Distances among Coenzyme and Metal Sites of $NADP^+$-Dependent Isocitrate Dehydrogenase Using Resonance Energy Transfer†

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ABSTRACT: When the substrate isocitrate–Mn^{2+} is present, the fluorescent nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) reacts irreversibly with pig heart $NADP^+$-specific isocitrate dehydrogenase at the coenzyme binding site on one subunit of the dimeric enzyme [Bailey, J. M., & Colman, R. F. (1985) *Biochemistry 24*, 5367–5377]. The modified enzyme, which retains partial activity, binds 1 mol of NADPH or 1 mol of the coenzyme analogue, reduced thionicotinamide adenine dinucleotide phosphate (TNADPH), per dimer. TNADPH quenches the fluorescence of enzyme-bound 2-BDB-TεA-2′,5′-DP with an efficiency of energy transfer of 9.8%. From this value and the spectral properties of the donor and acceptor chromophores, a distance of 32 Å was calculated as the average distance between coenzyme sites on the two subunits. Isocitrate dehydrogenase activity requires a divalent metal ion, such as Mn^{2+}, Co^{2+}, or Ni^{2+}. Co^{2+} and Ni^{2+} have absorption spectra that overlap the emission spectra of enzyme-bound 2-BDB-TεA-2′,5′-DP. In the presence of isocitrate, each of these two metal ions quenches the fluorescence of the enzyme-bound reagent with an efficiency of energy transfer of 28–29%. From this value and the spectral characteristics of the energy donor and acceptors, an average distance of 8.0 Å was estimated between the metal–isocitrate site and the labeled coenzyme site. These distances have provided constraints in formulating a model of the spatial arrangement of active-site ligands on isocitrate dehydrogenase.

The fluorescent nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP)[1] reacts irreversibly with porcine heart $NADP^+$-specific isocitrate dehydrogenase [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] at the coenzyme binding site of one subunit of the dimeric enzyme (Bailey & Colman, 1985). The modified enzyme is fluorescent with an emission peak at 424 nm.

Resonance energy transfer can be used to estimate the distance between two sites on a protein if suitable chromophores occupy these sites specifically and the emission spectrum of the energy donor overlaps the absorption spectrum of the energy acceptor. When applied to a wide variety of enzymes and macromolecular assemblies (Fairclough & Cantor, 1978; Stryer, 1978), this technique has provided valuable structural information about the relationship of sites in these systems. Examples of proteins for which such distance measurements have been made are chloroplast coupling factor 1 (Cantley & Hammes, 1975), pyruvate dehydrogenase complex (Angelides & Hammes, 1979), galactosyltransferase (O'Keeffe et al., 1980), glutamate dehydrogenase (Jacobson & Colman, 1984), phosphofructokinase (Craig & Hammes, 1980), G-actin (Miki & Wahl, 1985), and glutamine synthetase (Maurizi et al., 1986). The coenzyme analogue thionicotinamide adenine dinucleotide phosphate ($TNADP^+$) is a substrate for isocitrate dehydrogenase (Stein et al., 1963). Since the absorption spectrum of the reduced coenzyme analogue TNADPH overlaps the emission spectrum of 2-BDB-TεA-2′,5′-DP, measurement of the distance between the subunit coenzyme binding sites by fluorescence energy transfer is feasible.

Isocitrate dehydrogenase requires a divalent metal ion for activity, and several metals can fulfill this function, including Mn^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+} (Northrop & Cleland, 1970; Colman, 1972). Previous studies of the kinetics and binding of manganous ion by isocitrate dehydrogenase indicated that the divalent metal can bind to one site in the absence of substrate and to a different, but mutually exclusive, site in the presence of isocitrate (Ehrlich & Colman, 1976; Colman, 1983). As the absorption spectra of Co^{2+} and Ni^{2+} overlap the emission spectra of 2-BDB-Tεα-2′,5′-DP, the distance between the coenzyme site occupied by the fluorescent nucleotide analogue and the metal binding site can also be measured by resonance energy transfer.

In this paper the kinetics and stoichiometry of ligand binding to isocitrate dehydrogenase modified by 2-BDB-TεA-2′,5′-DP are compared to those of native enzyme in order to further characterize this enzyme with only one functional subunit. Additionally, fluorescence energy transfer is used to assess the distances between the incorporated 2-BDB-TεA-2′,5′-DP donor chromophore and either the reduced thionicotinamide adenine dinucleotide phosphate site or the Ni^{2+} (Co^{2+}) site.

Experimental Procedures

Materials and Methods. Porcine heart $NADP^+$-specific isocitrate dehydrogenase was purified as described by Bacon et al. (1981). The specific activity of the enzyme used in this study was 37–42 units/mg. Enzyme concentrations were determined from $E^{0.1\%}_{280nm} = 1.08$ (Johanson & Colman, 1981a,b). A subunit molecular weight of 58 000 (Colman et al., 1970) was used to calculate the concentration of enzyme subunits.

2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) was synthesized as

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[1] Abbreviations: 2-BDB-TεA-2′,5′-DP, 2-[(4-bromo-2,3-dioxobutyl)-thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate; PADPR, 2′-phosphoadenosine 5′-diphosphoribose; MES, 2-(N-morpholino)ethanesulfonic acid; $TNADP^+$ and TNADPH, oxidized and reduced thionicotinamide adenine dinucleotide phosphates, respectively; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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previously described (Bailey & Colman, 1985). DL-[U-^{14}C]Isocitrate was obtained from New England Nuclear Corp. Coenzymes (sodium salts), triethanolamine hydrochloride, MES (free acid), Tris (base), and DL-isocitrate were purchased from Sigma Chemical Co. Nickel sulfate, cobaltous sulfate, glycerol, and EDTA (disodium salt) were from Fisher Chemical Co., and $MnSO_4$ was from Mallinckrodt. Yeast glucose-6-phosphate dehydrogenase (grade 1) was from Boehringer Mannheim and was dialyzed before use against 50 mM Tris-HCl, pH 8.0.

