The average value of K^0(TPNH) = 0.4 ± 0.1 nM was calculated (Table IV). An analogous experiment was performed in 50 mM triethanolamine chloride buffer, pH 7.6, 10% glycerol, and 0.5 mM EDTA (I = 0.05 M) in the presence of 228 μM and 456 μM Rib-P_2-Ado-P (data not shown) and yielded K^0(TPNH) = 0.5 ± 0.1 nM, in a good agreement with the value obtained in MOPS buffer.

Stoichiometry of Binding of Nucleotide to Isocitrate Dehydrogenase. Nucleotides have been shown to quench protein fluorescence of many dehydrogenases. In contrast, we have observed an increase in the fluorescence of the pig heart isocitrate dehydrogenase induced by the interaction with TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2. A complete saturation of the enzyme with TPN or with Rib-P_2-Ado-P in triethanolamine buffer in the absence of sulfate resulted in a 50% (±3%) enhancement and with Ado(2′,5′)P_2 in a 41% (±3%) enhancement of the protein fluorescence emission at 350 nm by using an excitation wavelength of 300 nm. Figure 6 shows the fluorescence titration of 5.8 μM enzyme subunits in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA, with TPN and with Rib-P_2-Ado-P at 25 °C. From the intercept of the two linear portions of the titration curve a stoichiometry of about 0.5 mol per enzyme subunit is obtained for both ligands, corresponding to one molecule of nucleotide bound to the dimeric molecule of isocitrate dehydrogenase. In contrast, the stoichiometry of one TPN bound per monomer was obtained from the UV difference spectral titration of enzyme both in triethanolamine and in MOPS buffer, in the absence of sulfate (Figure 7). The spectral change $\Delta A_{278-289nm}$ was monitored during this titration (see TPN–enzyme absorption difference spectra in Figure 3). A molar extinction coefficient, $\Delta\epsilon_{278-289nm}$, of 1.71 mM^{-1} cm^{-1} was calculated for this spectral change. Stoichiometry binding of TPN is observed under these conditions, in contrast to the results obtained in the presence of 100 mM sulfate, indicating that binding of the oxidized coenzyme is much tighter at low ionic strength. In addition to spectral titrations, we performed ultrafiltration experiments as an independent method for determination of the number of nucleotide binding sites for TPN and for Rib-P_2-Ado-P. The results of these experiments, performed in triethanolamine buffer as described under Ex-

Table V: Determination of the Stoichiometry of Binding of TPN and Rib-P_2-Ado-P by Ultrafiltration[a]

ligand	enzyme subunits (μM)	total ligand (μM)	free ligand (μM)	bound ligand (μM)	bound ligand/enzyme subunit
TPN	15.2	43.6	28.8	14.9	0.98
TPN	18.0[b]	39.2	21.9	17.3	0.96
Rib-P_2-Ado-P	13.8	42.2	27.3	14.9	1.08
Rib-P_2-Ado-P	18.0[b]	38.8	21.2	17.6	0.98

[a] The ultrafiltration experiments were performed at 25 °C in 50 mM triethanolamine chloride buffer, pH 7.6, containing 0.5 mM EDTA, as described under Experimental Procedures. [b] Average of two experiments.

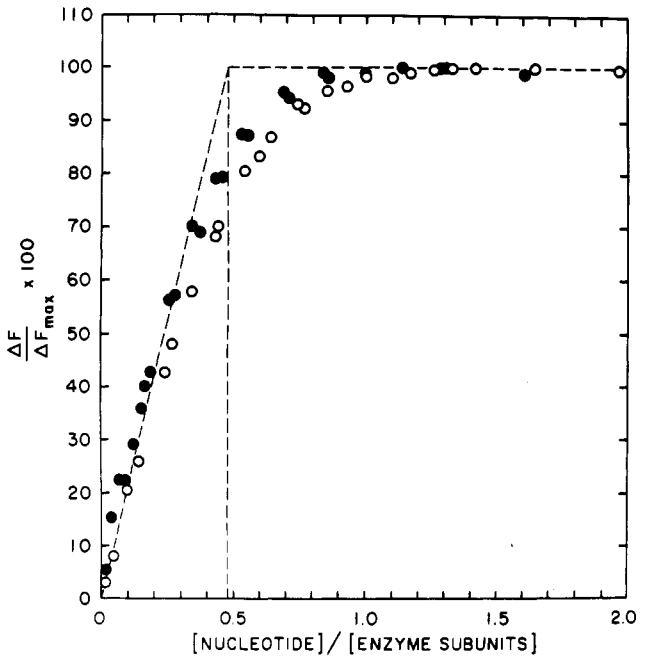

FIGURE 6: Fluorescence determination of the stoichiometry of binding of TPN and Rib-P_2-Ado-P to isocitrate dehydrogenase. The enzyme samples (5.8 μM subunit concentration) in 50 mM triethanolamine chloride buffer (pH 7.6), containing 10% glycerol and 0.5 mM EDTA, were titrated with the nucleotides (0–12 μM final concentrations) at 25 °C. The enhancement of protein fluorescence emission (ΔF) was recorded at 350 nm (excitation at 300 nm). The results of duplicate titrations with TPN (○) and with Rib-P_2-Ado-P (●) are plotted as percent of maximum change ($\Delta F/\Delta F_{max}$) vs. the nucleotide to enzyme subunit ratio. ΔF_{max} = 50% (±3%) for both ligands.

