Regulation of Diphosphopyridine Nucleotide-linked Isocitrte Dehydrogenase from Bovine Heart

BINDING OF INHIBITORY NUCLEOTIDES*

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SUMMARY

DPN-linked isocitrate dehydrogenase from bovine heart binds 1 molecule of DPNH per molecule of enzyme of 320,000 daltons with a concomitant 20-fold increase in nucleotide fluorescence. The fluorescence properties of this tightly bound DPNH (Kᵣ = 1.4 μM) are not affected by DPN⁺, isocitrate, MgSO₄, or ADP, either alone or in combinations. However, ATP, an inhibitor of isocitrate dehydrogenase, competitively displaces bound DPNH. Ultrafiltration or gel chromatography of the enzyme in the presence of DPNH reveals a total binding of 3.5 to 4.5 eq of nucleotide with a limiting dissociation constant of 14 μM obtained at near saturating ligand concentrations.

Fluorescence titration indicates that isocitrate dehydrogenase also binds 1 eq of TPNH with a Kᵣ of 0.9 μM. In contrast to the enzyme-DPNH complex, bound TPNH is not displaced by ATP and shows no evidence of energy transfer in its fluorescence excitation spectrum. Ultrafiltration experiments reveal at least two additional binding sites for TPNH.

The presence of DPNH does not affect the binding of TPNH, and DPNH binding is not altered by TPNH, indicating separate sites on the enzyme for the two types of pyridine nucleotides.

The catalytic activity of DPN-linked isocitrate dehydrogenase of bovine heart (threo-α,α-isocitrate + DPN⁺ → α-ketoglutarate + CO₂ + DPNH + H⁺; EC 1.1.1.41) is enhanced by ADP. The regulatory implications of adenine nucleotides at the level of isocitrate oxidation were recognized by several investigators in studies with purified enzymes from animal tissues (2-4) and microorganisms (5, 6). In the latter, the importance of the level of the energy charge of the adenine nucleotide pool has received substantial support by the investigations of Atkinson and associates (for review see Ref. 7). However, the DPN-linked isocitrate dehydrogenases from animal tissues and yeast differ in the severe inhibition of the former (2, 4, 8) by reduced pyridine nucleotides. Recent studies of kinetic parameters of purified enzyme (4) and observations with more integrated systems from liver (9, 10) suggest that the ratio of DPNH:DPN⁺ may influence the in vivo activity of the enzyme more than the level of the positive modifier ADP. For example, Williamson et al. (9) found that ethanol slowed the activity of the tricarboxylic acid cycle in perfused liver from starved rats. It was suggested that the increase in reduced pyridine nucleotides observed in the whole organ also occurred within mitochondria causing a shift in the malate to oxaloacetate equilibrium and an inhibition by reduced pyridine nucleotides of DPN-specific isocitrate dehydrogenase. König, Nicholls, and Garland (10) have shown that the oxidation of succinate and palmitoyl carnitine in intact rat liver mitochondria resulted in an inhibition of isocitrate oxidation and a concomitant increase of the intramitochondrial DPNH:DPN⁺ ratio. These observations, while not conclusive, do suggest the importance of reduced pyridine nucleotides in the in vivo regulation of DPN-linked isocitrate dehydrogenase.

The effect of the intramitochondrial oxidation-reduction potential on isocitrate oxidation may be mediated by both the DPN⁺:DPN⁺ ratio and the level of TPNH. Kinetic experiments with purified enzyme preparations have shown that whereas there is a competitive relationship between DPN⁺ and TPNH, TPNH (which is neither a substrate nor an inhibitor of DPN-linked isocitrate dehydrogenase) significantly potentiates DPNH inhibition (2, 4, 8).

DPNH and TPNH thus appear to be important in the regulation of cellular oxidation of isocitrate. The present investigations with techniques involving fluorescence measurements, ultrafiltration, and gel chromatography provide more direct evidence for the nature and extent of binding of these ligands to the DPN-linked isocitrate dehydrogenase protein. Two separate sets of binding sites have been determined in these experiments, one specific for DPNH and the other specific for TPNH. Preliminary reports have appeared on the binding of reduced pyridine nucleotides and of DPN⁺ to purified enzyme preparations from bovine heart (1) and yeast (11), respectively.

