Yeast Diphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase. Binding of Ligands*

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ABSTRACT: The binding of diphosphopyridine nucleotide (DPN^+), manganous ion, *threo*-$D_s(+)$-isocitrate, and adenosine 5′-monophosphate (AMP) by yeast DPN-specific isocitrate dehydrogenase (EC 1.1.1.41) has been studied by the technique of equilibrium dialysis. The native enzyme has been shown to contain eight similar, and possibly identical, subunits. Two binding sites per enzyme molecule were estimated from Scatchard plots for DPN^+, manganous ion, and AMP. Four binding sites, all with the same or similar intrinsic dissociation constant, were observed for isocitrate. Slopes of Hill plots of the binding results were 1.8 for manganous ion, 1.9 for AMP, 3.7 for isocitrate, and 1.0 for DPN^+. The binding of the positive modifier, AMP, is dependent on the presence of isocitrate. A specific binding site for adenosine triphosphate was identified from which the bound triphosphate could not be displaced by other nucleotides; conversely, adenosine triphosphate did not compete with the other bound nucleotides for their respective binding sites. However, either AMP or DPN^+ at high concentrations interferes with binding of the other. α-Ketoglutarate, a product of the reaction catalyzed by isocitrate dehydrogenase, did not displace bound isocitrate at pH 7.6, but at pH 6.5 could completely displace the bound substrate.

Various kinetic characteristics of the reaction catalyzed by DPN-specific isocitrate dehydrogenase (*threo*-$D_s(+)$-isocitrate + $DPN^+ \rightarrow$ α-ketoglutarate + DPNH + CO_2, EC 1.1.1.41) of yeast have been reported (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965). The enzyme demonstrates absolute requirements for DPN^+, a divalent metal ion such as Mg^{2+} or Mn^{2+}, and the threo-$D_s(+)$ isomer of isocitrate. AMP is a positive modifier that does not alter the maximum reaction velocity but increases the affinity of the enzyme for each of the other ligands. Kinetic analyses have also indicated that ligand binding to the enzyme is a highly cooperative process in which the binding of each individual ligand facilitates the binding of additional molecules of the same ligand as well as all of the others. When the concentration of each reaction component was varied singly, the slopes of Hill plots indicated that the reaction was second order with respect to Mg^{2+}, DPN^+, and AMP, and fourth order with respect to isocitrate (Atkinson *et al.*, 1965). Because of the linearity of the Hill slopes over wide ranges of reaction velocity, it was suggested that these orders might correspond to the actual numbers of binding sites.

With the availability of the purified enzyme (Barnes *et al.*, 1971), it became possible to test this suggestion. This paper reports the stoichiometries of ligand binding to yeast DPN isocitrate dehydrogenase and some ligand binding interactions, as measured by equilibrium dialysis. The results agree with the binding stoichiometries suggested by the kinetic behavior of the enzyme in crude preparations, but some cooperative interactions have apparently been lost in purification.

Materials and Methods

Yeast DPN isocitrate dehydrogenase, with a specific activity of 26.5 μmoles of DPNH formed per min per mg of protein at 30°, was isolated as described in the preceding paper (Barnes *et al.*, 1971). All unlabeled nucleotides were purchased from P-L Biochemicals, Inc., and were checked for purity prior to use; diammonium [^{14}C]AMP (37 Ci/mole) was obtained from International Chemical and Nuclear Corp.; [^{14}C]DPN^+ (20.6 Ci/mole) from Amersham-Searle Corp.; tetralithium [^{3}H]ATP (14.4 Ci/mole) from Schwarz BioResearch, Inc.; and carrier-free $^{54}MnCl_2$ (260 Ci/mole) from New England Nuclear Corp. The radiochemical purity of all labeled nucleotides was confirmed by paper chromatography employing suitable solvent systems (Reference Guide Catalog, 1970). The monopotassium salt of *threo*-$D_s(+)$-isocitrate was purchased from Sigma Chemical Co. and was tritiated by the catalytic exchange service of New England Nuclear Corp. The tritiated sample was subsequently purified in our laboratory by diluting 23 mg of the labeled material (1.25 Ci/mmole) with 542 mg of unlabeled *threo*-$D_s(+)$-isocitrate, then recrystallizing five times at pH 3.5 to a constant specific activity of 1.14 Ci/mole and 92% purity. Care was taken to minimize formation of the lactone (Vickery and Wilson, 1960). The final purity was determined enzymically, using purified isocitrate dehydrogenase.

Assays. Enzyme solutions were concentrated by ultrafiltration using XM-50 membranes and filtration units manufactured by Amicon Corp. Concentrated preparations, which were stored in 50% glycerol solutions at −20°, were dialyzed prior to use for 4 to 10 hr against 0.1 or 0.2 M Hepes[1] buffer

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $(S)_{0.5}$, the concentration of substrate required for half-maximal activity; $(M)_{0.5}$, the concentration of modifier required for half-maximal effect.