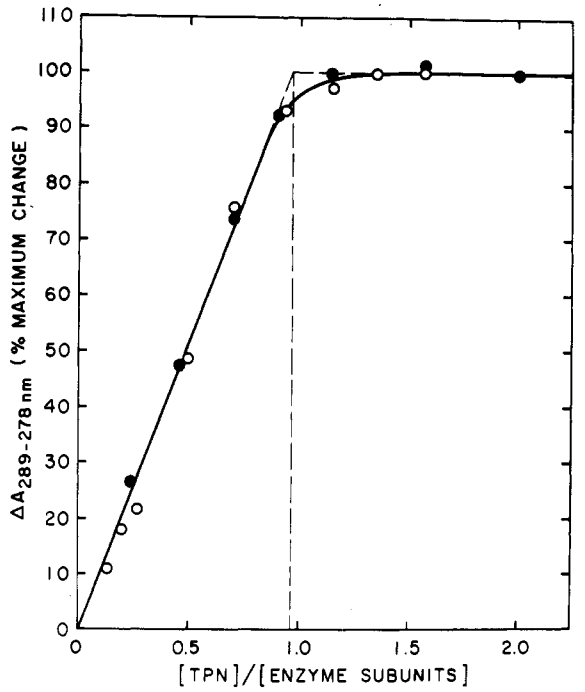

FIGURE 7: Spectrophotometric determination of the stoichiometry of binding of TPN to isocitrate dehydrogenase. The enzyme in 50 mM triethanolamine chloride (○) or 50 mM MOPS (●) buffer (pH 7.6), containing 100% glycerol and 0.5 mM EDTA, was titrated with TPN. Titrations were performed by sequential addition of ligand to the enzyme solution in the sample cuvette and to the buffer compartment of the reference cuvette. An identical volume of buffer was added to the second compartment of each split-compartment cell. The spectral difference between the maximum at 278 nm and the minimum at 289 nm, shown in the spectrum in Figure 3, was monitored during the titration. The maximum change of 0.019 ± 0.0005 A was obtained at total saturation of the enzyme with the ligand.

perimental Procedures, are presented in Table V. The stoichiometry of one ligand bound per enzyme subunit was obtained for both nucleotides by this method. The same result was obtained in MOPS buffer (data not shown). Under similar conditions one binding site per subunit was measured for TPNH, from difference absorption and fluorescence titrations described above. The possible origins of the differences in the number of binding sites obtained for TPN and Rib-P_2-Ado-P from various methods will be discussed later.

Determination of the Dissociation Constants. The dissociation constants for TPN, Rib-P_2-Ado-P, and for Ado(2′,5′)P_2 were determined from protein fluorescence titrations in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA, as described under Experimental Procedures. In contrast to the experiments described in the preceding section low enzyme concentrations were used to assure equilibrium conditions (subunit concentration ≤1 μM). The concentration of bound ligand was calculated by using equation 12 assuming 0.5 site per monomer according to the

$$[L]_{bound} = n\frac{\Delta F}{\Delta F_{max}}[P]_{total} \qquad (12)$$

stoichiometry determined from protein fluorescence titrations performed under stoichiometric conditions (Figure 6). A maximum fluorescence change ΔF_{max} of 50% for TPN and Rib-P_2-Ado-P and that of 41% for Ado(2′,5′)P_2 were used for these calculations. The data were analyzed in accordance with the Scatchard equation (eq 10). From the plot shown in Figure 8, dissociation constants of 0.29 μM and 0.15 μM were calculated for TPN and Rib-P_2-Ado-P, respectively. The dissociation constant of 1.3 μM was obtained in a similar manner for the Ado(2′,5′)P_2–enzyme complex under identical conditions (data not shown). Comparison of the dissociation constant for TPN (K_d = 0.29 μM) with that esimated in the presence of 100 mM sulfate (K_d = 100 μM; Table III) demonstrates a dramatic effect of salt on the affinity of this nucleotide for enzyme, in analogy to the effect observed previously for TPNH.