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EUTA, Tris, glucose 6-phosphate dehydrogenase, rabbit muscle lactate dehydrogenase, isocitrate lactone, glucose 6-phosphate, glycine, and dithiothreitol were from Sigma. Yeast alcohol dehydrogenase was from Boehringer Mannheim, acetylypyridine DPNH and acetylypyridine TPN+ from P-L Biochemicals, and Sephadex G-50 from Pharmacia. Glycerol and inorganic salts were analytical reagent grade from Mallinckrodt Chemical Works. Carboxyl-labeled [14C]TPN+ was from Amer sham-Searle, DPN+ labeled with 3H in the adenosine portion from New England Nuclear, and Norit A (acid washed) from Flainstil Laboratories, Inc.

DEAE-cellulose (DE-32) from Whatman for chromatography of DPNH was washed sequentially with 0.5 M NaOH, 0.5 M NaCl, water, and with 0.1 M sodium phosphate at pH 7.2 until the effluent was at pH 7.2. Columns were equilibrated with 0.005 M sodium phosphate at pH 7.2 before use.

Preparation of Labeled Nucleotides—Tritiated DPNH was prepared by the reduction of PHDPN+ in the presence of alcohol dehydrogenase (12). Semicarbazide hydrochloride (0.5 mmole), DPN+ (1 μmole), and 30 μl of ethanol (0.5 mmole) were added to 1 ml of 0.1 M sodium glycinate at pH 10. The solution was adjusted to pH 10.5 and 3 μCi of [3H]DPN+ followed by 2 μl of a 30 mg per ml suspension of alcohol dehydrogenase were added. The reduction, monitored at 340 nm, occurred instantaneously. The enzyme was denatured by placing the vessel containing the reaction mixture in boiling water for 4 min. The reaction mixture was diluted into 19 ml of 0.01 M sodium phosphate buffer at pH 7.2, followed by 0.05 M NaCl in the above buffer until in each case the absorbance of the effluent at 340 nm and 260 nm was less than 0.005. The reduced nucleotide was then eluted with 0.25 M NaCl, 0.005 M sodium phosphate at pH 7.2. Fractions were combined which had ratios of absorbance at 260 nm:340 nm of less than 2.6. The combined solutions containing 0.60 to 0.70 μmole of [3H]DPN+ usually exhibited a 260 nm:340 nm absorbance ratio of 2.4 to 2.5. This ratio remained constant for 3 to 4 days at 0°C; the solution was used in the experiments during this period.

[14C]Labeled TPNH was prepared by the enzymatic reduction of [14C]TPN+. Glucose 6-phosphate (10 μmoles), unlabeled TPN+ (1 μmole), 2 μCi of [14C]TPN+, MgCl2 (20 μmoles), and approximately 10 μg of glucose 6-phosphate dehydrogenase were incubated. The absorbance at 340 nm was followed until there was no further change. The reaction mixture was then adjusted to pH 10.5, placed in a boiling water bath for 3 min, diluted with 17 ml of 0.01 M NaHCO3, and chromatographed on a column of DEAE-cellulose as described above. The product had a 260 nm:340 nm absorbance ratio of 2.5.

Methods

DPN-linked isocitrate dehydrogenase, which was prepared and assayed as described previously (13), was stored at -70°C in a solution containing 10% glycerol, 5 mM sodium phosphate (pH 7.2), and 0.1 mM dithiothreitol. The molar concentration of isocitrate dehydrogenase was calculated from the protein content as determined by the biuret method (14), and a molecular weight of 320,000 (13). Enzyme preparations used in these studies had a specific activity1 of 24,000 to 27,000 nmols of DPN+ reduced per mg of protein per min at 95°C, migrated as a single band on disc electrophoresis, and contained less than 0.5% carbohydrate as determined by the phenol-sulfuric acid method (16).