TABLE I: Requirements for Binding of [^{14}C]AMP to DPN$^+$-Isocitrate Dehydrogenase.[a]

Unlabeled Ligand	Act. Bound (dpm)	r[b]
4 mM *threo*-D_s(+)-Isocitrate	5918	1.61
4 mM Citrate	5549	1.51
1 mM DPN$^+$	225	0.06
4 mM $MgSO_4$	475	0.13
None	221	0.06

[a] Binding was estimated by equilibrium dialysis as described in the text. The ligand compartment initially contained 0.56 nmole of [^{14}C]AMP (specific activity 7.80×10^7 dpm/μmole), other ligands to yield the concentrations indicated after equilibration between the chambers, and water to 20 μl. The protein compartment contained 14 μg of enzyme in 20 μl of 0.1 M Hepes–3 mM dithiothreitol (pH 7.6). [b] Moles of AMP bound per mole of enzyme.

(pH 7.6), containing 3 mM dithiothreitol. Protein concentrations were measured colorimetrically by the method of Klungsøyr (1969), with modifications previously described (Barnes *et al.*, 1971), using lyophilized pure isocitrate dehydrogenase as a standard.

Isocitrate dehydrogenase activity was determined spectrophotometrically as described in the accompanying paper (Barnes *et al.*, 1971). Concentrations of AMP were determined on the basis of a molar absorptivity of 15,400 at 259 nm. Concentrations of DPN$^+$ were determined enzymically with isocitrate dehydrogenase.

Binding Procedures. The binding of labeled nucleotides by isocitrate dehydrogenase was measured by equilibrium dialysis in microdialysis chambers constructed from 1-in. diameter Lucite rod with dimensions described by Englund *et al.* (1969). The membrane was cleaned by boiling in a 65 mM $NaHCO_3$–20 mM EDTA solution for 10 min, rinsed exhaustively with glass-distilled water, and stored at 5° in 70% ethanol. Before use the membrane was rehydrated, then blotted dry. The volume of protein solution and ligand solution added to each of the chambers on opposite sides of the membranes was 20 μl. All experiments were run at 30° in a circulating water bath. After an equilibration period of 3.5–4 hr, two samples of 8 and 4 μl were withdrawn from each chamber and transferred to vials containing 3 ml of scintillation solvent. The scintillator fluid was prepared by diluting 500 ml of Biosolv III (Beckman) with 2.5 l. of toluene containing 12 g of Omnifluor. Samples were counted in a Packard Model 2008 scintillation spectrometer.

In some experiments, a sample of isocitrate dehydrogenase was removed from the dialysis cell after equilibration to measure enzymatic activity. No decrease in activity was observed.

The difference in radioactivity between the protein and ligand chambers of the cell was assumed to represent enzyme-bound ligand. The activity in the ligand chamber was used to calculate the free-ligand concentration. The total counts from protein and ligand chambers after equilibration was compared with the activity added initially, and recoveries usually exceeded 97%. Throughout these studies a molecular weight of 300,000 for yeast DPN isocitrate dehydrogenase (Barnes *et al.*, 1971) has been used to calculate the molar ratios of bound ligand to enzyme. No corrections for Donnan effects appeared necessary in view of the buffer concentration employed in the experiments.

Results

Analysis of the Data. Data obtained from the binding of small ligands to specific sites on macromolecules have usually been presented graphically according to the mass law binding equation of Scatchard (1949) (eq 1), where r is the number of

$$\frac{r}{c} = \frac{n - r}{k} \tag{1}$$

moles of ligand bound per mole of enzyme, c is the concentration of free ligand, n is the number of binding sites per mole of enzyme, and k is the dissociation constant for the protein–ligand complex. Thus, when there are no interactions among multiple binding sites, *i.e.*, when the sites are identical and independent, a plot of r/c *vs.* r is linear. Extrapolation of the linear plot to infinite free-ligand concentration (the intercept on the r axis) gives an estimate of the value of n. For ligands that are bound cooperatively, however, Scatchard plots are curved (Frieden and Colman, 1967; Sarngadharan *et al.*, 1969) and extrapolation is ambiguous. In such cases, a plot of $r/c^{n'}$ *vs.* r (eq 2) should be linear and extrapolation to the r intercept unambiguous. The value of n' to be used in eq 2 may

$$\frac{r}{c^{n'}} = \frac{n - r}{k} \tag{2}$$

be determined by a Hill plot of the binding results.[2]

Binding of AMP by Isocitrate Dehydrogenase. As shown by earlier kinetic experiments (Atkinson *et al.*, 1965), binding of each ligand by yeast DPN isocitrate dehydrogenase is facilitated by the presence of other ligands. This finding was confirmed by the dialysis binding experiments. Indeed, the affinity of the enzyme for each individual ligand in the absence of others is quite low, and reliable measurements of binding constant under those conditions were not attainable with the concentrations of the enzyme which were available. Consequently, binding of each ligand was always measured in the presence of one or more other ligands that increased the affinity of the enzyme for the labeled compound. The results in Table I show a specific requirement for isocitrate (or its isomer, citrate) for binding of [^{14}C]AMP by the enzyme. Neither the other substrate, DPN$^+$, nor the cofactor, Mg^{2+}, was effective, thus suggesting an absolute requirement for isocitrate in order for effector to bind. In the absence of isocitrate we could not detect binding of AMP even at 0.1 mM, three times the level used in the experiments of Table I. The dependence of [^{14}C]AMP binding on the concentration of iso-

