Equilibrium Binding of Coenzymes and Substrates to Nicotinamide-Adenine Dinucleotide Phosphate-Linked Isocitrate Dehydrogenase from Bovine Heart Mitochondria

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1. The stoichiometries and affinities of ligand binding to isocitrate dehydrogenase were studied at pH 7.0, mainly by measuring changes in NADPH and protein fluorescence. 2. The affinity of the enzyme for NADPH is about 100-fold greater than it is for NADP⁺ in various buffer/salt solutions, and the affinities for both coenzymes are decreased by Mg²⁺, phosphate and increase in ionic strength. 3. The maximum binding capacity of the dimeric enzyme for NADPH, from coenzyme fluorescence and protein-fluorescence measurements, and also for NADP⁺, by ultrafiltration, is 2 mol/mol of enzyme. Protein-fluorescence titrations of the enzyme with NADP⁺ are apparently inconsistent with this conclusion, indicating that the increase in protein fluorescence caused by NADP⁺ binding is not proportional to fractional saturation of the binding sites. 4. Changes in protein fluorescence caused by changes in ionic strength and by the binding of substrates, Mg²⁺ or NADP⁺ (but not NADPH) are relatively slow, suggesting conformation changes. 5. In the presence of Mg²⁺, the enzyme binds isocitrate very strongly, and 2-oxoglutarate rather weakly. 6. Evidence is presented for the formation of an abortive complex of enzyme–Mg²⁺–isocitrate–NADPH in which isocitrate and NADPH are bound much more weakly than in their complexes with enzyme and Mg²⁺ alone. 7. The results are discussed in relation to the interpretation of the kinetic properties of the enzyme and its behaviour in the mitochondrion.

Many aspects of both the kinetic mechanism and the metabolic roles of NADP⁺-linked isocitrate dehydrogenase [*threo*-D₃-isocitrate–NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.42] are uncertain, in spite of much work on the enzyme from several tissues. Studies of the equilibrium binding of coenzymes and substrates to isocitrate dehydrogenase from bovine heart mitochondria are described here. The enzyme was recently obtained in apparently homogeneous form from this tissue, and shown to be dimeric with mol.wt. 90000 (MacFarlane *et al.*, 1977a), in contrast with the enzyme from pig heart, reported to consist of a single polypeptide chain with mol.wt. 58000 (Colman, 1972a). The affinities and stoichiometries reported here have intrinsic interest and are also needed for the mechanistic interpretation of both steady-state and transient kinetic studies, some of which are described in the following paper (Dalziel *et al.*, 1978). The data are also important in relation to the forms of the enzyme present in the tissue and its metabolic functions and control in the mitochondrion, as will be discussed.

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A preliminary account of some of this work has been published (MacFarlane *et al.*, 1977b).

Experimental

Isocitrate dehydrogenase

The purification of the enzyme from bovine heart mitochondria, and determinations of the protein concentrations and specific activities of enzyme solutions, were carried out as described by MacFarlane *et al.* (1977a). Before each experiment enzyme samples were dialysed for 3–4 h at 3°C against the required buffer; for some experiments the enzyme was first dialysed overnight against 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA, in which it is stable. Except where otherwise stated, 0.1 M-EDTA was present in all the buffer solutions used. All enzyme concentrations are expressed as the molar concentrations of active enzyme. In general, enzyme concentrations were calculated from the measured specific activity and the specific activity (39 μmol of NADPH formed/min per mg) and molecular weight (90000) of the pure enzyme (MacFarlane *et al.*, 1977a). For fluorescence titrations with NADPH for the estimation of dissociation constants, the enzyme concentrations were determined by stoichiometric

titrations with high concentrations of enzyme, as described below.