CD Spectra. In order to evaluate the extent of conformational changes which were indicated by the large enhancement of protein fluorescence caused by the binding of TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2, we have compared the circular dichroism spectra of 1.5 μM isocitrate dehydrogenase in the absence and in the presence of 1.5 μM Rib-P_2-Ado-P. The 200–240-nm spectral region was examined because it is sen-

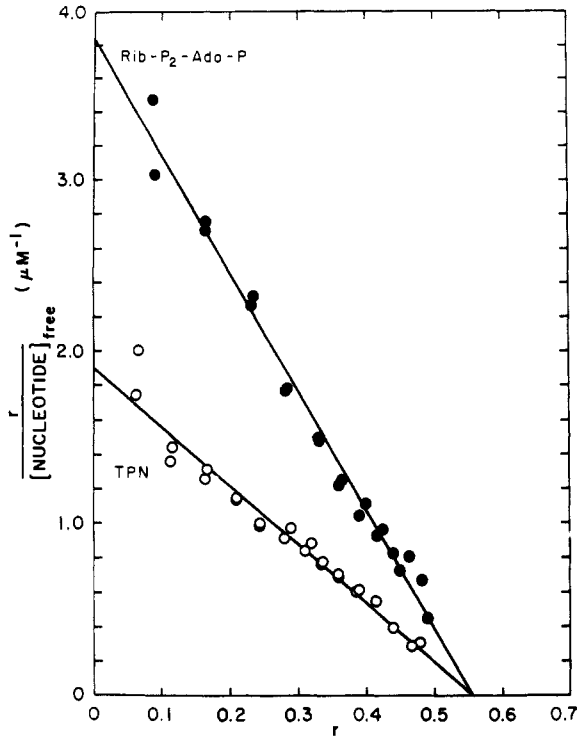

FIGURE 8: Determination of ligand dissociation constants for TPN (○) and for Rib-P_2-Ado-P (●). Protein fluorescence titrations (in duplicate) were performed as described in the legend to Figure 6, at 0.9 μM isocitrate dehydrogenase subunits in 50 mM triethanolamine chloride buffer (pH 7.6), containing 10% glycerol and 0.5 mM EDTA, at 25 °C. The lines are least-squares fits to the Scatchard equation (eq 10). The dissociation constants, of K_d = 0.15 ± 0.01 μM for Rib-P_2-Ado-P and of K_d = 0.29 ± 0.01 μM for TPN, were calculated, assuming 0.5 nucleotide binding sites per subunit (Figure 6).

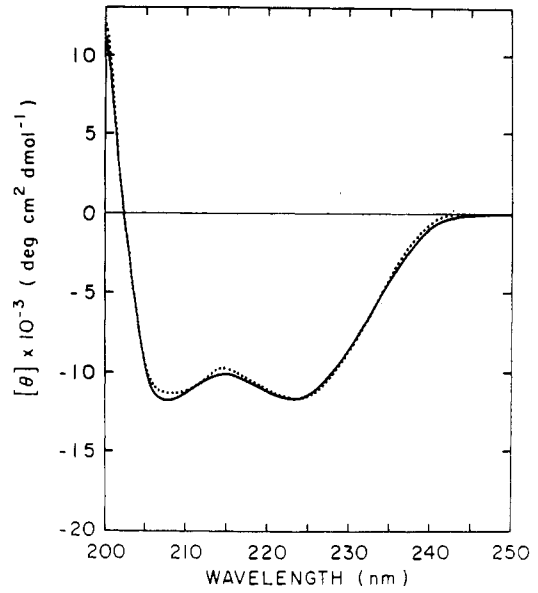

FIGURE 9: Far-UV CD spectra of 1.5 μM subunits of isocitrate dehydrogenase in 50 mM Tris-HCl buffer (pH 7.6), containing 10% glycerol, in the absence (—) and in the presence (···) of 1.5 μM Rib-P_2-Ado-P, with the base line subtracted. At these concentrations, 75% of the enzyme binding sites are saturated with ligand, assuming that there is one binding site per subunit and the K_d = 0.15 μM for Rib-P_2-Ado-P.

sitive to changes in secondary structure (Greenfield & Fasman, 1969). Spectra were measured in 50 mM Tris-HCl buffer (pH 7.6), containing 10% glycerol. Triethanolamine buffer was not transparent in the entire UV region and therefore could not be used. In order to test the comparability of the results obtained in these two buffers, we performed the fluorescence titration of the enzyme with Rib-P_2-Ado-P, as a control. Fluorescence enhancement was observed in Tris-HCl buffer, as well, with a maximum change of 40% corresponding to saturation of 0.5 site per dimer (data not shown). The dissociation constant determined in Tris buffer, K_d = 0.12 μM, was similar to that obtained for Rib-P_2-Ado-P in triethanolamine buffer (K_d = 0.15 μM). This agreement justified further comparisons of the results obtained in the two buffers. The spectra of the enzyme alone at 1.5 μM subunit concentration and of enzyme–Rib-P_2-Ado-P complex (with 75% of the enzyme sites occupied by ligand) are shown in Figure 9. The differences between these two spectra are within the experimental error and indicate that there is not appreciable change in the peptide backbone conformation. Thus, changes observed in protein fluorescence are most likely the result of local conformational changes, which are not detectable by CD and absorbance measurements. From the enzyme spectrum in Figure 9, an α-helix content of 27% was calculated from eq 13, according to Greenfield & Fasman (1969).