Synthesis of TNADPH. Thionicotinamide adenine dinucleotide phosphate ($TNADP^+$) was synthesized by an exchange reaction, catalyzed by porcine brain NAD^+ glycohydrolase (Sigma), between thionicotinamide and $NADP^+$ as described by Stein et al. (1963) except for a change in the final precipitation of $TNADP^+$. After elution from a column of AG1-X2 (100–200 mesh) with ammonium formate, the fractions corresponding to $TNADP^+$ were pooled, made 10% in trichloroacetic acid, and precipitated with cold acetone. The yellow oil was washed repeatedly with methanol until the wash was clear. Addition of 60 mL of methanol and 2-propanol (1:1) precipitated $TNADP^+$ as a pale yellow powder. Evaporation under reduced pressure gave $TNADP^+$ in a 27% yield. TNADPH was synthesized from $TNADP^+$ by enzymatic reduction using glucose-6-phosphate dehydrogenase (Wright & Takahashi, 1977). To a 1-mL solution containing 2.6 mM $TNADP^+$, 50 mM glucose 6-phosphate (Sigma), and 50 mM Tris-HCl, pH 8.0, was added glucose-6-phosphate dehydrogenase to a final concentration of 21 μg/mL. Because of the reported photolability of reduced $TNADP^+$ (Shifrin, 1963), the synthesis of TNADPH was performed in the dark. After incubation for 20 min at 25 °C, the absorption spectrum of reduced $TNADP^+$ (Stein et al., 1963) indicated that the conversion was complete. The reaction mixture was then applied to a 1 × 6 cm column of DE-52 (Whatman) equilibrated with 0.01 M LiCl at 4 °C. The column was eluted with a linear gradient (100 mL of 0.01 M LiCl and 100 mL of 0.5 M LiCl). Fractions (1.4 mL) were collected. TNADPH eluted between fractions 64 and 84 and was located by its characteristic yellow color. This pool was concentrated to 5 mL by evaporation under reduced pressure and precipitated with 200 mL of a cold mixture consisting of 1 part ethanol and 4 parts acetone. The yellow precipitate was washed with acetone and then with diethyl ether and dried with a stream of nitrogen. The reduced coenzyme was stored in the dark at −80 °C in this form. No decomposition was detected by absorption spectra after this procedure. TNADPH was dissolved in the appropriate buffer immediately prior to use and kept in the dark and on ice for the duration of a particular experiment. An extinction coefficient of 11 700 M^{-1} cm^{-1} at 400 nm (Stein et al., 1963) was used to determine the concentration of TNADPH solutions.

Determination of Isocitrate Dehydrogenase Activity. Enzyme assays, performed at 25 °C, were based on the rate of appearance of NADPH as measured by the absorbance at 340 nm on a Gilford Model 240 spectrophotometer (full scale, 0.1 *A* unit) (Colman, 1968). The initial rate measurements were performed in a 1-mL solution containing as standard concentrations 30 mM triethanolamine hydrochloride buffer (pH 7.4), 0.1 mM $NADP^+$, 4 mM DL-isocitrate, and 2 mM $MnSO_4$. For K_m determinations, the concentration of either coenzyme or isocitrate was varied and the other substrate was maintained at the standard concentration.

Preparation of 2-BDB-TϵA-2′,5′-DP-Modified Enzyme. Isocitrate dehydrogenase (1.0 mg/mL) was incubated with 75 μM 2-BDB-TϵA-2′,5′-DP in the presence of 4 mM isocitrate and 2 mM $MnSO_4$ at 25 °C in 0.10 M triethanolamine hydrochloride buffer, pH 7.0, in a total volume of 10 mL. The incubation was allowed to continue for 90 min, after which time the solution was made 0.2 M in dithiothreitol. The enzyme was then extensively dialyzed at 4 °C against three changes of 2 L of 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA. The dialyzed enzyme was centrifuged at 4 °C for 20 min at 14 000 rpm to remove any precipitated or denatured protein, and the concentration was determined by the Bio-Rad protein assay based on the method of Bradford (1976). Standard protein solutions were prepared with unmodified isocitrate dehydrogenase in the same buffer. Incorporation of 2-BDB-TϵA-2′,5′-DP into isocitrate dehydrogenase was measured both by the fluorescence intensity of modified enzyme (Bailey & Colman, 1985) and by quantitation of phosphorus (Colman et al., 1984).

Ultrafiltration Measurements. Protein and ligand solutions of known concentrations were mixed in a total volume of 2 mL and placed in an Amicon ultrafiltration cell, Model 3, 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 for 5–10 min. The initial concentration and concentration of free nucleotide in the filtrate were measured spectrophotometrically by using an extinction coefficient of 18 000 M^{-1} cm^{-1} for $NADP^+$ at 259 nm, 15 400 M^{-1} cm^{-1} for 2′-phosphoadenosine 5′-diphosphoribose (PADPR) at 259 nm, 6200 M^{-1} cm^{-1} for NADP at 340 nm, and 11 700 M^{-1} cm^{-1} for TNADPH at 400 nm. The concentration of isocitrate was determined from the specific radioactivity of [U-^{14}C]isocitrate with a Packard Tricarb liquid scintillation counter.

NADPH Binding Measured by Fluorescence Titration. The binding of NADPH to modified enzyme was measured, in a cuvette maintained at 25 °C, in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA by using a Perkin-Elmer MPF-3 spectrofluorometer operated in the ratio mode. Fluorescence titrations with NADPH were made at the excitation wavelength of 360 nm and a bandwidth of 5 nm and at the emission wavelength of 440 nm and a bandwidth of 8 nm. The fluorescence of bound NADPH was determined by titrating a 0.91 μM solution of NADPH with a concentrated enzyme solution containing the same NADPH concentration as that of the titrated sample. The enzyme concentration varied from 0.25 to 8.28 μM. A value of 6 for the fluorescence enhancement factor H was calculated as described by Mas and Colman (1985) and is defined as $H = (F_{bound}/F_{free}) - 1$, where F_{bound} and F_{free} are the molar fluorescence of bound and free NADPH, respectively. The K_d for NADPH was measured by titrating, with identical increments of NADPH, two parallel samples containing enzyme solution or buffer alone. The enzyme concentration was maintained constant at 3.32 μM by titrating with a NADPH solution containing the same concentration of the enzyme as that of the titrated sample. The NADPH concentration was varied from 0.16 to 3.74 μM.

The concentration of bound NADPH at each point on the titration curve was calculated from the relationship

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

where F and F_0 represent the observed fluorescence in the presence and absence of enzyme, respectively.[2] The data were

[2] We would like to point out that this equation was printed incorrectly as eq 9 in the paper by Mas and Colman (1985).

analyzed in terms of the Scatchard equation:

$$r/[L]_{free} = n/K_d - r/K_d \quad (2)$$

where $[L]_{free}$ is the free NADPH concentration, r is the moles of NADPH bound per mole of enzyme subunit, n is the maximum number of binding sites per subunit, and K_d is the dissociation constant for the enzyme–NADPH complex.