Coenzymes, substrates and salts

NADP⁺ and NADPH were from Boehringer Corp. (London) Ltd. (Lewes, Sussex BN7 1LG, U.K.). DL-Isocitrate (trisodium salt) and *threo*-D₅-isocitrate (monopotassium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concentrations of NADP⁺, NADPH and *threo*-D₅-isocitrate were determined by enzymic assay (Londesborough & Dalziel, 1968). Solutions of 2-oxoglutaric acid, purchased as the free acid from Boehringer Corp., were neutralized with Tris base. Triethanolamine hydrochloride, obtained from Boehringer Corp., was recrystallized with water for some experiments to remove fluorescent impurities. All other chemicals were of A.R. quality from Fisons Scientific Apparatus (Loughborough, Leics., U.K.).

Fluorescence titrations

Fluorescence measurements were made with a Farrand spectrofluorimeter, Mk I (Farrand Optical Co., New York, NY, U.S.A.) in quartz cells (1 cm × 1 cm) in a cell-holder thermostatically controlled at 25°C. For measurements of NADPH fluorescence, excited at 340 nm and emitted at 415 nm, the slit widths were equivalent to 10 nm for the excitation monochromator and 20 nm for the emission monochromator. For measurements of protein fluorescence, excited at 300 nm and emitted at 340 nm, the entrance slit of the excitation monochromator was decreased to give a bandwidth of 1 nm to minimize photo-destruction of the enzyme. Buffer solutions were filtered (Millipore, 0.22 μm) and enzyme solutions were centrifuged or filtered (Millipore, 5 μm) to minimize light-scattering. Fluorescence titrations were carried out by successive additions of samples (5–25 μl) of ligand solution to the enzyme solution, and, in studies of NADPH binding by coenzyme-fluorescence enhancement, to a second cell containing an equal volume (3 ml) of buffer solution. Calibrated micro-pipettes were used, and the ligand solution was introduced on a glass stirrer.

Calculations of fractional saturation

All measurements were corrected for the fluorescence and light-scattering obtained with buffer solution alone, and for the small dilutions during the titrations. Corrections for changes of absorption of the exciting radiation by the added ligand were also made, when necessary, from titrations with *N*-acetyltryptophan as fluorophore in place of enzyme.

Two methods were used to calculate the fractional saturation of the enzyme-binding sites with

NADPH from measurements of NADPH fluorescence. In Method I, the ratio of the fluorescence intensity of the enzyme solution (F_p) to that of the buffer solution (F) after each addition of NADPH to the two solutions was used to calculate the concentration of enzyme-bound NADPH from:

$$[\text{NADPH}]_{\text{bound}} = \frac{[\text{NADPH}]_{\text{total}}}{Q-1} \left(\frac{F_p}{F} - 1 \right)$$

In this equation, Q is the ratio of the specific fluorescence intensities of bound and free NADPH, and was estimated from a separate titration of a high concentration of enzyme with NADPH or of NADPH with enzyme (Melzi-D'Eril & Dalziel, 1973).

In Method II, the difference between the fluorescence intensities of the enzyme and buffer solutions, $\Delta F = F_p - F$, after each addition of NADPH was used, by equating the fractional saturation of the enzyme binding sites to $\Delta F / \Delta F_{\text{max}}$, where ΔF_{max} is the limiting value of ΔF approached at high concentrations of NADPH (Stinson & Holbrook, 1973). This method was also used for studies of ligand binding by protein fluorescence measurements; F is then the fluorescence intensity of the enzyme solution before addition of ligand.

For NADPH-fluorescence titrations, Method I was used when the binding was relatively weak and high concentrations of NADPH were needed, because it is less dependent on corrections for light-absorption by the ligand than is Method II. The latter was used for titrations of low enzyme concentrations, because it is less affected by errors in the measurement of small values of F , the fluorescence of NADPH in the absence of enzyme.

Calculations of dissociation constants

For the binding of a single ligand at independent and identical sites, the fractional saturation of the total sites, α , is related to the free ligand concentration $[L]$ and the dissociation constant K_{EL} by the mass-action relationship:

$$K_{\text{EL}} = \left(\frac{1-\alpha}{\alpha} \right) [L] \quad (1)$$

or

$$\alpha = 1 - K_{\text{EL}} \frac{\alpha}{[L]} \quad (2)$$

K_{EL} was evaluated from eqn. (2) by plotting α against $\alpha/[L]$ (Scatchard *et al.*, 1957).