Fluorescence Measurements—Fluorescence emission, excitation, and polarization measurements were performed with a recording spectrophotometer constructed in this laboratory. The excitation wave length was selected by a monochromator (0.25 meter, Jarrell Ash) from the emission of a xenon lamp (PEK 75 watt powered by a Hewlett Packard 8274 A supply). A quartz lens system (Bausch and Lomb) focused the excitation light on a standard 1-cm quartz fluorescence cuvette containing 1 ml of sample. After isolation by a monochromator or interference filter, the fluorescence emission was monitored with an EMI photomultiplier (6256SA, EMI Electronics) and an operational amplifier circuit described previously (17). A voltage proportional to the emission intensity was displayed on a recorder (Bristol) or a digital voltmeter (Heath Instrument Co.). A nominal band width of 5 nm for excitation and emission was used in all experiments. Fluorescence polarization measurements employed polarizing filters in the excitation and emission beams as outlined by Chen and Bowman (18). Spectra reported are not corrected for lamp, monochromator, and photomultiplier response. The temperature of the solution during the fluorescence measurement was held at 25 ± 0.1°C by a Tamson refrigerated circulating water bath. Absorption spectra were determined in a Cary model 14 spectro photometer fitted with a thermostated cuvette compartment.

Determination of Binding Capacity and Affinity of Enzyme by Ultrafiltration—An ultrafiltration technique described by Paulus (10) was used to measure the binding of radioactive nucleotides to isocitrate dehydrogenase. A solution (0.5 ml) containing enzyme (44 μg), 0.17 M NaCl, and labeled DPNH or TPNH was filtered through a membrane (UM-10, Amicon Corp.) in an ultrafiltration cell (Metallglas) at 22-23°C. The enzyme and bound ligand were retained by the filter while free ligand passed through. The membrane was removed from the apparatus, placed in a scintillation vial containing 0.2 ml of water, and agitated for approximately 10 min. A solution (10 ml) containing 0.3 g of p-bis[2-(5-phenyloxazolyl)]benzene, 6 g of 2,5-diphenyloxazole, and 50 ml of Bioisol BBS-2 (Beckman Instruments, Inc.) per liter of toluene was added, and the samples were counted in a liquid scintillation spectrometer (Nuclear-Chicago). The radioactivity retained on the membrane allows a direct calculation of the amount of ligand bound. The dissociation constant and the number of binding sites were obtained by the Scatchard plot (20).

\[
\frac{r}{n} = \frac{K_{eq}}{A}
\]

1 The specific activity of the enzyme used here is essentially the same as that of preparations used previously. The values reported then (13) were based on protein determined by the method of Warburg and Christian (15) which in the case of this enzyme is about one-half of that found by the biuret method (13, 14). The latter has been adopted as the basis of the protein values in the present report.
where \( r \) is the moles of bound ligand per mole of enzyme, \( (A) \) is the concentration of free ligand, \( K_D \) is the dissociation constant for the ligand, and \( n \) is the number of sites. The plot of \( r \) against \( r/A \) is linear if the sites are identical and independent. The slope is \(-K_D\) and the ordinate intercept is \( n \).

**Gel Filtration**—The Sephadex gel filtration technique (21) used for determining the coenzyme-binding capacity of isocitrate dehydrogenase is similar to that described by Pfleiderer and Auriechio (22). Most experiments were performed on Sephadex G-50 columns (0.8 x 20 cm) equilibrated with a buffer containing 0.005 M sodium phosphate at pH 7.2, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, 10^{-4} M ADP, 10% (v/v) glycerol, and DPNH (20 to 150 \( \mu \)M). Isocitrate dehydrogenase (4 to 8 mg) in 1.0 ml was applied to the column which was then developed with the equilibrating buffer at 22-23°C. Smaller columns (0.6 x 10 cm) and proportionally reduced amounts of enzyme also gave satisfactory results. The absorbance at 340 nm, enzyme activity, and protein concentration of the effluent fractions were determined. The absorbance at 340 nm showed a peak containing the enzyme-DPNH complex followed by a trough which corresponded to the amount of DPNH bound by the isocitrate dehydrogenase. The difference between the concentration of DPNH in the enzyme-containing fractions and that contained in the equilibrating buffer corresponds to the concentration of bound DPNH. It was shown in separate experiments that the molar absorbance of DPNH is not altered on binding to the enzyme.

**Determination of Binding Capacity and Affinity of Enzyme by Fluorescence Enhancement**—Unless noted otherwise all fluorescence titrations were performed in the presence of 5 mM sodium phosphate at pH 7.2, 0.1 mM dithiothreitol, 10% (v/v) glycerol, and 1.8 \( \mu \)M isocitrate dehydrogenase (0.58 mg per ml). The fluorescence emission of reduced pyridine nucleotides was determined in the presence and absence of enzyme at ligand concentration ranging from 0.5 \( \mu \)M to 30 \( \mu \)M. The fluorescent enhancement was used to calculate the amount of enzyme-bound ligand after making corrections for volume changes, internal absorption (if applicable), and protein emission.