$$\% \ \alpha\text{-helix} = \frac{[\theta]_{208nm} - 4000}{33\,000 - 4000} \qquad (13)$$

The spectrum of pig heart isocitrate dehydrogenase (Figure 9) exhibits great similarity to the CD spectrum published previously for the bovine liver enzyme (Carlier & Pantaloni, 1973).

Effect of Substrate on Nucleotide Binding. The binding constant for TPN determined previously by Ehrlich & Colman (1975) in the presence of 60 mM sulfate ions (K_d = 49 μM) was about 10-fold higher than the K_m of 4.6 μM for TPN measured at lower ionic strength in the presence of 2 mM $MnSO_4$ and 4 mM DL-isocitrate (Colman, 1968). This led to a hypothesis that manganese isocitrate strengthens the binding of TPN (Ehrlich & Colman, 1975). Differences in binding of TPN in the presence and in the absence of the substrate were also suggested on the basis of our affinity labeling studies of the nucleotide binding sites using the dialdehyde analogue of the coenzyme (Mas & Colman, 1983). ^{31}P NMR studies did not reveal any changes in the spectrum of Rib-P_2-Ado-P–enzyme complex upon addition of magnesium isocitrate, suggesting that the environment of the 2′-phosphate was unchanged in the presence of the substrate (Mas & Colman, 1984). Comparison of binding constants for the nucleotides determined under various conditions show that high ionic strength weakens binding of nucleotides by several orders of magnitude. It was therefore of interest to reexamine the effect of manganese isocitrate on the coenzyme binding. The dissociation constants determined in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA (I = 0.05 M) were compared to the inhibition constants for Rib-P_2-Ado-P and Ado(2′,5′)P_2 determined from kinetic competition experiments performed under similar conditions, as described under Experimental Procedures. Striking similarity of the UV difference spectra and fluorescence changes, and the similar binding affinities found for TPN and Rib-P_2-Ado-P, make the latter compound an excellent analogue of the oxidized coenzyme. Ado(2′,5′)P_2 also exhibits comparable spectral effects, and its binding constant is only 4-fold weaker than that of TPN. Both nucleotides can thus reasonably be used to study the effect of manganese isocitrate on the binding of coenzyme to isocitrate dehydrogenase.

The K_m for TPN of 3.2 ± 0.2 μM determined in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol, 2 mM $MnCl_2$, and 4 mM DL-isoctrate (I = 0.08 M)

Table VI: Equilibrium Constants and Gibbs' Free Energy Changes for the Binding of Nucleotides to Isocitrate Dehydrogenase in the Absence and Presence of Manganese Isocitrate[a]

nucleotide	K_d (μM)	$-\Delta G°$ (kJ/mol)	K_I (μM)	$-\Delta G°$ (kJ/mol)
TPN	0.29	36.5	[b]	
Rib-P_2-Ado-P	0.15	38.9	7.9	29.1
Ado(2′,5′)P_2	1.3	33.6	158.0	21.7
TPNH	0.0005[c]	53.0		

[a] The dissociation constants (K_d) for TPN, Rib-P_2-Ado-P, and Ado-(2′,5′)P_2 were determined from protein fluoresence titrations. The inhibition constants (K_I) were determined kinetically in the presence of saturating concentrations of manganese isocitrate. All measurements were performed in 50 mM triethanolamine chloride buffer (pH 7.6), containing 10% glycerol and 0.5 mM EDTA, at 25 °C, as described in the text; $\Delta G° = -RT \ln K$; 1 cal = 4.18 J. [b] The Michaelis constant of 3.2 μM was determined for TPN from kinetic measurements. [c] Determined indirectly in the same buffer, as described in the text.

at 25 °C (data not shown), agrees well with the values determined previously under similar conditions (Ehrlich & Colman, 1978; Mas & Colman, 1983). This K_m value is about 10 times higher than the dissociation constant for TPN, K_d = 0.29 μM, determined in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA (I = 0.05 M). The result obtained previously ($K_m < K_d$) by Ehrlich & Colman (1975) was apparently due to the presence of 60 mM sulfate ions in the buffer solution used for the determination of the dissociation constant for TPN. Rib-P_2-Ado-P and Ado(2′5′)P_2 are competitive inhibitors with respect to TPN. Inhibition constants of 7.9 ± 0.5 μM for Rib-P_2-Ado-P and 158 ± 9 μM for Ado(2′,5′)P_2 were obtained from plots of the apparent K_m vs. inhibitor concentration (data not shown). The comparison of the dissociation constants and the inhibition constants, as well as the corresponding values of free energies of the binding, are shown in Table VI.