[2] The Hill equation has recently been used mainly with kinetic data, in the form $v/(V_m - v) = (S)^{n'}/K'$. If converted into the symbols used in the Scatchard equation, this becomes $r/(n - r) = c^{n'}/k'$, which is an algebraic variant of eq 2. Thus, when the exponential term n' in the Hill equation is evaluated by the usual logarithmic plot, the value obtained (the slope of the Hill plot) is the appropriate exponent for substitution into eq 2 in order to linearize the Scatchard plot. Only the case of infinitely cooperative binding will the slope of the Hill plot equal n, the number of binding sites; hence the exponent is designated n' to distinguish it from n, which is to be estimated by the Scatchard extrapolation. Small changes in the value of n' do not affect the Scatchard plot strongly, however, and in this paper n' values were for convenience rounded up to the next larger whole number when Scatchard plots were constructed.

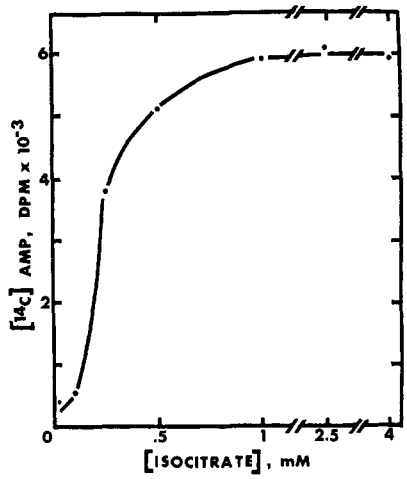

FIGURE 1: Binding of [^{14}C]AMP to yeast DPN isocitrate dehydrogenase as a function of isocitrate concentration. The ligand compartment initially contained 0.56 nmole of [^{14}C]AMP (specific activity 7.80 $\times$ 10^7 dpm/μmole), *threo*-D_s(+)-isocitrate to yield the concentrations indicated after equilibration between the two chambers, and water to a final volume of 20 μl. The protein chamber contained 15 μg of enzyme in 20 μl of 0.1 M Hepes–3 mM dithiothreitol (pH 7.6). Other details are described in the text.

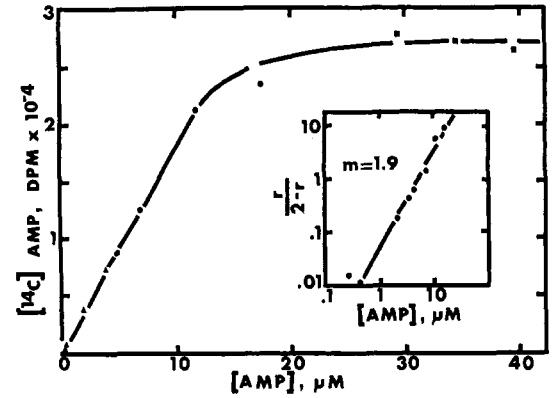

FIGURE 2: Binding of [^{14}C]AMP by yeast DPN isocitrate dehydrogenase. The ligand compartment initially contained 80 nmoles of *threo*-D_s(+)-isocitrate, varying levels of [^{14}C]AMP (specific activity 7.80 $\times$ 10^7 dpm/μmole), and water to a final volume of 20 μl. The protein chamber contained 62 μg of enzyme in 20 μl of 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). The abscissa indicates the concentration of free AMP after equilibration. The inset depicts the same data in the form of a Hill plot, using a value of 2 for n. Points designated by ■ on the main curve are not included in the inset figure.

citrate is shown in Figure 1. At an AMP concentration of 28 μM, half of the AMP binding sites were occupied when the isocitrate concentration was approximately 230 μM. In subsequent binding experiments involving [^{14}C]AMP, 2 mM isocitrate was provided in order to ensure maximal binding of the nucleotide.

The curves of Figure 2 show the binding of [^{14}C]AMP to isocitrate dehydrogenase in the presence of 2 mM isocitrate. Sigmoidicity in the binding curve indicates cooperative interaction between AMP sites. This is shown more definitely by the slope of the inset Hill plot. The corresponding Scatchard curve in Figure 3 (curve B) for these results plotted as r/c^2 *vs.* r is linear and extrapolates to 1.7 binding sites for AMP at infinite concentration of free AMP. The Scatchard and Hill plots yield estimates of $(M)_{0.5}$ for the enzyme–AMP complex under these conditions of 7.2 and 7.5 μM, respectively. Estimates of n by extrapolation have consistently ranged from 1.5 to 1.7 in numerous experiments. The isolated enzyme does not appear to have any nucleotides associated with it since its A_{280}/A_{260} absorbance ratio is 1.79.