The data were analyzed by a modification of the Klotz equation as developed by Stockell (23)

\[
\frac{d}{p} = \frac{K_D}{e} + n
\]

where \( d \) is the total concentration of ligand, \( e \) is the total molar concentration of protein, \( p \) is the fraction of enzymatic sites bound multiplied by \( e \), \( K_D \) the dissociation constant, and \( n \) the number of binding sites. A plot of \( d/p \) versus \( 1/(e-p) \) is linear if the binding sites are equivalent and independent. The slope corresponds to \( K_D \) while the ordinate intercept is \( n \).

**RESULTS**

**Spectral Properties of Isocitrate Dehydrogenase and Its Complex with DPNH**

The absorption spectrum of isocitrate dehydrogenase is characteristic of a simple unconjugated protein exhibiting a single maximum at 277 nm with a 280 nm:260 nm ratio of 1.8; charcoal treatment of the protein failed to increase the 280 nm:260 nm ratio indicating the absence of readily dissociable nucleotide. The absorbance of 0.58 (at 277 nm) per mg of protein (protein determined by the biuret method (14)) indicates a low content of aromatic amino acids.

**Fluorimetry**—The dramatic increase in the fluorescence of enzyme-bound DPNH has been used to quantitatively evaluate the dissociation constant and the number of nucleotide-binding sites.
The validity of the method was confirmed by the demonstration of multiple binding sites at a site which is separate from the catalytic site. Since the results consistently showed equimolecular binding of DPNH to isocitrate dehydrogenase, it was desirable to determine whether the conditions selected for fluorescence titration would detect more than one binding site for reduced pyridine nucleotides per molecule of protein. The validity of the method was confirmed by the demonstration of multiple binding sites in other pyridinoproteins. For example, the procedure indicated the binding of 3.8 to 4.1 molecules of DPNH per molecule of rabbit muscle or bovine heart lactate dehydrogenase, values in excellent agreement with those reported in the literature (31).

Displacement of Bound DPNH—While the substrates of isocitrate dehydrogenase failed to displace bound DPNH, fluorescence titrations in the presence of ATP revealed that this inhibitor competitively displaced the reduced cofactor (Fig. 4). The dissociation constant for the binding of ATP was calculated to be about 0.1 mM from the following equation which expresses competition by two ligands for identical and independent binding sites on a macromolecule (32).

\[
K_D = \frac{(P_A)K_{d}(A)}{K_{d}(A) - (K_{d}(A)(P_A) + (P_A))}
\]

\[K_D\] is the dissociation constant for ATP, \(K_d\) is the association constant of DPNH, \((P_A)\) is the concentration of bound DPNH, \((A)\) is the concentration of free DPNH, \((B_0)\) is the total concentration of ATP, \((P_d)\) is the total concentration of protein, and \(n\) the number of binding sites. The \(K_D\) for ATP determined fluorimetrically thus is in reasonable agreement with the kinetically determined \(K_D\) of 0.15 mM (2).

The acetylpyridine derivative of DPNH showed no enhanced fluorescence emission or polarization in the presence of isocitrate dehydrogenase under conditions where a spectrally distinct complex was formed with DPNH. Acetylpyridine DPNH was ineffective in displacing bound DPNH when the analog was present at 10 times the concentration of DPNH. However, when the ratio of acetylpyridine DPNH to DPNH was 100 to 500, measurable displacement of bound DPNH was observed. These results are consistent with kinetic data indicating that the reduced acetylpyridine analog is only weakly inhibitory to isocitrate dehydrogenase.

Ultrafiltration and Gel Chromatography—While the existence of one binding site for DPNH has been established by fluorescence measurements, the possibility remained that additional DPNH-binding sites may exist which do not lead to an alteration of the fluorescence properties of the bound ligand. Such binding sites should be detectable by procedures which do not rely on a fluorescence end point. Equilibrium dialysis, frequently used for such determinations, was impractical since this enzyme was denatured extensively during the equilibration period. Chromatography on columns of Sephadex G-50, equilibrated with buffers containing DPNH, led to minimal inactivation of the enzyme and showed binding of more than 1 molecule of nucleotide. The results of such experiments as shown in Fig. 5 have been plotted according to the method of Scatchard (20) and indicate, upon extrapolation to infinite concentration of ligand, that 4 molecules of DPNH are bound per molecule of the enzyme. These data are not sufficiently precise to allow firm conclusions about the identity or nonidentity of the binding sites corresponding to \(r \geq 2.5\). However, from the slope of the line shown in Fig. 5 a limiting value of \(K_D\) of 14 \(\mu\)M can be calculated which is significantly different from a \(K_D\) of 1.3 \(\mu\)M determined by fluorimetry (Fig. 3). These binding constants compare with values for apparent \(K_D\) for DPN\(^+\) of 80 \(\mu\)M and \(K_D\) for DPNH of 40 \(\mu\)M observed previously in kinetic experiments (2).