TNADPH Binding Measured by Ultrafiltration. The binding of TNADPH to modified enzyme was measured at 25 °C in the above buffer by ultrafilation using an Amicon Model 3 cell with a Diaflo PM-10 membrane. A constant protein concentration of 5.85 μM in a total volume of 1.2 mL was used with the TNADPH concentration varying from 1.5 to 7 μM. About 0.4 mL of the filtrate was collected following an initial equilibration of the solution for 5–10 min. The fluorescence emission at 500 nm of filtrate was compared to the fluorescence of known concentrations of TNADPH to calculate the concentration of free and bound coenzyme. The data were analyzed in terms of eq 2.

Fluorescence Measurements. The quantum yields of free 2-BDB-TεA-2′,5′-DP and 2-BDB-TεA-2′,5′-DP-modified enzyme were measured in a thermostated cuvette (25 °C) in a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a corrected spectr accessory. Quantum yields of 0.043 for modified enzyme and 0.034 for free 2-BDB-TεA-2′,5′-DP were obtained by the comparative method of Parker and Reese (1960) using a quantum yield of 0.70 for a standard solution of quinine sulfate in 0.1 N H_2SO_4 at 25 °C (Scott et al., 1970).

The steady-state fluorescence polarization of 2-BDB-TεA-2′,5′-DP-modified enzyme was measured at 25 °C with an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The excitation and emission wavelengths were 302 and 428 nm, respectively. The polarization P was defined as

$$P = (F_{\parallel} - F_{\perp})/(F_{\parallel} + F_{\perp}) \quad (3)$$

where $F_{\parallel}$ and $F_{\perp}$ are the fluorescence intensities when the emission polarizer is oriented in the parallel and perpendicular directions, respectively, relative to the excitation polarizer. A correction was made for the unequal transmission of horizontally and vertically polarized light by the emission monochromator at the emission wavelength of 428 nm. A value of 0.14 was measured for the polarization of enzyme-bound 2-BDB-TεA-2′,5′-DP. The limiting polarization of immobilized 2-BDB-TεA-2′,5′-DP, determined in 70% glycerol at dry ice/ethanol temperature, was 0.34.

The distance between sites was measured in accordance with the Förster theory of energy transfer (Förster, 1959). The distance R is inversely related to the efficiency of energy transfer E according to

$$R = R_0(E^{-1} - 1)^{1/6} \quad (4)$$

where R_0 is the distance at which E is 50%. The value of R_0 is calculated from the spectral properties of the energy donor and acceptor by

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6}\ \text{Å} \quad (5)$$

(Stryer, 1978) where n is the refractive index of the medium, Q_D is the quantum yield of the donor in the absence of the acceptor, κ^2 is an orientation factor dependent on the relative orientation of the donor and acceptor transition dipoles, and J is the spectral overlap integral of donor fluorescence and acceptor absorption. The spectral overlap, J, is calculated by using numerical integration (Stryer, 1978):

$$J = \sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda / \sum F_D(\lambda)\Delta\lambda \quad (6)$$

and integrating by 5-nm intervals. In the calculation of R_0, 1.4 was used as the refractive index of water, n. The orientation factor κ^2 was assumed to be $^2/_3$, which is a calculated value for donor and acceptor dipoles rotating rapidly compared to the fluorescence lifetime of the donor (Förster, 1959). Energy-transfer measurements were carried out by titrating 2-BDB-TεA-2′,5′-DP-modified enzyme with TNADPH and monitoring the decrease in emission at 428 nm (excitation 302 nm). The efficiency of energy transfer is given by

$$E = 1 - Q_{DA}/Q_D \quad (7)$$

(Fairclough & Cantor, 1978) where Q_{DA} and Q_D are the quantum yields of enzyme-bound 2-BDB-TεA-2′,5′-DP in the presence and absence of TNADPH, respectively. The wavelength dependence of bound 2-BDB-TεA-2′,5′-DP excitation and emission is unaffected by the presence of TNADPH; thus, the ratio of fluorescence emission at 428 nm (excitation 302 nm) F_{DA}/F_D is proportional to the ratio of the quantum yields and was used to calculate the efficiency E. Free 2-BDB-TεA-2′,5′-DP was also titrated with TNADPH to evaluate the possibility of quenching due to trivial transfer. Distance measurements between the bound fluorescent nucleotide analogue and metal binding sites were measured by titrating both enzyme-bound and free 2-BDB-TεA-2′,5′-DP with either Co^{2+} or Ni^{2+} and comparing the efficiency of quenching in the absence or presence of enzyme.

Results

Ligand Binding and Kinetic Constants for 2-BDB-TεA-2′,5′-DP-Modified Isocitrate Dehydrogenase. 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TεA-2′,5′-DP) has been shown to function as an affinity label for the coenzyme site of $NADP^+$-dependent isocitrate dehydrogenase (Bailey & Colman, 1985). In the presence of the substrate isocitrate–Mn^{2+}, this fluorescent nucleotide analogue reacts specifically and irreversibly at the coenzyme binding site of one subunit of the enzyme dimer. As prepared under the conditions described under Experimental Procedures, this partially modified enzyme (0.5 mol of BDB-TεA-2′,5′-DP/mol of subunit) exhibits 24% residual activity rather than the 50% expected for independent subunits. This observation implies the existence of an interaction between the identical subunits, as we noted earlier (Bailey & Colman, 1985). The incorporation of reagent was measured both by the fluorescence and by the phosphorus content of modified enzyme as described under Experimental Procedures; the two techniques gave the same value of 0.5 mol of 2-BDB-TεA-2′,5′-DP/mol of subunit.

The stoichiometry of binding of various ligands was examined in order to further characterize this modified enzyme. The results of ultrafiltration experiments performed at pH 7.6 in triethanolamine hydrochloride buffer are presented in Table I. A stoichiometry of 0.5 mol of ligand/mol of subunit was obtained for both NADPH and isocitrate. Under similar conditions with unmodified enzyme, one binding site per subunit was observed for NADPH (Mas & Colman, 1985) and isocitrate (Colman, 1969). In contrast, little or no binding of $NADP^+$ or PADPR by modified enzyme could be demonstrated (Table I), although native enzyme binds both of these nucleotides to the extent of one mol/mol of subunit with dissociation constants of 0.29 and 0.15 μM, respectively (Mas & Colman, 1985).