Binding of Manganese Ion by Isocitrate Dehydrogenase. Magnesium or manganous ion is required as a cofactor in the reaction catalyzed by DPN-specific isocitrate dehydrogenase. The maximal velocity of the reaction is about twice as great in the presence of Mn^{2+} as when Mg^{2+} is used. Earlier kinetic studies suggested that two Mg^{2+} binding sites may participate in the catalyzed reaction. Metal ion binding to isocitrate dehydrogenase was investigated using $^{54}Mn^{2+}$ in the presence of 2 mM *threo*-D_s(+)-isocitrate and 1 mM AMP. The binding data shown in Figure 4 are corrected for complex formation between Mn^{2+} ion and isocitrate, as indicated in the figure legend. The inset Hill plot of Figure 4 and the Scatchard plot of Figure 3 (curve C) are alternate treatments of the same results. Extrapolation of the Scatchard plot indicates the presence of two Mn^{2+}-binding sites. The slope of the Hill plot (1.8) and the linearity of the modified Scatchard plot with r/c^2 as the ordinate demonstrate cooperativity between these binding sites. Estimates of $(S)_{0.5}$ for Mn^{2+} from the two plots are 38 μM (Hill) and 30 μM (Scatchard).

Binding of threo-D_s(+)-Isocitrate by Isocitrate Dehydrogenase. Plots of the velocity of the reaction catalyzed by yeast DPN isocitrate dehydrogenase as a function of isocitrate concentration are sigmoid, demonstrating a high degree of cooperativity. Slopes of Hill plots approach 4 (Atkinson *et al.*, 1965). The kinetic Hill slope for the purified enzyme used in the current binding studies was 3.6 in the absence of the positive modifier AMP and 2.6 in the presence of 1 mM AMP. Because of the relatively low affinity of the enzyme for isocitrate in the absence of modifiers, it was not feasible to study the binding of the substrate alone with the concentrations of enzyme which were available. Therefore, all isocitrate binding trials were run

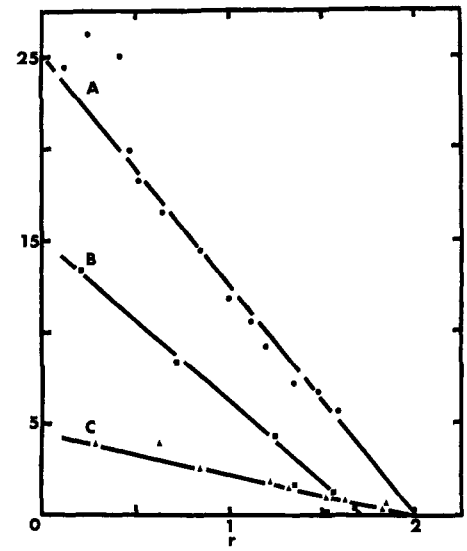

FIGURE 3: Scatchard plots of the binding of DPN^+, AMP, and Mn^{2+} to yeast DPN isocitrate dehydrogenase. Curve A: binding of [^{14}C]-DPN^+; the ordinate scale is r/c $\times$ 10^{-3}, M^{-1}. Curve B: binding of [^{14}C]AMP; the ordinate scale is r/c^2 $\times$ 10^{-10}, M^{-2}. Curve C: binding of ^{54}Mn; the ordinate scale is r/c^2 $\times$ 10^{-11}, M^{-2}. These plots correspond to results from Figures 7, 2, and 4, respectively. Points designated by ▲ in Figure 2 and Figure 4 do not appear in this figure.

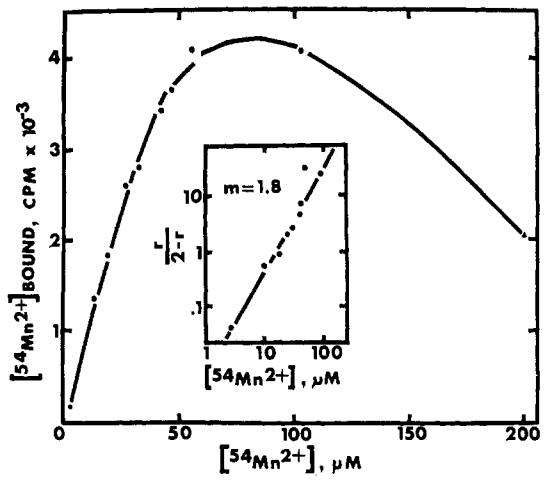

FIGURE 4: Binding of $^{54}Mn^{2+}$ by yeast DPN isocitrate dehydrogenase. The ligand compartment initially contained 80 nmoles of *threo*-D_5-(+)-isocitrate, 40 nmoles of AMP, varying levels of $^{54}MnSO_4$ (specific activity 5.14×10^6 cpm/μmole), and water to a final volume of 20 μl. The abscissa indicates the concentration of free $^{54}Mn^{2+}$ after equilibration. The protein chamber contained 124 μg of enzyme in 20 μl of 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). The inset is a Hill plot of the same results, using a value of 2 for n. The point designated by ▲ is not included in the inset. Concentrations of uncomplexed Mn^{2+} ion were estimated from the total concentrations of Mn^{2+} and isocitrate using a pK of 3.06 for dissociation of the Mn^{2+}–isocitrate complex (Grzybowski *et al.*, 1970).