The binding of NADPH to the enzyme has also been studied by coenzyme-fluorescence titrations in the presence of fixed concentrations of NADP⁺ and of isocitrate in order to estimate the affinities of the enzyme for these ligands. If the concentration of such an additional ligand, M , is much larger than the enzyme concentration, so that its total and

free concentrations can be equated, the dissociation constant K_{EM} for its compound with the enzyme can be calculated from the apparent dissociation constant ($K_{app.}$) for NADPH so obtained. Two cases may be distinguished.

(a) If the second ligand M competes with NADPH (L) for the same site on the enzyme, then as shown by Stinson & Holbrook (1973):

$$K_{EL} \left(1 + \frac{[M]}{K_{EM}} \right) = \left(\frac{1-\alpha}{\alpha} \right) [L]$$

and comparison with eqn. (1) shows that, if K_{EL} is known, K_{EM} can be calculated from:

$$K_{app.} = K_{EL} \left(1 + \frac{[M]}{K_{EM}} \right) \quad (3)$$

(b) If M combines at a different site from L, so that it can form a ternary complex EML, as well as a binary complex EM, it can be shown that at equilibrium:

$$\frac{K_{EL} \left(1 + \frac{[M]}{K_{EM}} \right)}{\left(1 + \frac{K_{EL}[M]}{K_{EM}K_{EML}} \right)} = \left(\frac{1-\alpha}{\alpha} \right) [L] = K_{app.} \quad (4)$$

Calculation of K_{EM} from $K_{app.}$ requires that K_{EML} , the dissociation constant for L from the ternary complex EML, be known, as well as K_{EL} . Inspection of eqn. (4) shows that K_{EML} can be obtained as the limiting value of $K_{app.}$ approached with sufficiently high concentrations of M, such that $K_{EM} \ll [M]$ and $K_{EM}K_{EML}/K_{EL} = K_{ELM} \ll [M]$. This analysis is based on that applied by Reynolds *et al.* (1970) to the kinetics of inactivation of liver alcohol dehydrogenase by iodoacetate.

Ultrafiltration

The stoichiometry of NADP⁺ binding to the enzyme was measured by ultrafiltration as described by Paulus (1966), with the use of Diaflo PM10 membranes (Amicon Corp., Lexington, MA, U.S.A.). Analyses for NADP⁺ were made enzymically (Londesborough & Dalziel, 1968). Control experiments without enzyme showed that there was no significant retention of free NADP⁺ by the membrane.

Ultracentrifugation

Sedimentation-equilibrium measurements were made by the meniscus-depletion method (Yphantis, 1964) in a Spinco model E ultracentrifuge equipped with mechanical speed control and Rayleigh interference optics. A 12mm six-channel carbon-filled Epon centrepiece was used with sapphire windows. Rotor AN-D was operated at 29500 rev./min for 22h at 21.7°C. The protein concentration was 0.34mg/ml.

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Results and Discussion

Studies of coenzyme binding by enhanced NADPH fluorescence

Studies of the effect of adding isocitrate dehydrogenase on the fluorescence emission spectrum of NADPH, excited at 340nm, showed that binding to the enzyme shifts the emission maximum from 455nm to 428nm, and increases the quantum yield 10-fold at 428nm. At 415nm, the intensity of emission is increased 15-fold, and this wavelength was chosen for quantitative studies of the binding by fluorescence titrations.