Kinetic constants for $NADP^+$ with both native and modified enzymes are shown in Table II. Modification with 2-BDB-TεA-2′,5′-DP has no effect on the K_m for $NADP^+$ but causes a marked reduction in V_{max}. This result indicates that the inactivation observed is due to an effect on V_{max}. The apparent

Table I: Determination of the Stoichiometry of Ligand Binding to 2-BDB-TεA-2′,5′-DP-Modified Isocitrate Dehydrogenase[a]

ligand	enzyme subunits (μM)	total ligand (μM)	free ligand (μM)	mol of bound ligand/ mol of enzyme subunit
NADPH	30.5	86.8	72.3	0.48
isocitrate[b]	24.5	97.6	84.5	0.54
$NADP^+$	30.5	61.3	58.3	0.10
PADPR	18.4	42.6	42.5	0.005

[a] Isocitrate dehydrogenase was modified in the presence of isocitrate and $MnSO_4$ as described under Experimental Procedures. The binding experiments were performed by ultrafiltration at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA as described under Experimental Procedures. [b] The binding of [^{14}C]isocitrate was measured in the presence of 2 mM $MnSO_4$.

Table II: Kinetic and Binding Parameters for Native and 2-BDB-TεA-2′,5′-DP-Modified Isocitrate Dehydrogenase[a]

enzyme	kinetics: ligands	kinetics: K_m (μM)	direct binding[b] (mol of bound TNADPH/ mol of enzyme subunit)
native	$NADP^+$	10.5[c]	1.04
	$TNADP^+$	26.2[c]	
	isocitrate	7.7	
modified	$NADP^+$	10.3[d]	0.53
	$TNADP^+$	40.9[d]	
	isocitrate	7.9	

[a] Isocitrate dehydrogenase was modified by 2-BDB-TεA-2′,5′-DP in the presence of isocitrate and $MnSO_4$ as described under Experimental Procedures. [b] Stoichiometry of TNADPH binding was determined by ultrafiltration at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA as described under Experimental Procedures. [c] The V_{max} with $TNADP^+$ as coenzyme is 1.2% that observed with $NADP^+$. Maximum velocities were obtained by extrapolation of Lineweaver–Burk plots to infinite pyridine nucleotide concentrations. [d] The V_{max} with $TNADP^+$ as coenzyme is 1.9% that observed with $NADP^+$.

discrepancy of an unchanged K_m for $NADP^+$ with little observed binding is considered under Discussion. Similar effects were observed for isocitrate, which has K_m values of 7.7 and 7.9 μM for native and modified enzymes, respectively, with the same V_{max} values as observed by varying the concentration of $NADP^+$.

The kinetic constants for thionicotinamide adenine dinucleotide phosphate ($TNADP^+$) were also measured (Table II) in order to evaluate $TNADP^+$ as a probe for the coenzyme site of unmodified subunit. Similar K_m values for $TNADP^+$ were observed for native and modified isocitrate dehydrogenases, and with both enzymes, the V_{max} was 1–2% that observed with $NADP^+$. The stoichiometry of binding of the reduced coenzyme analogue (TNADPH), as determined by ultrafiltration, is shown in Table II. The observation of 1 TNADPH site/subunit for native enzyme and 0.5 site/average subunit for modified enzyme agrees with the results found for NADPH.

The reduced coenzyme, NADPH, upon binding to native isocitrate dehydrogenase, exhibits a sevenfold increase in fluorescence intensity (Mas & Colman, 1985) as well as a blue shift from 470 (free NADPH) to 440 nm (Ehrlich & Colman, 1975; Mas & Colman, 1985). This fluorescence enhancement has been used under a variety of conditions to measure the K_d for the NADPH–isocitrate dehydrogenase complex. Although 2-BDB-TεA-2′,5′-DP-modified enzyme binds only 1 mol of NADPH/mol of enzyme dimer, the bound NADPH exhibits the same enhancement factor and blue shift as observed for native enzyme. This result suggests that the environment around the reduced nicotinamide ring is not greatly changed in the coenzyme site which remains after modification

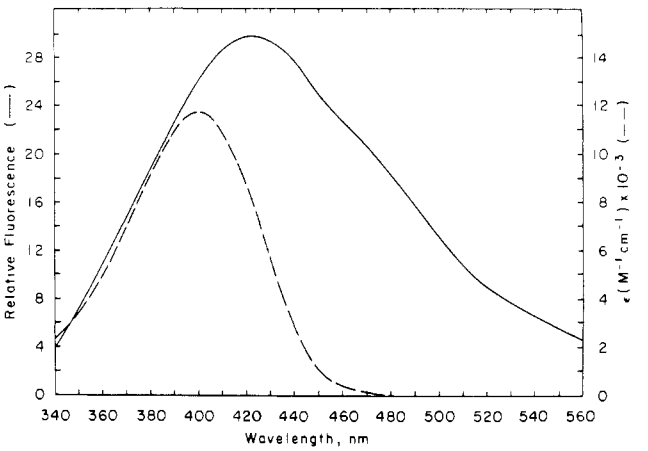

FIGURE 1: Overlap of the corrected fluorescence emission spectrum of 2-BDB-TεA-2′,5′-DP-modified enzyme excited at 302 nm (—) with the absorption spectrum of TNADPH (– –) in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA.

of the enzyme by 2-BDB-TεA-2′,5′-DP. By use of this fluorescence enhancement and the methods described under Experimental Procedures, K_d = 0.57 μM was calculated for NADPH, with a maximum of 0.49 binding sites/subunit. This compares to K_d = 0.0005 μM for the native enzyme–NADPH complex (Mas & Colman, 1985).

The fluorescence emission spectrum of reduced thionicotinamide adenine dinucleotide phosphate, TNADPH, is not shifted or enhanced on binding to either native or modified isocitrate dehydrogenase. The K_d for the complex of TNADPH and 2-BDB-TεA-2′,5′-DP-modified enzyme was therefore assessed by ultrafiltration, using 5.85 μM isocitrate dehydrogenase subunits in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA at 25 °C. The data were analyzed by least squares in accordance with eq 2 to give K_d = 0.90 μM with n = 0.51 (correlation coefficient 0.98).