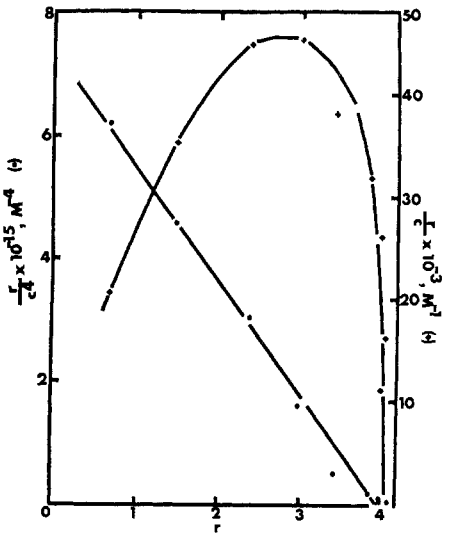

FIGURE 6: Scatchard plot of the binding of [^{3}H]*threo*-D_5(+)-isocitrate by yeast DPN isocitrate dehydrogenase. The results presented in Figure 5 were used to generate these two curves. The curved plot results from the usual plot of r/c *vs.* r (Scatchard, 1949). The linear plot presents the same results utilizing the Hill slope as a linearizing factor (see text).

in the presence of a saturating concentration (1 mM) of AMP. The sigmoid binding curve of Figure 5 demonstrates cooperative binding, which is further shown by the binding Hill plot slope of 3.7. The modified Scatchard plot in Figure 6, when m of eq 2 has a value of 4, is linear and intersects the abscissa at 3.9 binding sites per enzyme molecule. This figure strikingly illustrates the usefulness of the Hill slope as a linearizing factor for Scatchard plots of binding results when binding is cooperative. The linearity of the Hill and the modified Scatchard plots suggests that all isocitrate binding sites have the same or similar intrinsic dissociation constants. Estimates of $(S)_{0.5}$ for isocitrate in the presence of 1 mM AMP are: from the Hill plot, 50 μM; from the Scatchard plot, 52 μM.

Binding of DPN$^+$ by Isocitrate Dehydrogenase. Unlike the other ligands whose binding to isocitrate dehydrogenase was investigated, DPN^+ was not bound cooperatively. This is in contrast to kinetic results obtained with crude preparations, for which slopes of Hill plots with respect to DPN^+ approached 2 (Atkinson *et al.*, 1965). Table II summarizes the requirements for binding of [^{14}C]DPN^+ to DPN isocitrate dehydrogenase. At 0.3 mM, DPN^+ was not bound to the enzyme. Neither 1 mM AMP nor 1 mM AMP plus 4 mM Mg^{2+} (these are kinetically saturating concentrations routinely employed in enzyme assays)

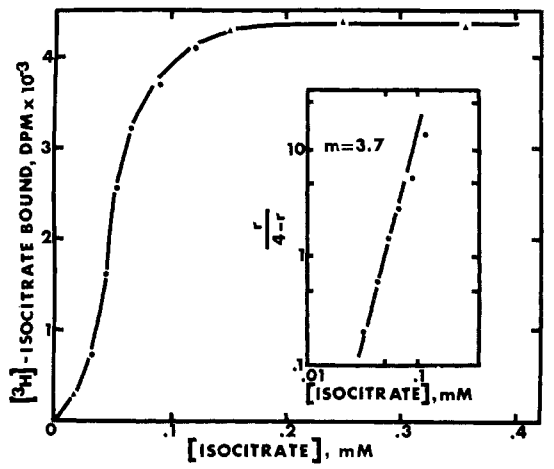

FIGURE 5: Binding of [^{3}H]*threo*-D_5(+)-isocitrate by yeast DPN isocitrate dehydrogenase. One compartment initially contained 40 nmoles of AMP, [^{3}H]*threo*-D_5(+)-isocitrate (specific activity 2.53×10^6 dpm/μmole), and water to a final volume of 20 μl. The opposite chamber contained 127 μg of enzyme in 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). The abscissa indicates the concentration of free isocitrate after equilibration. The inset is a Hill plot of the same results, using a value of 4 for n. The points designated by ▲ are not included in the inset.

TABLE II: Requirements for Binding of [^{14}C]DPN^+ to DPN Isocitrate Dehydrogenase.[a]

Unlabeled Ligand	Act. Bound (dpm)	r[b]
None	37	0
1 mM AMP	383	0.21
1 mM AMP + 4 mM Mg^{2+}	227	0.13
0.4 mM Isocitrate	3304	1.8
0.4 mM Isocitrate + 4 mM Mg^{2+}	2037	1.1
4 mM Mg^{2+}	192	0.10

[a] Binding was estimated by equilibrium dialysis as described in the text. The ligand compartment initially contained 12 nmoles of [^{14}C]DPN^+ (specific activity 5×10^6 dpm/μmole), other ligands to yield the concentrations indicated after equilibration between the two chambers, and water to 20 μl. The protein compartment contained 0.11 mg of enzyme in 20 μl of 0.1 M Hepes–3 mM dithiothreitol (pH 7.6). [b] Moles of DPN^+ bound per mole of enzyme.

TABLE III: Ligand-Binding Parameters for Yeast DPN Isocitrate Dehydrogenase.