The far-ultraviolet CD spectra of the ternary complex of the enzyme (1.5 μM), Rib-P_2-Ado-P (1.5 μM), and saturating concentrations of manganese isocitrate (data not shown) were indistinguishable from the spectra of the enzyme alone or of the binary complex with Rib-P_2-Ado-P, shown in Figure 9, indicating the absence of major conformational changes in the ternary complex as well. No significant changes in the protein fluorescence emission at 350 nm were caused by the addition of saturating concentration of manganese isocitrate alone or manganese isocitrate followed by Rib-P_2-Ado-P (75 μM) to 1 μM enzyme in 50 mM triethanolamine chloride buffer, pH 7.6, containing 10% glycerol at 25 °C (data not shown). These results contrast with the 50% enhancement in protein fluorescence caused by the nucleotide alone. The enhancement caused by Rib-P_2-Ado-P was completely abolished by the addition of saturating concentrations of the substrate, manganese isocitrate. These observations suggest that there are differences in the conformation of the enzyme in its ternary complex with manganese isocitrate and nucleotide, as compared to that in the binary enzyme–nucleotide complex. The lack of an effect of the substrate on the fluorescence of pig heart isocitrate dehydrogenase is in contrast to a 30% quenching observed for the bovine heart enzyme (Reynolds et al., 1978).

Discussion

It has been demonstrated from the spectral titrations conducted under conditions of stoichiometry binding that there is one TPNH binding site per subunit of pig heart TPN-specific isocitrate dehydrogenase. This result, obtained by two independent methods, absorption spectroscopy (Figure 2) and fluorescence, indicates that the enzyme used in our studies, purified according to the procedure of Bacon et al. (1981), is fully active and does not contain tightly bound nucleotides. The dissociation constant for TPNH determined from fluorescence titrations in 50 mM MOPS buffer, pH 7.6, containing 10% glycerol, 0.5 mM EDTA, and 100 mM sulfate (K_d = 1.2 μM), is 2 orders of magnitude lower than the dissociation constant estimated for TPN (K_d = 122 μM) under identical conditions.

The pH dependence of the dissociation constants for TPNH is consistent with our conclusion from ^{31}P NMR studies (Mas & Colman, 1984) that the nucleotides bind to the enzyme in their unprotonated form. The apparent increase in the dissociation constants observed in the presence of 20 mM Mn^{2+} over the pH range 5.8–7.6 can be ascribed to a decrease in the concentration of metal-free TPNH, in agreement with the earlier observation of Ehrlich & Colman (1975) at pH 7.4. Recalculation of the dissociation constants, determined in the absence and in the presence of Mn^{2+}, with the assumption that only the metal-free, fully ionized species binds to the enzyme, in both cases yields a relatively invariant K_d over the pH range studied (compare lines a and b of Figure 5). This result confirms the validity of our assumption and indicates the lack of involvement in nucleotide binding of amino acid residues with pK_a values in this range. Some contribution of a group(s) which becomes protonated below pH 5.8 may be implied from the slight decrease in K_d with decreasing pH. The amino acid residues involved in the predominant electrostatic interactions with negatively charged nucleotide are apparently protonated over the pH range 5.8–7.6. This suggests that lysyl and/or arginyl residues are present in the nucleotide binding site. The interaction of the 2′-phosphate moiety of TPNH, TPN, and Rib-P_2-Ado-P with a positively charged group(s) on the enzyme has previously been suggested from the ^{31}P NMR studies of the binary complexes (Mas & Colman, 1984).

An effect of ionic strength on the activity coefficients of a charged multivalent ligand and a macromolecule can be expected on the basis of the theory of electrostatic interactions. A thermodynamic analysis of ion effects on the binding equilibria of proteins and nucleic acids has been published (Record et al., 1978). The dramatic effect of ionic strength on the dissociation constants for TPNH demonstrated in this study suggests significant contribution of electrostatic interactions to the energy of binding. A decrease in the K_d, of 3 orders of magnitude, results from the decrease of ionic strength by 0.3 M (Table II). Pig heart isocitrate dehydrogenase has a net positive charge at pH 7.6 (Colman, 1968). Moreover, the presence of positively charged residues in the 2′-phosphate binding site was inferred from our ^{31}P NMR studies (Mas & Colman, 1984). The net charge of TPNH is 4– at this pH, with the 2′-phosphate group in its dianionic form. In agreement with simple electrostatic theory, a maximum interaction between two molecules bearing opposite charges is observed at low ionic strength. The Debye–Hückel screening effect of counterions at high ionic strength leads to weakening of these attractive forces. As a result, a decrease in the dissociation constant for TPNH is observed upon decreasing the ionic strength from I = 0.336 M (K_d = 0.45 μM and K_d = 1.2 μM in the presence of 300 mM NaCl and of 100 mM Na_2SO_4, respectively) to I = 0.036 M (K_d = 0.4 nM in the absence of chloride and sulfate salts). In addition to the ionic strength effect on the binding of TPNH to isocitrate dehydrogenase, we have demonstrated a weak interaction of sulfate ions with the nucleotide binding sites, with an inhibition constant of 57 ± 3 mM. This interaction has been shown to affect equally