Ultratilfiltration experiments have provided further support for

\[J. I. Heron and R. A. Harvey, unpublished observation.\]
the existence of more than one DPNH-binding site. Filtration of isocitrate dehydrogenase in the presence of nearly saturating concentrations of [3H]DPNH (80 μM) lead to retention of radioactivity on the membrane which corresponded to 2.5 to 3.5 molecules of the nucleotide per molecule of enzyme. Separate experiments showed that the amount of labeled DPNH retained on the filter was proportional to enzyme concentration and that nonspecific binding of [3H]DPNH to the filter did not occur. Interaction between isocitrate dehydrogenase and [3H]DPNH (20 μM) was not affected by isocitrate (4 mM), magnesium acetate (9 mM), TPN+ (95 μM), and TPNH (50 μM). However, high concentrations of DPN+ (0.1 mM to 0.2 mM) partially displaced the labeled DPNH from the enzyme.

Effect of Protein Concentration on Number of DPNH-binding Sites—One binding site for DPNH was found by fluorescence titration with 1.8 μM isocitrate dehydrogenase. Much higher protein concentrations were used with the gel chromatography and ultrafiltration techniques (10 μM to 20 μM protein in gel chromatography) which established the presence of four nucleotide-binding sites. It became important to exclude concentration-dependent protein-protein interactions as the cause of the differential stoichiometry, particularly since previous studies have shown that isocitrate dehydrogenase participates in a monomer-dimer equilibrium in the presence of ADP (13). Anderson and Weber (31) reported that if the activity coefficient of an enzyme is independent of protein concentration, dilution of a solution of the enzyme-ligand will produce no change in n. Under such conditions a linear relationship should obtain between nucleotide fluorescence and total protein concentration.

The initial enzyme concentration before ultrafiltration is approximately 0.3 μM. However, during the ultrafiltration process the enzyme collects on the surface of the membrane resulting ultimately in a thin layer of concentrated protein.

Fig. 3 (left). Stockell plots of data from Fig. 2. A straight line was fitted to the points by unweighted least squares. Values of n (intercept) and KD (slope) were calculated as described under “Methods.”

Fig. 4 (center). Stockell plot for DPNH binding in the presence of ATP. Experimental details and calculations are described under “Methods.”

Fig. 5 (right). Scatchard plot of DPNH binding determined by gel filtration. The straight line was fitted to the points by unweighted least squares. The conditions of chromatography and calculations are described under “Methods.”

Fig. 6. The effect of protein concentration on the fluorescence of DPNH. The fluorescence emission of a solution containing 5 mM phosphate buffer at pH 7.2, 0.1 mM ADP, 0.1 mM EDTA, 0.1 mM dithiothreitol, 97 μM DPNH, and 17.2 μM isocitrate dehydrogenase (5.5 mg per ml) was measured. The enzyme-DPNH solution was diluted with a buffer solution containing DPNH (37 μM), equivalent to the concentration of free DPNH calculated for the original solution. The fluorescence was measured after each dilution. All other conditions were as described under “Methods.”

Fig. 6 shows that this is the case, i.e., DPNH binding determined by fluorescence enhancement is independent of enzyme concentration over a range which includes the low and high protein concentrations used in the fluorescence method and in gel chromatography, respectively.

It can be calculated from the KD of the enzyme monomer-dimer equilibrium (13) that the percentage of monomer varies from 60% to 20% over the range of protein concentrations reported in Fig. 6. While the experimental conditions used in determining pro-
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Fig. 7. Stockell plot of binding of TPNH determined by fluorescence titration. The experimental details are described under "Methods."

Fig. 8. Scatchard plot of the binding of [14C]TPNH determined by ultrafiltration. Experimental details are described under "Methods."