Labeled Ligand	Other Ligands	Hill Slope	$(S)_{0.5}$ (μM) Hill	$(S)_{0.5}$ (μM) Scatchard
	From Equilibrium Binding Experiments[a,b]			
AMP	4 mM Isocitrate	1.9	8.0	7.5
Mn^{2+}	4 mM Isocitrate and 2 mM AMP	1.8	38	30
Isocitrate	2 mM AMP	3.7	50	52
DPN^+	6 mM Isocitrate	1.0	85	80
	From Kinetic Experiments[c]			
AMP	4 mM Mg^{2+}, 0.4 mM DPN^+, and 0.2 mM Isocitrate	1.5	6	
Mn^{2+}	4 mM Isocitrate, 2 mM AMP, and 0.4 mM DPN^+	1.0	0.95	
Isocitrate	1 mM AMP, 4 mM Mg^{2+}, and 0.4 mM DPN^+	2.6	18	
DPN^+	3 mM Isocitrate, 1 mM AMP, 4 mM Mg^{2+}	1.0	210	

[a] Conditions for binding experiments are given in the legends for Figures 2–9. [b] Hill slopes and $(S)_{0.5}$ values were estimated by linear least-squares analysis of the binding results. [c] From Barnes *et al.* (1971).

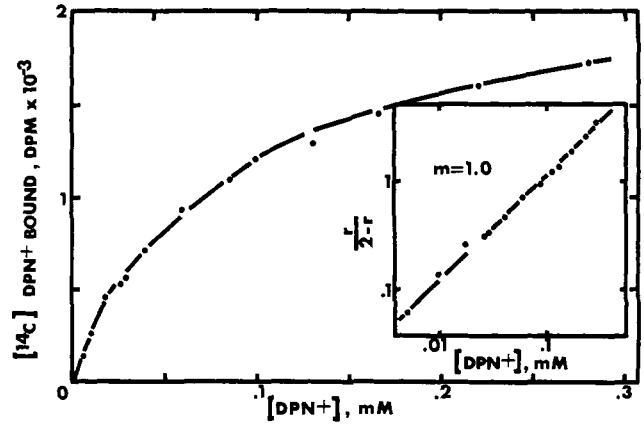

FIGURE 7: Binding of [^{14}C]DPN^+ by yeast DPN isocitrate dehydrogenase. The ligand compartment initially contained 120 nmoles of *threo*-D_s(+)-isocitrate, varying levels of [^{14}C]DPN^+ (specific activity 5.0×10^6 dpm/μmole), and water to a final volume of 20 μl. The abscissa indicates the concentration of free [^{14}C]DPN^+ after equilibration. The protein compartment contained 65 μg of enzyme in 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). The inset is a Hill plot of the same results, using a value of 2 for n.

supported significant binding of the substrate. Only isocitrate facilitated binding of DPN^+. The simultaneous addition of Mg^{2+} antagonized this binding. The metal ion alone did not elicit binding of DPN^+. No binding of DPN^+ at concentrations up to 10 mM (data not shown) was observed in the absence of isocitrate.

Subsequent binding experiments were carried out in the presence of 2 mM isocitrate. The binding curve of Figure 7, the corresponding Hill plot slope of unity, and the Scatchard plot in Figure 3 (curve A) indicate the presence of two independent and noninteracting binding sites for DPN^+ on the purified enzyme. Estimates of $(S)_{0.5}$ for DPN^+ in the presence of 2 mM isocitrate are: Hill plot, 85 μM; Scatchard plot, 80 μM. Table III summarizes the binding parameters for all ligands studied, and compares them with the results obtained in kinetic studies.

Competition Experiments. Yeast DPN isocitrate dehydrogenase responds to variation in the adenylate energy charge in the way expected for enzymes involved in the regeneration of ATP (Atkinson, 1968a,b). In order to determine whether this response is due to competition among the adenylates for a single type of binding site, or whether more than one type of nucleotide binding site is involved, competition experiments were performed among ATP, AMP, and DPN^+. Some of these experiments are summarized in Table IV. The addition of isocitrate increased the binding of ATP. A Scatchard plot of the ATP binding results indicated the presence of only one ATP binding site. Magnesium ion antagonizes binding of ATP. Neither AMP nor DPN^+ at concentrations ten and five times, respectively, that of ATP competes with the nucleotide triphosphate. Conversely, ATP at 83 times the concentration of AMP does not displace the bound monophosphate and ATP at 3 times the concentration of DPN^+ does not decrease the binding of DPN^+. These experiments strongly suggest that ATP binds to a specific site on isocitrate dehydrogenase and that the energy charge response is due to interactions elicited

TABLE IV: Competition among Nucleotides for Sites on Yeast DPN^+ Isocitrate Dehydrogenase.[a]

Labeled Ligand	Unlabeled Competing Ligand	Other Unlabeled Ligands	r[b]
0.10 mM ATP	None	None	0.47
0.10 mM ATP	None	2 mM Isocitrate	1.06
0.10 mM ATP	1 mM ATP	2 mM Isocitrate	0.94
0.10 mM ATP	0.5 mM DPN^+	2 mM Isocitrate	0.97
0.10 mM ATP	None	2 mM Isocitrate and 4 mM Mg^{2+}	0.33
0.03 mM AMP	None	2 mM Isocitrate	1.58
0.03 mM AMP	2.5 mM ATP	2 mM Isocitrate	1.51
0.03 mM AMP	2.4 mM DPN^+	2 mM Isocitrate	0
0.3 mM DPN^+	None	2 mM Isocitrate	2.13
0.3 mM DPN^+	1 mM ATP	2 mM Isocitrate	2.14
0.3 mM DPN^+	1 mM AMP	3 mM Isocitrate	1.47

[a] Binding was estimated by equilibrium dialysis as described in the text. Both the isotopically labeled and unlabeled ligands of each experiment were added to one compartment to give the concentrations indicated after equilibration between the two chambers. The opposite compartment contained 20 μl of enzyme solution previously dialyzed against 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). Protein concentrations varied between 3.0 and 3.6 mg per ml. The specific activities of the labeled ligands were the same as indicated in the legends for Figures 2–7. [b] Moles of isotopically labeled ligand bound per mole of enzyme.