the binding of TPNH at pH 5.8 and at pH 7.6 (Table I). The binding of TPN is also affected by sulfate. The binding affinity for TPN in the presence of 100 mM sulfate ions decreases about 380-fold as compared to about a 1000-fold change for TPNH. The comparison of the UV difference spectra of the binary complexes of isocitrate dehydrogenase with TPN and TPNH measured in the presence and in the absence of 100 mM sulfate does not reveal major spectral differences (Figures 1 and 3, respectively). There is also no indication from the effect of sulfate on protein fluorescence of conformational changes that might have been caused by the sulfate: the titration of 1 μM enzyme with sulfate resulted in a weak, linear quenching of protein fluorescence emission at 350 nm (about 6% at 2 mM sulfate concentration) (M. T. Mas and R. F. Colman, unpublished data). Thus, rather than to a major conformational change, weakening of the binding of the nucleotides in the presence of sulfate is best ascribed to the effect of the ionic strength increase and to weak competition for the coenzyme binding site. The effect of ionic strength and phosphate ions on the binding of coenzymes to bovine heart enzyme has been reported previously by Reynolds et al. (1978) and Fatania et al. (1982). The specific interaction of sulfate has also been demonstrated for other dehydrogenases (Rutter, 1957; Liljas & Rossmann, 1974; Olden & Pettersson, 1982).

The presence of one nucleotide binding site per enzyme subunit has been found for TPN and Rib-P_2-Ado-P from the UV difference spectral titrations and from ultrafiltration experiments (Figure 7 and Table V, respectively). Interestingly, the protein fluorescence titration performed under the same conditions with TPN and Rib-P_2-Ado-P (Figure 6), as well as with Ado(2′,5′)P_2, gave an apparent stoichiometry of one site per dimeric molecule of the enzyme or 0.5 site per peptide chain. A similar discrepancy among the results of various methods has previously been reported by Reynolds et al. (1978) for the bovine heart enzyme. In constrast to the ultrafiltration technique, from which stoichiometry is obtained by measurement of the concentration of free ligand in an equilibrium mixture of protein and nucleotide, the spectroscopic methods used in this study rely on the observations of the spectral changes corresponding to perturbations of protein and/or nucleotide chromophores in such a mixture.

It is known from numerous studies of conformational transitions in proteins that the results of various optical methods, including fluorescence and absorption, frequently do not change coordinately (Chen et al., 1969). In general, the major structural changes are revealed by most methods. However, fluorescence is much more sensitive to the changes in the local environment of the chromophore than is absorption. The enhanced effect on fluorescence is produced by changes in solvent composition and by neighboring polar and charged groups. The effect of distant groups can affect fluorescence of a chromophore via the energy-transfer mechanism, without changing its absorption characteristics. The difference between the effect of solvent on fluorescence and absorption spectra results from the different time scale of the two processes. The fluorescence lifetime of the excited state is several orders of magnitude longer than the time required for excitation ($\sim 10^{-15}$ s). Certain events, which are too slow to influence absorption properties, can occur during the lifetime of the excited state (10^{-9}–10^{-8} s) and influence the fluorescence spectra; these might include reorientation of solvent molecules around a chromophore in the more polar excited state, perturbations in acid–base equilibria, and local conformational changes (Van Duuren, 1963; Chen et al., 1969; Crooks, 1978; Cantor & Schimmel, 1980).