Measurement of Energy Transfer between Coenzyme Sites on Isocitrate Dehydrogenase. The absorption spectrum of TNADPH overlaps substantially with the corrected emission of 2-BDB-TεA-2′,5′-DP-modified enzyme, as shown in Figure 1. The emission maximum of enzyme-bound 2-BDB-TεA-2′,5′-DP (424 nm) is slightly shifted from that of free 2-BDB-TεA-2′,5′-DP (428 nm), while the absorption spectra of enzyme-bound TNADPH is unchanged from that of the free compound. The spectral overlap, J, was calculated from Figure 1 by using the overlap integral (eq 6) and integrating by 5-nm intervals. A spectral overlap value of 1.63×10^{-14} M^{-1} cm^3 was calculated for the donor–acceptor pair of 2-BDB-TεA-2′,5′-DP and TNADPH. From the spectral overlap and eq 5, a value of 22 Å was calculated for R_0 (the distance at which the efficiency of energy transfer is 50%). The quenching of covalently bound 2-BDB-TεA-2′,5′-DP upon the reversible binding of TNADPH is shown in Figure 2. The percentage decreases in the fluorescence of the modified enzyme is plotted as a function of the fraction of maximum TNADPH sites occupied at a given TNADPH concentration (calculated on the basis of K_d = 0.90 μM). The maximum quenching of fluorescence occurs when all the TNADPH sites are occupied; extrapolation of the line in Figure 2 to r/n = 1 yields 9.8% as the efficiency of energy transfer from enzyme-bound 2-BDB-TεA-2′,5′-DP to TNADPH. Since there is spectral overlap of the two chromophores and quenching of the fluorescence by TNADPH is observed, it is assumed that energy transfer occurs. A value of 32 Å can be calculated as

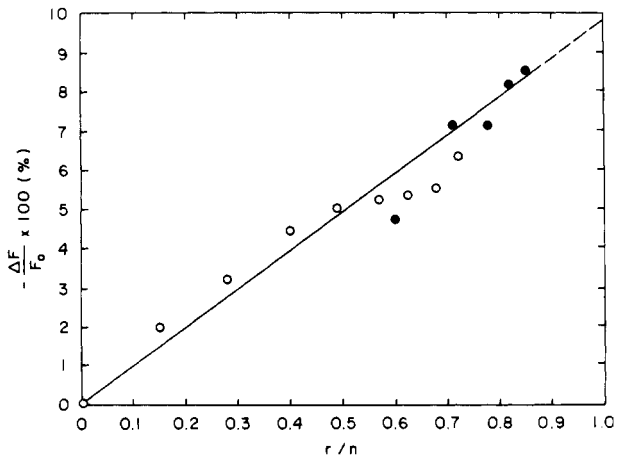

FIGURE 2: Percent decrease in fluorescence as a function of the fraction of TNADPH sites occupied (r/n). The titration of 2.20 (●) and 4.25 μM (○) modified enzyme containing 0.5 mol of 2-BDB-TϵA-2′,5′-DP/mol of subunit was carried out at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol and 0.5 mM EDTA. ΔF was calculated from the difference between the fluorescence observed in the absence (F_0) and presence of varying concentrations of TNADPH (1–6 μM). A value of 0.5 was used for n, the maximum number of binding sites per subunit. A K_d of 0.90 μM was used for the TNADPH-modified enzyme complex to calculate the moles of TNADPH bound per mole of enzyme subunit, r. The line drawn was obtained by a least-squares fit and extrapolated to $r/n = 1$ to give a maximum efficiency of TNADPH quenching equal to 9.8%.

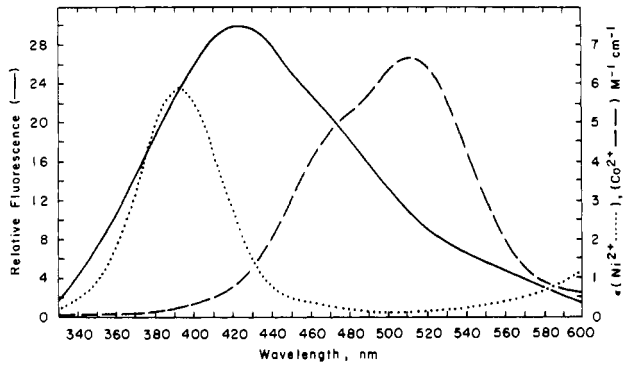

FIGURE 3: Overlap of the corrected fluorescence emission of 2-BDB-TϵA-2′,5′-DP-modified enzyme excited at 302 nm (—) with the absorption spectra of 0.12 M Ni^{2+} (···) and 0.12 M Co^{2+} (– –) in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol.

the average distance between the coenzyme sites from the energy-transfer efficiency and eq 4. Titration of free 2-BDB-TϵA-2′,5′-DP with free TNADPH in the concentration range 0–6 μM caused no change in donor fluorescence, indicating that the quenched fluorescence of enzyme-bound 2-BDB-TϵA-2′,5′-DP in the presence of TNADPH was due to TNADPH bound to the enzyme.

Measurement of the Energy Transfer between the Coenzyme and Metal Binding Sites. The absorption spectra of Ni^{2+} and Co^{2+} exhibit overlap with the corrected emission spectrum of 2-BDB-TϵA-2′,5′-DP-modified enzyme, as shown in Figure 3. (The absorption spectra of free $CoSO_4$ and $NiSO_4$, dissolved in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol, were used for these comparisons.) When metal titrations were performed in the presence of isocitrate, the absorption spectra of 0.06 M metal ions in the presence of 0.12 M isocitrate were used to calculate the overlap integrals. The calculated overlap integrals, J, from eq 6, were 5.42×10^{-18} M^{-1} cm^3 and 1.46×10^{-17} M^{-1} cm^3 for the pairs 2-BDB-TϵA-2′,5′-DP–Ni^{2+} and 2-BDB-TϵA-2′,5′-DP–Co^{2+}, respectively. Overlap integrals for the pairs 2-BDB-TϵA-2′,5′-DP–Ni^{2+}–isocitrate and 2-BDB-TϵA-2′,5′-DP–Co^{2+}–isocitrate were calculated to be 7.93×10^{-18} M^{-1} cm^3 and 2.88×10^{-17} M^{-1} cm^3, respectively. From the overlap integrals and eq 5, an R_0 of 5.7 Å was calculated for Ni^{2+} and an R_0 of 6.8 Å was calculated for Co^{2+} in the absence of isocitrate, while R_0 values of 6.1 Å for Ni^{2+} and 7.6 Å for Co^{2+} were calculated in the presence of isocitrate. Figure 4 shows the quenching of the fluorescence of enzyme-bound 2-BDB-TϵA-2′,5′-DP by titration with Ni^{2+} or Co^{2+} in the absence and presence of isocitrate. When enzyme-bound 2-BDB-TϵA-2′,5′-DP was titrated by Ni^{2+} (Figure 4A) or Co^{2+} (Figure 4B) in the absence of isocitrate (open circles), no more quenching was observed than in the case of free 2-BDB-TϵA-2′,5′-DP. This result suggests that the metal binding site on isocitrate dehydrogenase is more than 12 Å from the bound 2-BDB-TϵA-2′,5′-DP, since no energy transfer attributable to metal bound to the enzyme could be detected. In contrast, when these titrations were performed in the presence of 4 mM isocitrate (Figure 4), a quenching attributable to enzyme-bound metal was observed. The quantum yield and emission spectrum of enzyme-bound 2-BDB-TϵA-2′,5′-DP were unaffected by the presence of 4 mM isocitrate and 2 mM $MnSO_4$, indicating that the quenching observed with Ni^{2+} and Co^{2+} is due to energy transfer. Since the wavelength dependence of excitation and emission of enzyme-bound 2-BDB-TϵA-2′,5′-DP was not altered by the presence of Ni^{2+} and/or Co^{2+} plus isocitrate, the ratio of fluorescence at 428 nm (F_{DA}/F_D) is proportional to the quantum yields and was used to calculate the efficiency, E. From Figure 4 an efficiency of energy transfer equal to 28% for Ni^{2+} and 29% for Co^{2+} was measured, allowing calculation of the distance between bound 2-BDB-TϵA-2′,5′-DP and the metal binding site in the presence of isocitrate. From eq 4 a distance between these sites of 7.1 Å was calculated from Ni^{2+} titration and 8.8 Å from Co^{2+} titration. The average of these two measurements, 8.0 Å, was used as the best estimate of the distance between the metal and coenzyme analogue sites.