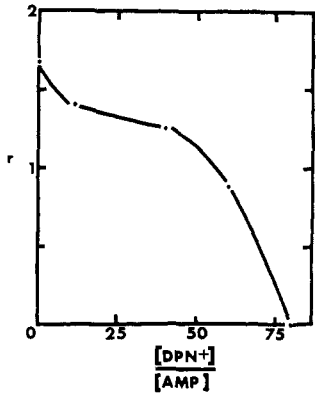

FIGURE 8: Competition between DPN^+ and [^{14}C]AMP in binding to yeast DPN isocitrate dehydrogenase. The ligand compartment initially contained 80 nmoles of *threo*-D_s(+)-isocitrate, 1.2 nmoles of [^{14}C]AMP (specific activity 7.80×10^7 cpm/μmole), DPN^+ to yield the [DPN^+]:[AMP] ratios indicated after equilibration between the two chambers, and water to a final volume of 20 μl. The protein compartment contained 51 μg of enzyme in 20 μl of 0.2 M Hepes–3 mM dithiothreitol (pH 7.6).

by positive and negative modifiers associated with independent binding sites.

In contrast, either AMP or DPN^+ at a high concentration interferes with binding of the other. Thus 2.4 mM DPN^+ abolishes the binding of [^{14}C]AMP at a concentration of 30 μM. Conversely, 1 mM AMP decreases the binding of [^{14}C]DPN^+ at 0.3 mM concentration. The concentration dependence of DPN^+ displacement of bound AMP is shown in Figure 8.

The reaction catalyzed by yeast DPN isocitrate dehydrogenase is reversible at pH 6.5, although the rate of the reverse reaction is very slow (Hathaway and Atkinson, 1963). The capacity of α-ketoglutarate, a product of the forward reaction, to displace enzyme-bound [^{3}H]isocitrate was investigated at both pH 7.6 and 6.5. Direct binding of labeled α-ketoglutarate could not be observed because of the very low affinity of the enzyme for this ligand. These experiments are summarized in Table V. At isocitrate concentrations of 0.3 mM and 72 μM, 5.33 mM α-ketoglutarate did not compete detectably with isocitrate binding at pH 7.6. At pH 6.5, the enzyme bound the same amount of [^{3}H]isocitrate at each concentration as was observed at pH 7.6. However, at this lower pH, 5.33 mM α-ketoglutarate completely displaced the bound isocitrate.

TABLE V: Competition between *threo*-D_s(+)-Isocitrate and α-Ketoglutarate for Sites on Yeast DPN Isocitrate Dehydrogenase.[a]

Isocitrate (mM)	α-Ketoglutarate (mM)	r[b]
	pH 7.6	
0.3	0	3.8
0.3	5.33	3.9
0.072	0	2.2
0.072	5.33	2.3
	pH 6.5	
0.3	0	4.0
0.3	5.33	0.0
0.072	0	2.0
0.072	5.33	0.1

[a] Binding was estimated by equilibrium dialysis as described in the text. The ligand compartment initially contained 40 nmoles of AMP and [^{3}H]*threo*-D_s(+)-isocitrate (specific activity 2.53×10^6 dpm/μmole) and α-ketoglutarate to give the concentrations indicated after equilibration between the two chambers. The protein compartment contained 42 μg of enzyme in 20 μl of 0.2 M Hepes–3 mM dithiothreitol (pH 7.6). [b] Moles of isocitrate bound per mole of enzyme.

Discussion

The binding studies reported in this paper confirm the stoichiometries inferred from earlier kinetic observations (Atkinson *et al.*, 1965), indicating that there are four binding sites for isocitrate and two each for DPN^+, AMP, and divalent metal ion on each molecule of yeast DPN isocitrate dehydrogenase. Comparison of the kinetic properties of the purified enzyme with the activity in crude extracts shows that the cooperativity of binding has been reduced somewhat during purification (Barnes *et al.*, 1971); nevertheless the purified enzyme retains a high degree of cooperativity. Indeed, with the levels of enzyme that we could feasibly use and the specific radioactivities that were available, we could not detect binding of any ligand in the absence of all others.