The observed effect of a ligand on the fluorescence of a protein containing large numbers of aromatic amino acid residues such as isocitrate dehydrogenase is an average of direct and/or indirect perturbations of individual fluorophores. The net change can therefore be negative, positive, or zero. In most cases, quenching of protein fluorescence by nucleotides has been observed for dehydrogenases, including quenching of the fluorescence of TPN-specific isocitrate dehydrogenase by TPNH. The enhancement of protein fluorescence by TPN and by its "fragments", rather than the expected quenching, suggests that the observed effect is the result of conformational changes of the enzyme, induced by nucleotide binding. Direct interaction of nucleotide with a fluorophore within the binding site would be expected to quench its fluorescence (Velick, 1958; Iewibo & Weiner, 1972). If such an interaction occurs in our case, it might have been overcome by a large increase in the fluorescence emission, caused by indirect effects. No information is available at present about the location of aromatic amino acid residues with respect to the nucleotide binding sites of TPN-specific isocitrate dehydrogenase. The maximum fluorescence change observed upon saturation of half of the nucleotide binding sites suggests that the binding of the first molecule of ligand is responsible for the total observed change, while binding to the second subunit is not detectable by fluorescence. Under similar conditions saturation of both sites is evident from the absorption difference titration and ultrafiltration measurements. It seems likely that the conformational change revealed by fluorescence, but not by other methods, might be of local nature, leading to subtle perturbations of the enzyme fluorophores. In agreement with this hypothesis, the CD spectrum of the binary complex of the enzyme with Rib-P_2-Ado-P does not reveal major structural changes in the polypeptide backbone (Figure 9).

The fluorescence enhancement accompanying the formation of the binary complexes of isocitrate dehydrogenase with Rib-P_2-Ado-P and Ado(2′,5′)P_2 can be completely eliminated by the substrate, manganese isocitrate. The effect of the substrate alone on protein fluorescence is negligible. Furthermore, addition of Rib-P_2-Ado-P or Ado(2′,5′)P_2 to the solution containing the binary enzyme–substrate complex does not cause substantial changes in protein fluorescence. These observations indicate that conformational differences exist between the binary and ternary complexes of TPN-specific isocitrate dehydrogenase. It appears that the conformational change induced by Rib-P_2-Ado-P and Ado(2′,5′)P_2 (and presumably that observed for TPN) can occur only in the absence of the substrate. The influence of the substrate on nucleotide binding has also been demonstrated by the results of the kinetic experiments. The inhibition constants determined by Rib-P_2-Ado-P (7.9 μM) and for Ado(2′,5′)P_2 (158 μM) are significantly higher than the dissociation constants determined for the binary complexes from the fluorescence titrations (0.15 μM and 1.3 μM, respectively). The dissociation constant for TPN is about 10-fold lower than the K_m determined under comparable conditions (0.3 μM and 3.2 μM, respectively). A similar difference between the direct and kinetic estimates of the equilibrium constants was observed for the nucleotide complexes of other dehydrogenases (Dalziel, 1975). The results obtained for TPN might be related to the fact that isocitrate weakens the binding of the coenzyme, in analogy to the effect on the binding of coenzyme fragments.

Several lines of evidence indicate that TPN, Rib-P_2-Ado-P, and TPNH bind to the same site on the enzyme. The evidence for the similar environment of the 2′-phosphate group in the binary complexes with TPN, TPNH, and Rib-P_2-Ado-P has

been presented in our previous study by using ^{31}P NMR spectroscopy (Mas & Colman, 1984). The UV difference spectra for the binary complexes of isocitrate dehydrogenase with the nucleotides are remarkably similar (Figure 3). The analogous effect of TPN, Rib-P_2-Ado-P, and Ado(2′,5′)P_2 on the fluorescence of the enzyme strongly suggests a similar mode of interaction of the adenosine ribose fragment of these compounds with isocitrate dehydrogenase. The presence of the nicotinamide ring in TPN apparently does not contribute to the observed fluorescence change. Evidently, the binding of the adenosine ribose part of the coenzyme molecule can by itself bring about the observed conformational change of the enzyme. The importance of the phosphoadenosine ribose fragment to the binding of coenzymes to TPN-specific isocitrate dehydrogenase has been suggested previously on the basis of the kinetic competition studies by Ehrlich & Colman (1975). It was shown in those experiments that the nicotinamide mononucleotide binds extremely weakly to the enzyme (K_I > 10 mM). The methods used in the present study did not provide evidence for a direct interaction of the oxidized nicotinamide of TPN with the enzyme. In fact, binding of 2′-phosphoadenosine 5′-diphosphoribose is about twice as strong as TPN. A similar result, suggesting the lack of effect of the oxidized nicotinamide ring on the binding of coenzyme, has been observed for lactate dehydrogenase (Schmid et al., 1978; Hinz et al., 1978) and for dihydrofolate reductase (Matthews et al., 1979). However, the specific interaction of the nicotinamide ring of TPN with the latter enzyme has clearly been demonstrated from NMR studies (Hyde et al., 1980). Direct interaction of the reduced nicotinamide ring of TPNH with TPN-specific isocitrate dehydrogenase can be deduced from the absorption difference spectrum of the binary complex (in the 340-nm region), from the quenching of protein fluorescence, and from the enhancement of TPNH fluorescence. Moreover, the dissociation constant for the reduced coenzyme is several orders of magnitude lower than that for TPN and Rib-P_2-Ado-P. Tighter binding of the reduced than of the oxidized coenzyme has been observed for many dehydrogenases (Dalziel, 1975; Schmid et al., 178; Hinz et al., 1978; Birdsall et al., 1980; Subramanian & Ross, 1978; Grau, 1982). In analogy to other dehydrogenases, the free energies of binding for the oxidized nucleotide or its fragments containing adenosine ribose are very similar (Table VI). The differences are very small, within about 1 kcal/mol. In contrast, $\Delta G°$ for TPNH is much more negative. It has been proposed that the presence of the positive charge on the nitrogen of the oxidized nicotinamide ring is responsible for weaker binding of the oxidized compared to reduced coenzyme (Subramanian et al., 1978; Danenberg et al., 1978). Additional contributions, probably of conformational origin, have been suggested by Birdsall et al. (1980), from NMR studies of dihydrofolate reductase–nucleotide complexes. The higher binding affinity of TPNH might suggest unfavorable interactions of the positively charged nicotinamide ring with isocitrate dehydrogenase and/or the effect of the lower neg negative charge of TPN. However, the dissocation constants for TPN and Rib-P_2-Ado-P are very similar. This indicates that the higher affinity of TPNH is attributable to the contribution of the reduced nicotinamide to the binding interaction.