Fluorimetry TPNH is not an inhibitor of the DPN-specific isocitrate dehydrogenase; however, TPNH potentiates the inhibition by DPNH of the enzyme from heart (2) and liver (4) and presumably interacts with the enzyme. Such an interaction is supported by the observation that the fluorescence emission of TPNH was markedly enhanced in the presence of isocitrate dehydrogenase (Fig. 1B). In contrast to DPNH, bound TPNH did not show an excitation peak at 285 nm (Fig. 1A), i.e. there is no energy transfer through the protein. Fluorimetric titrations, similar to those described for DPNH, indicated a single binding site for TPNH with a $K_D$ of 0.89 $\mu$M (Fig. 7). The values of $K_D$ and $n$ were the same in the presence or absence of the positive modifier ADP (0.86 mM) or of the inhibitor ATP (1 mM).

Ultrafiltration—At least two more binding sites, in addition to the one shown fluorimetrically, could be detected by the ultrafiltration technique (Fig. 8). The scatter of the data shown in Fig. 8 makes uncertain the precise value of $n$; it is possible that, in analogy to DPNH, 4 molecules of TPNH are bound per molecule of protein. The binding of [14C]TPNH (25 $\mu$M) to isocitrate dehydrogenase was not altered by the presence of DPNH (100 $\mu$M), DPN$^+$ (96 $\mu$M), or the acetylpyridine derivative of TPNH (89 $\mu$M). Acetylpyridine TPNH may not be bound significantly to the enzyme since it failed to exhibit enhanced fluorescence emission or polarization in the presence of isocitrate dehydrogenase and, in contrast to TPNH, did not potentiate the inhibition by DPNH of enzyme activity.

Independence of DPNH- and TPNH-binding Sites

The interaction of DPNH and of TPNH with isocitrate dehydrogenase is similar in a number of respects. Thus, while these ligands are bound to multiple sites on the enzyme, fluorescence enhancement corresponding to only one binding site is observed with each of the reduced pyridine nucleotides. Nevertheless, binding of DPNH and TPNH must occur at different sites on the enzyme. As shown in Fig. 9, initial partial saturation of the protein with DPNH does not prevent further enhancement of fluorescence by subsequently added TPNH (Fig. 9). Furthermore, TPNH does not displace DPNH from the enzyme since the energy transfer from the protein (Fig. 11) which is displayed at the excitation wave length of 290 nm, but not by TPNH, is not diminished in intensity by subsequent addition of TPNH to the DPNH-enzyme complex (Fig. 9B). Although not shown here, it has also been observed, that when the sequence of addition is reversed from that reported in Fig. 9, evidence for binding of DPNH and TPNH at separate sites is obtained. Thus, initial saturation of the enzyme by TPNH does not prevent the further enhancement of fluorescence of
of the exciting incident light energy. If one assumes that the fluorescence shown in Figs. 3 and 7, respectively.

of these nucleotides, values of $n$ and $K_0$ can be calculated. The fluorescence is most entirely due to the absorption of the exciting light by DPN$^+$. However, binding of these molecules to the protein to the bound DPNH. A detailed discussion is not pronounced energy transfer from the excited aromatic residues of the excited states. However, the fluorimetric data suggest that this large increase in quantum yield is accompanied by a pronounced effect of various ligands on protein fluorescence is shown in Fig. 10. I so citrate up to a final concentration of 2.7 mM had no effect. DPN$^+$ lowered fluorescence, but this decrease is almost entirely due to the absorption of the exciting light by DPN$^+$ (inner filter effect).

The decrease in protein fluorescence by DPNH and TPNH shown in Fig. 10 is in excess of that predicted from absorption of the exciting incident light energy. If one assumes that the decrease in protein fluorescence is proportional to the binding of these nucleotides, values of $n$ and $K_B$ can be calculated.

The constants for DPNH ($n = 1.1; K_B = 3 \mu M$) and TPNH ($n = 0.9; K_B = 2 \mu M$) are similar to those obtained from nucleotide fluorescence shown in Figs. 3 and 7, respectively.