The most striking and unexpected example of cooperativity, and one that could not be revealed by kinetic observations, is the requirement of isocitrate for binding of AMP. Since the function of the AMP site appears to be regulation of the affinity of the enzyme for the substrates, and especially for isocitrate, it is surprising that substrate binding should be a prerequisite for binding of the modifier. Although there are apparently only two catalytic sites on the enzyme molecule, as indicated by the stoichiometry of binding of DPN^+ and Mn^{2+}, isocitrate is bound at four sites. Possibly two noncatalytic sites are specifically linked to AMP binding. However, the binding results are compatible with a similar intrinsic binding affinity for isocitrate at all four sites. The significance of these observations remains unexplained, but they are not wholly unprecedented. Substrate dependence for binding of negative modifiers has been observed in at least two cases. Fructose 1,6-diphosphate is required for binding of AMP by fructose 1,6-diphosphate phosphatase (Watanabe *et al.*, 1968), and L-alanine, a feedback inhibitor of glutamine synthetase, binds only in the presence of glutamine (Ginsburg, 1969).

Cooperative interactions tend to be lost or weakened during purification, as reported, for example, in the cases of aspartate transcarbamylase (Weitzman and Wilson, 1966) and fructose 1,6-diphosphate phosphatase (Sarngadharan *et al.*, 1969), and as observed in early attempts at purification of yeast DPN isocitrate dehydrogenase. It seems likely that slight perturbations of the enzyme structure during isolation procedures, resulting perhaps in weakened subunit interactions, can account for such differences. Each step in the purification was therefore evaluated for preservation of cooperative kinetics, as well as for yield in terms of catalytic activity. The procedure finally adopted (Barnes *et al.*, 1971) preserved the cooperativity of binding of isocitrate and AMP nearly intact, but cooperativity of DPN^+ binding was totally lost. In crude extracts, the reaction is second order with respect to DPN^+, as previously reported (Atkinson *et al.*, 1965). The purified enzyme, as expected, binds 2 moles of DPN^+/mole, but both kinetic and ligand-binding experiments with the purified enzyme indicate no cooperativity

in DPN^+ binding. Since cooperativity between isocitrate sites and even cooperativity between isocitrate and DPN^+ sites (Table II) is retained, the loss of cooperative interaction between DPN^+ sites is perplexing.

For most regulatory enzymes thus far studied by binding techniques, the number of bound substrates or effectors corresponds to the number of subunits comprising the active enzyme. Thus, glutamate dehydrogenase binds 8 moles of GTP/mole of enzyme (Frieden and Colman, 1967); adenylylated glutamine synthetase binds manganous ions at 12 high-affinity sites (Denton and Ginsburg, 1969), covalently binds 12 adenylyl groups to tyrosyl residues (Kingdon *et al.*, 1967; Shapiro and Stadtman, 1968), and binds 12 moles each of AMP and L-tryptophan per mole of enzyme (Ginsburg, 1969); fructose 1,6-diphosphate phosphatase binds four substrate and four AMP molecules per molecule of enzyme (Sarngadharan *et al.*, 1969; Pontremoli *et al.*, 1968); and aspartate transcarbamylase binds six molecules of CTP per enzyme molecule (Winlund and Chamberlin, 1970; Hammes *et al.*, 1970). The stoichiometries found for ligands binding to yeast DPN^+ isocitrate dehydrogenase do not show such a clear correspondence to its eight-subunit structure. The evidence for a single ATP-binding site is especially unexpected and difficult to rationalize.

Although the competition binding experiments indicate that DPN^+ and AMP can compete rather weakly for their respective binding sites, the concentration of either required for displacement of the other is probably too high to be of physiological significance. This effect may, however, be related to the report by Cennamo *et al.* (1970) that, at high concentrations of isocitrate where the action of AMP as a positive modifier is obscured, AMP at a high concentration can be shown by kinetic experiments to compete weakly with DPN^+. In contrast, ATP does not compete with either AMP or DPN^+, and appears to bind at a specific independent site.

The binding parameters, estimated by equilibrium binding and by kinetic techniques, compiled in Table III cannot be compared directly. In order that the concentrations of ligands can be specified, the binding experiments must be done under conditions that do not allow the enzyme-catalyzed reaction to proceed–that is, at least one component of the kinetic assay mixture must be omitted. Thus binding and kinetic experiments cannot be carried out under identical conditions. The differences in $(S)_{0.5}$ values in Table III must reflect this fact. The two approaches yield very similar estimates of affinity for AMP, and the threefold difference in $(S)_{0.5}$ values for isocitrate probably results from the presence of Mg^{2+} and DPN^+ in the kinetic assay. The very large difference between the values for Mn^{2+} is difficult to rationalize, since the only component of the kinetic assay mixture that was absent from the solution used in the binding assay was 0.4 mM DPN^+. The two- to threefold difference in the estimates for DPN^+ may be due in part to the weak competition between AMP and DPN^+ discussed above. The Hill slopes (a function of the number of sites and the degree of cooperativity of binding) obtained by the two approaches are similar for AMP, isocitrate, and DPN^+. However Mn^{2+} is bound cooperatively, but no cooperativity is seen in the kinetic experiments. This discrepancy, together with the very large difference in $(Mn^{2+})_{0.5}$ values, merits further study.

DPN isocitrate dehydrogenase from beef heart has recently been reported to have a molecular weight similar to that of the yeast enzyme, to contain eight subunits (Giorgio *et al.*, 1970), and to bind four molecules each of DPNH and TPNH (Harvey *et al.*, 1970). The TPN-specific enzyme from pig heart has a much lower molecular weight (58,000) and has only one binding site for isocitrate (Colman, 1969). No modifiers have been reported for the TPN enzyme.

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