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Effects of Replacement of the Hydroxyl Group of Cholesterol and Tocopherol on the Thermotropic Behavior of Phospholipid Membranes†

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ABSTRACT: The role of the hydroxyl groups of cholesterol and tocopherol in mediating their interaction with phospholipid bilayers has been a subject of considerable interest. We have examined this question by using derivatives of cholesterol and tocopherol in which the hydroxyl group is esterified to succinate. The hemisuccinate esters of cholesterol and α-tocopherol can be readily incorporated into phospholipid membranes and in fact can by themselves form closed membrane vesicles as demonstrated by the encapsulation of [^{3}H]sucrose. The thermotropic behavior of mixtures containing each succinate ester and phospholipid was studied by differential scanning calorimetry. The effect of cholesteryl hemisuccinate on the thermotropic properties of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylethanolamine is very similar to that of cholesterol. This indicates that the 3β-OH is not required for the formation of a cholesterol-phospholipid complex. In mixtures of tocopherol acid succinate and phospholipids the peak transition temperature is progressively shifted to lower temperatures as the mole fraction of α-tocopherol succinate is increased, while the enthalpy of the transition is only slightly affected. At a tocopherol succinate/phospholipid molar ratio of 9/1 a phase transition is still detectable. A comparison between tocopherol succinate and tocopherol indicates that the substitution of the hydroxyl group reduces the interaction of tocopherol with phospholipids to a small but measurable extent. Thus, the hydroxyl group of tocopherol is more important than the hydroxyl group of cholesterol in influencing their interactions with phospholipids.

The biological functions attributed to cholesterol and α-tocopherol have stimulated a considerable number of studies on their physicochemical properties and their interactions with phospholipids. In the case of cholesterol, up to 50 mol % can be dissolved in phosphatidylcholine membranes (Ladbrooke et al., 1968; Lecuyer & Dervichian, 1969). Below this limit cholesterol and lecithin form complexes of varying mole ratios that have been discussed in a number of recent reviews (Demel & de Kruijff, 1976; Presti et al., 1982). Although not as well studied, a complex between tocopherol and polyunsaturated phospholipids has been proposed by Diplock & Lucy (1973). Moreover, tocopherol, like cholesterol, can significantly broaden the gel-liquid-crystalline phase transition in phospholipid membranes (Massey et al., 1982) and, in the case of bilayers composed of unsaturated phospholipids, reduce the permeability of small molecules (Diplock et al., 1977).

The structural features of cholesterol that have been considered to be important for the formation of the phospholipid complex include the planar α face of the molecule, the acyl chain of between five and seven carbons, and the β-OH group which has been suggested to participate in a hydrogen bond with a polar component of the phospholipid (Brockerhoff, 1974; Huang, 1977; Presti et al., 1982). In the case of tocopherol the hydroxyl group has been suggested to form a hydrogen bond with one of the oxygen atoms of the phospholipid (Srivastava et al., 1983), while the phytanoyl chain has been considered to play a role in the tocopherol-unsaturated phospholipid interaction (Diplock & Lucy, 1973).

We had used derivatives of cholesterol and tocopherol modified at the hydroxyl group to prepare lipid vesicles which are destabilized at low pH (Ellens et al., 1984; M.-Z. Lai and F. C. Szoka, unpublished results) and became interested in the question of the role of the hydroxyl group in the interaction of these compounds with phospholipids. Reports in the literature concerning a number of hydrophilic substituents of the β-OH group of cholesterol indicated that such derivatives in phospholipids membranes behave in many respects like cholesterol (Lyte & Shinitzky, 1979; Shinitzky et al., 1979; Co-

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