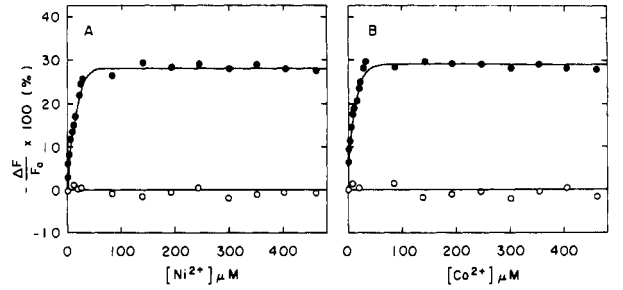

FIGURE 4: Plot of quenching of the fluorescence of 2-BDB-TϵA-2′,5′-DP-modified enzyme (428-nm emission, 302-nm excitation) as a function of total (A) Ni^{2+} and (B) Co^{2+} concentration. The titration of 2.75 μM modified enzyme containing 0.5 mol of 2-BDB-TϵA-2′,5′-DP/mol of subunit was conducted at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.6, containing 10% glycerol in the absence (○) and presence of 4 mM isocitrate (●). The $-\Delta F/F_0 \times 100$ is the net percentage decrease in fluorescence of enzyme-bound 2-BDB-TϵA-2′,5′-DP by metal ion after correction for the effect of metal ion on the fluorescence of free 2-BDB-TϵA-2′,5′-DP (1.38 μM). F_0 is the fluorescence observed for enzyme-bound 2-BDB-TϵA-2′,5′-DP in the absence of metal ion.

From the data shown in Figure 4 in the presence of isocitrate, a dissociation constant of 10 μM for Ni^{2+} and 8 μM for Co^{2+} can be estimated. These values are in good agreement with literature values of 6.7 μM for Ni^{2+} and 11 μM for Co^{2+} (Northrop & Cleland, 1970). Furthermore, the difference in the observed distances in the absence and presence of the substrate isocitrate is consistent with the conclusion from the

binding and kinetic studies of Ehrlich and Colman (1976) that, while no more than one metal site per subunit is occupied at any time, metal binds to different (presumably mutually exclusive) sites on the enzyme in the absence and presence of isocitrate.

Discussion

In order to interpret distances obtained from fluorescence in terms of a geometric model, the stoichiometry and uniqueness of donor and acceptor sites must be known. The fluorescent nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (2-BDB-TϵA-2′,5′-DP) has been shown to be an affinity label for the coenzyme binding site of $NADP^+$-specific isocitrate dehydrogenase and to be incorporated to the extent of 0.5 mol of reagent per peptide chain when the reaction is conducted in the presence of isocitrate and Mn^{2+} (Bailey & Colman, 1985). A single peptide has been isolated from a tryptic digest of enzyme modified when isocitrate and Mn^{2+} were present, and the appearance of this modified peptide could be prevented by including either $NADP^+$ or NADPH in the reaction mixture (Bailey & Colman, 1987). These results suggest that reaction of 2-BDB-TϵA-2′,5′-DP at a distinct location, a coenzyme binding site, causes inactivation.

Isocitrate dehydrogenase has been shown to exist as a dimer of two identical subunits both before and after modification with 2-BDB-TϵA-2′,5′-DP (Bailey & Colman, 1985). As a result, the incorporation of 0.5 mol of reagent/mol of subunit observed for enzyme modified in the presence of isocitrate and Mn^{2+} can best be explained as the reaction of 2-BDB-TϵA-2′,5′-DP at the coenzyme site of one subunit of the enzyme dimer, leaving the coenzyme site on the other subunit unmodified. This allows distance measurements to be made between the coenzyme sites.

Isocitrate dehydrogenase modified to the extent of 0.5 mol of reagent/mol of subunit has a residual activity of only 24%, rather than the expected 50%. This observation indicates that there is interaction between the identical subunits: modification of the coenzyme site of one subunit inactivates that subunit and causes a substantial decrease in the activity of the unmodified subunit. To further characterize this modified enzyme, the stoichiometry of ligand binding was examined and compared to that of native enzyme. Native enzyme binds 1 mol of NADPH, $NADP^+$, isocitrate, and PADPR per mole of subunit (Colman, 1969; Mas & Colman, 1985), whereas 2-BDB-TϵA-2′,5′-DP-modified enzyme binds 0.5 mol of NADPH and 0.5 mol of isocitrate–Mn^{2+} per mole of subunit. The stoichiometry of 0.5 mol of NADPH/mol of subunit is consistent with the postulate that 2-BDB-TϵA-2′,5′-DP modifies a coenzyme binding site on one of the two subunits. Since the K_m for $NADP^+$ was unchanged by modification with 2-BDB-TϵA-2′,5′-DP, the failure to observe appreciable binding by $NADP^+$ and the coenzyme fragment, PADPR, was unexpected. This result may be understood in terms of the observation that the dissociation constant for the enzyme–NADPH complex is increased from 0.0005 μM for native enzyme to 0.57 μM for modified enzyme: modification by 2-BDB-TϵA-2′,5′-DP at the coenzyme site of one subunit causes a 1000-fold weakening in NADPH binding at the coenzyme binding site on the second unmodified subunit. Since $NADP^+$ and PADPR bind to the native enzyme with K_d values of 0.29 and 0.15 μM, respectively (Mas & Colman, 1985), one might predict K_d values of 150–290 μM for these two nucleotides and the modified enzyme; little or no binding of $NADP^+$ or PADPR to modified enzyme could therefore have been expected under the conditions used. [A marked difference between the K_m and K_d for $NADP^+$ has been noted before (Mas & Colman, 1985) and presumably is due to an influence on K_m of additional kinetic parameters in the catalytic mechanism.] This weakening of binding at the second subunit by modification of the first may explain the biphasic kinetics of inactivation observed in the absence of ligands (Bailey & Colman, 1985). Modification of the coenzyme binding site of the first subunit by 2-BDB-TϵA-2′,5′-DP may substantially weaken the binding constant of 2-BDP-TϵA-2′,5′-DP for the coenzyme binding site of the second subunit, thus accounting for the lack of reaction at the coenzyme binding site of the second subunit.