**DISCUSSION**

Binding of DPNH to isocitrate dehydrogenase leads to a shift in the excitation and emission maxima of the coenzyme and to an increase in fluorescence intensity. While qualitatively similar changes in spectra have been reported for a number of dehydrogenases, isocitrate dehydrogenase is unique in that the DPNH fluorescence increases 10- to 20-fold on binding to the enzyme. This increase in quantum yield is accompanied by pronounced energy transfer from the excited aromatic residues of the protein to the bound DPNH. A detailed discussion is not yet feasible in the absence of information about the lifetimes of the excited states. However, the fluorimetric data suggest that bound DPNH is shielded from radiationless thermal deactivation in the excited state and that, at least at this site, spatially favored orientations exist between DPNH and one or more aromatic amino acids in the isocitrate dehydrogenase protein.

The binding of DPNH to isocitrate dehydrogenase is also unusual in that only one of the four potential binding sites alters the fluorescence properties of the bound DPNH. It is not clear whether the DPNH-binding site which can be demonstrated fluorimetrically is functionally different from the three additional binding sites revealed by gel chromatography or ultrafiltration. However, several observations suggest that the DPNH-enzyme interaction accompanied by altered fluorescence does not occur at the catalytically active site. Thus, though kinetic experiments show a competitive relationship between DPN$^+$ and DPNH with values of $K_m$ and $K_i$ of the same order of magnitude (2), DPNH is not displaced readily by DPN$^+$ from the fluorimetrically detectable site. It may be that this specific site, where binding of the inhibitor ATP also occurs (ATP can displace bound DPNH competitively (Fig. 4)), has a regulatory rather than a catalytic function. The experimental conditions of binding and kinetic measurements are sufficiently different to make such a conclusion tentative. Nevertheless, the binding of DPNH to such a regulatory site (instead of product binding at the catalytic site) may have a bearing on the apparently aberrant behavior of DPNH with purified liver DPN-linked isocitrate dehydrogenase. It was observed in these experiments (4) that, while the apparent $K_m$ of DPN$^+$ is changed by varying concentrations of isocitrate and by the positive modifier ADP, the $K_i$ of DPNH is essentially uninfluenced by these substances. This observation is not consistent with a mechanism in which DPN$^+$ and DPNH are bound to the same site on the enzyme.

It is noteworthy that direct interaction of ATP with the enzyme has been demonstrated in the absence of added metal ions (Fig. 4), since there has been some uncertainty whether inhibition by ATP is attributable only to chelation of $Mg^{2+}$ (38) or to an additional more specific effect of the nucleotide on the enzyme (9).

The binding of TPNH while superficially similar to that of DPNH (e.g. binding at multiple sites of which only one alters the fluorescence of bound reduced pyridine nucleotide) occurs at distinctly different sites (e.g. the failure of TPNH to exhibit energy transfer through the protein (Fig. 1A) and to be displaced by ATP (cf. Fig. 4) or DPNH (Fig. 9)). The previous observations that TPNH potentiates the inhibitory action of DPNH (2, 4, 8) suggest that there should be an interaction between the TPNH-binding site(s) and at least one DPNH-binding site. However, the presence of TPNH did not markedly alter the stoichiometry or dissociation constant(s) of DPNH, and DPNH appeared to be without effect on TPNH binding either fluorimetrically or by the ultrafiltration technique. It may be that the procedures used in the present studies, especially the ultrafiltration and gel filtration techniques, are not sufficiently precise to detect subtle interactions between the two types of coenzyme sites or that incubation conditions permitting catalytic activity will be necessary to reveal changes in binding characteristics at these sites.

The enzymically active species of bovine heart DPN-linked isocitrate dehydrogenase of molecular weight 390,000 contains eight polypeptide chains which appear equivalent in molecular weight (13), whereas it has been found here that 4 molecules of DPNH and 3 to 4 molecules of TPNH are bound per molecule of...
enzyme. There are a number of well studied dehydrogenases, such as malic dehydrogenase (22, 34), horse liver alcohol dehydrogenase (35, 36), and lactate dehydrogenase (31) which show equivalence between the number of subunits and the number of pyridine nucleotide-binding sites. However, enzymes have also been reported containing fewer coenzyme-binding sites than the number of apparently identical subunits, e.g. threonine-sensitive homoserine dehydrogenase (37). Bovine heart DPN-specific isocitrate dehydrogenase thus falls in the category of enzymes containing fewer binding sites for each DPNH and TPNH than subunits; however, the number of binding sites for total reduced pyridine nucleotides may be equal to the number of subunit polypeptide chains.

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