The observation that modified enzyme binds only 0.5 mol of isocitrate–Mn^{2+}/mol of enzyme subunit may initially seem surprising. However, the adenosine moiety of the bound coenzymes, $NADP^+$ and NADPH, as well as the coenzyme fragments, PADPR and 2′,5′-ADP, was determined to be in the syn conformation by intramolecular ^1H–^1H nuclear Overhauser effects (Ehrlich & Colman, 1985). It seems reasonable to expect that the adenosine ring of bound 2-BDB-TϵA-2′,5′-DP is also in a syn conformation, which may position the reactive bromoketo group in the vicinity of the nicotinamide ring of $NADP^+$. It is possible that while the adenine ribose part of the reagent is physically in the coenzyme binding site, the modified amino acid may be located close to the substrate binding site. The observation that the substrate, isocitrate–Mn^{2+}, binds to only one subunit of the enzyme dimer, presumably the unmodified one, is consistent with this proposal. Although isocitrate–Mn^{2+} does not protect againt modification by 2-BDB-TϵA-2′,5′-DP, it does significantly slow the rate of modification by this reagent. The rate constant of inactivation in the presence of isocitrate is 35% that seen in its absence (Bailey & Colman, 1985).

The spectral properties of the coenzyme analogue, reduced thionicotinamide adenine dinucleotide phosphate (TNADPH), are suitable for use as an energy acceptor with enzyme-bound 2-BDB-TϵA-2′,5′-DP as donor in energy-transfer measurements. TNADPH has been shown to bind to modified enzyme with the same stoichiometry as NADPH and with a binding constant similar to that of NADPH to modified enzyme. Additionally, $TNADP^+$ is relatively as effective a coenzyme, when compared to $NADP^+$, for the modified enzyme as it is for the native enzyme. These results imply that $TNADP^+$ and TNADPH are good probes of the coenzyme site of isocitrate dehydrogenase.

The data presented indicate that TNADPH quenches the fluorescence of enzyme-bound 2-BDB-TϵA-2′,5′-DP. The modified enzyme was also titrated with $NADP^+$, which has no spectral overlap with 2-BDB-TϵA-2′,5′-DP. Upon addition of $NADP^+$, the decrease in fluorescence emission at 428 nm (excitation 320 nm) was no more than 2% at concentrations up to 1 mM (high relative to the presumed K_d of 150–290 μM), indicating that conformational changes of the enzyme upon ligand binding may, at most, make only a minor contribution to the fluorescence quenching.

The accuracy of a distance measured by fluorescence energy transfer is limited by the uncertainty in the orientation factor κ^2 used in calculating R_0. In theory, κ^2 may have any value between 0 and 4, depending on the relative orientations of the donor emission and acceptor absorption dipoles (Fairclough & Cantor, 1978). If both donor and acceptor dipoles have complete rotational freedom during the lifetime of the donor in the excited state, a value of $^2/_3$ for κ^2 is used in the calculation of R_0 (Förster, 1959). Probes relatively free to rotate can often be assumed to have random orientations.

Fluorescence polarization measurements can give information about the rotational mobility of a fluorescent label and thus some idea of the uncertainty in κ^2 (Dale et al., 1979). The limiting polarization of a chromophore is expected to be 0.5 if its electronic transitions are characterized by a single transition (Haas et al., 1978). The limiting polarization of 0.34 calculated for 2-BDB-TεA-2′,5′-DP is indicative of mixed polarizations (i.e., they are characterized by more than one electronic transition) (Haas et al., 1978) and is in good agreement with limiting polarizations found for other ethenoadenosine nucleotides (Secrist et al., 1972; Maurizi et al., 1986). For comparison, a polarization of 0 would be expected for a chromophore than can assume all orientations during the lifetime of the donor excited state. The polarization of enzyme-bound 2-BDB-TεA-2′,5′-DP was determined to be 0.14, indicating that although this reagent is covalently bound, it still retains a considerable degree of rotational mobility. If at least one member of a donor–acceptor pair has some rotational mobility, the uncertainty in the distances calculated with $\kappa^2 = {}^2/_3$ is probably not greater than 10% due to uncertainty in orientation (Haas et al., 1978). Furthermore, the range of variation of κ^2 is markedly decreased for chromophores with multiple electronic transitions (such as 2-BDB-TεA-2′,5′-DP) as compared with corresponding cases in which the donor and acceptor are characterized by a single dipole each (Steinberg, 1975). This uncertainty due to orientation is even less when the chromophore metals Ni^{2+} and Co^{2+} are used as acceptors, since these metals have electronic transitions along all three perpendicular directions (Stryer, 1978).

The distance between the coenzyme binding sites on the two subunits of dimeric isocitrate dehydrogenase was determined as 32 Å from the quenching of 2-BDB-TεA-2′,5′-DP donor fluorescence upon addition of TNADPH. This value represents the distance between 2-BDB-TεA-2′,5′-DP covalently bound at the coenzyme binding site of one subunit and TNADPH reversibly bound at the coenzyme binding site of the second subunit. As the distance measured is theoretically from the center of each chromophore, the 32 Å is actually the distance between the ethenoadenosine ring and the thionicotinamide ring of TNADPH. The intramolecular ^1H–^1H nuclear Overhauser effects demonstrated that NADPH can bind to isocitrate dehydrogenase in a stacked conformation, as distinguished from the more open conformation assumed by enzyme-bound $NADP^+$ (Ehrlich & Colman, 1985). Since enzyme–TNADPH has a dissociation constant similar to that of enzyme–NADPH, it seems reasonable to assume that TNADPH binds in a folded conformation as well. Thus, the distance measured may actually be from the ethenoadenosine ring to the region of the adenine ring of TNADPH.

Isocitrate dehydrogenase requires a divalent metal ion for activity. Several metal ions will function as activators (Northrop & Cleland, 1970; Colman, 1972). These include Zn^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+}. Co^{2+} and Ni^{2+} have spectral properties which are suitable for use as energy acceptors from donor enzyme-bound 2-BDB-TεA-2′,5′-DP. In the absence of isocitrate, no quenching of 2-BDB-TεA-2′,5′-DP was observed as a result of either Ni^{2+} or Co^{2+} binding to the enzyme. This result suggests that the metal binding site must be >12 Å from bound 2-BDB-TεA-2′,5′-DP; because of the small R_0 for these metals, 12 Å is the largest distance one could accurately measure for these donor–acceptor pairs using resonance energy transfer. In contrast, in the presence of isocitrate, an average distance of 8.0 Å was measured with either Ni^{2+} or Co^{2+}. These distinct distances, in the absence (>12 Å) and presence of isocitrate (8 Å), indicate that metal

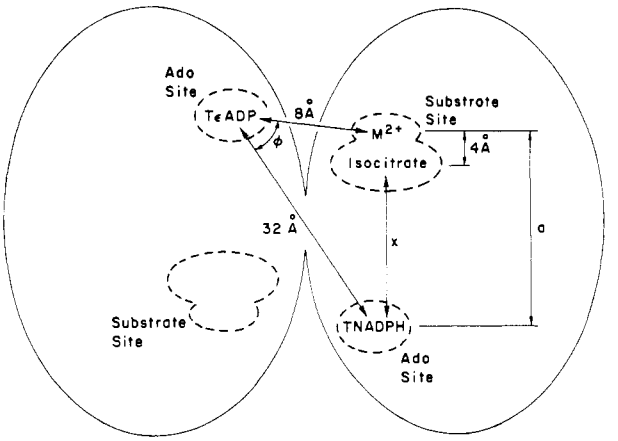

FIGURE 5: Schematic representation of the spatial arrangement of active-site ligands for $NADP^+$-specific isocitrate dehydrogenase.

ion binds to a different site depending on whether isocitrate is present and thus support the earlier studies of the kinetics and chemical modification of isocitrate dehydrogenase (Ehrlich & Colman, 1976; Colman, 1983).

Since isocitrate–Mn^{2+} has been shown to bind to only one subunit of the modified enzyme (presumably the unmodified one), the distance of 8 Å could be interpreted as that between the adenine portion of the coenzyme site of one subunit, occupied by 2-BDB-TεA-2′,5′-DP, and the metal binding site in the presence of isocitrate on the other unmodified subunit. That both coenzyme and isocitrate–Mn^{2+} are binding to the unmodified subunit of the dimeric enzyme is suggested by the observation that the modified enzyme which can bind 0.5 mol of isocitrate/mol of subunit and 0.5 mol of coenzyme/mol of subunit retains significant activity.

A model for the spatial relationship of these sites to one another is shown schematically in Figure 5. The angle ϕ, which is not known, defines the distance (a) between the metal binding site M and the adenine portion of the coenzyme binding site occupied by TNADPH. The minimum possible distance between these two sites would be 24 Å (32 Å −8 Å) when $\phi = 0°$. The maximum distance (of 40 Å) between the TNADPH site and the metal binding site would occur when $\phi = 180°$; however, this is highly unlikely, since then either the metal iron or the TNADPH would have to be positioned on the same subunit as 2-BDB-TεA-2′,5′-DP. A distance of 33 Å can be calculated for a if ϕ equals 90° and the relationship of sites occupied by 2-BDB-TεA-2′,5′-DP, metal ion, and TNADPH is described by a right triangle. This may be a reasonable upper limit for ϕ if the metal ion and TNADPH binding sites are both to be on the unmodified subunit. A second metal–isocitrate binding site is drawn in Figure 5 on the same subunit as 2-BDB-TεA-2′,5′-DP. Since native enzyme binds 2 mol of metal–isocitrate/enzyme dimer (Villafranca & Colman, 1972), this second metal–isocitrate site must exist, and its proposed location is based on symmetry considerations. Monod et al. (1965) have postulated that the subunits, and therefore the ligand binding sites, of an enzyme like isocitrate dehydrogenase, which consists of two equivalent polypeptide chains, must be associated in an isologous manner and possess at least one axis of symmetry.

The distance between Mn^{2+} and the C-2 hydrogen in the isocitrate–Mn^{2+} complex was estimated by NMR to be 4 Å on the basis of proton relaxation rates (Villafranca & Colman, 1974). If the substrate isocitrate is positioned on the enzyme between the metal binding site and the TNADPH site as shown in Figure 5, the distance (designated as x) between the locus of hydride transfer and the adenine portion of the co-

enzyme site (occupied by TNADPH) can now be estimated for a series of ϕ values. At $\phi = 0°$, x is 20.0 Å. At low angles this value increases slowly with increasing ϕ (e.g., to 21.0 Å at 25° and to 22.4 Å at 40°), but at ϕ values larger than 45°, the change in x is more marked (e.g., 29.0 Å at 90°).

The ^{31}P NMR studies indicate that the 2′-phosphate of both $NADP^+$ and NADPH bind to the same site on isocitrate dehydrogenase (Mas & Colman, 1984). The difference in binding between these two coenzymes lies in their conformation: NADPH is in a stacked conformation, while $NADP^+$ is in an open conformation (Ehrlich & Colman, 1985). It is postulated here that $NADP^+$, when bound to isocitrate dehydrogenase, in the presence of substrate (isocitrate–Mn^{2+}), is in an open extended conformation such that the nicotinamide ring is located near isocitrate, and as catalysis occurs and the coenzyme becomes reduced, it then assumes a folded, stacked conformation.

A comparison of the X-ray structures of several dehydrogenases, including lactate dehydrogenase, liver alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase indicates that NAD^+ is bound in an open extended conformation and that the adenine ring is in a hydrophobic cavity about 20 Å from the center of hydride transfer (Bränden & Eklund, 1980). Values in the isocitrate dehydrogenase model (Figure 5) of the angle ϕ from 0° to 40° yield estimates for x that are close to the crystallographic distance (20 Å) between the adenine and the nicotinamide rings determined for other dehydrogenases. Thus, the intersubunit distances between the ethenoadenosine and the metal–isocitrate or between the two coenzyme sites estimated by resonance energy transfer measurements provide the basis of arranging the ligand sites on isocitrate dehydrogenase in a model that is consistent with the known data on this and related enzymes.

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