The stoichiometry of binding was determined by titration of 113nm-enzyme in 0.05M-triethanolamine chloride buffer, pH7.0, with NADPH (Fig. 1). The end point of this titration, at 210nm-NADPH, corresponds to a binding capacity of 1.9mol of NADPH/mol of enzyme. The ratio of the initial slope of the titration curve in the presence of enzyme to that of the control titration without enzyme (16.9) is the fluorescence enhancement factor, Q. A number of such stoichiometric titrations with high concentrations of enzyme were made in phosphate and triethanolamine chloride buffers, with and without the addition of NaCl and MgCl₂ (Table 1), and all gave similar results. The average and ranges of values from all these measurements were 2.1 ± 0.3 mol of NADPH/mol of enzyme and $Q = 16.5 \pm 1.5$. These results confirm and extend those of MacFarlane *et al.* (1977a).

The large fluorescence enhancement was used to study the binding of NADPH quantitatively by titrations with smaller enzyme concentrations. The results of two titrations in 0.05M-triethanolamine chloride buffer, pH7.0, containing 0.1M-NaCl, with and without the addition of 5mM-MgCl₂, are shown

Fig. 1. Fluorescence titration of isocitrate dehydrogenase with NADPH

The increases in fluorescence, excited at 340nm and emitted at 415nm, accompanying the addition of NADPH to 113nm-enzyme in 0.05M-triethanolamine chloride buffer, pH7.0 (○), and to buffer alone (●) are shown.

in Fig. 2, plotted as described by Scatchard *et al.* (1957). They show that the affinity of the enzyme for NADPH is decreased by Mg^{2+} , and that in the presence of this activator the results are consistent with coenzyme binding at identical and independent sites. In the absence of activator, the binding is too tight to allow measurements below 70% saturation.

Estimates of the dissociation constant from these and other titrations in various buffer/salt media are given in Table 1. The uncertainty of values of 40 nM or more is estimated as $\pm 30\%$, and that of smaller

Fig. 2. Scatchard *et al.* (1957) plots of the equilibrium binding of NADPH to isocitrate dehydrogenase

The fractional saturation (α) was determined from coenzyme-fluorescence titrations. The conditions were 25 nM-enzyme in 0.05 M-triethanolamine chloride/0.1 M-NaCl buffer, pH 7.0 (○); the effect of the presence of 5 mM- $MgCl_2$ is also shown (●).

values as $\pm 100\%$. In none of the experiments did the lines plotted according to eqn. (2) show significant curvature, but it must be emphasized that only in the presence of $MgCl_2$, or with ionic strengths greater than 0.2, was the binding sufficiently weak to permit measurements at substantially less than 50% saturation. In spite of these limitations, the results show clearly that the binding of NADPH is weakened specifically by phosphate, and by Mg^{2+} (except in 0.1 M-phosphate buffer), as well as by increase in ionic strength. The great effect of ionic strength, noted by Londesborough (1969), may be attributed to changes of the activity coefficient of the multivalent ion NADPH (Londesborough & Dalziel, 1968), whereas phosphate presumably competes for the coenzyme-binding site. The effect of the metal activator is open to several possible interpretations, because it is known to chelate the coenzymes (Colman, 1972b; Apps, 1973) and to combine with the enzyme from bovine heart (P. W. Kuchel & K. Dalziel, unpublished work) and from pig heart (Villafranca & Colman, 1972).

The affinity of the enzyme for $NADP^+$ was estimated indirectly from the apparent increase of the dissociation constant for NADPH obtained from titrations in the presence of $NADP^+$, by using eqn. (3). The results (Table 1) show that the dissociation constant for $NADP^+$ is greater, by two orders of magnitude, than that for NADPH, under all the conditions studied, and is similarly affected by phosphate, Mg^{2+} and increase in ionic strength. The dissociation constant for $NADP^+$ was also estimated by protein-fluorescence titrations, as will be described below. The values obtained, shown in

Table 1. Effects of ionic strength, phosphate and Mg^{2+} on the dissociation constants of coenzyme compounds of isocitrate dehydrogenase at pH 7.0 and 25°C

The values were estimated from titrations of the enzyme with NADPH and measurements of NADPH fluorescence. NaCl was added to vary the total ionic strength as indicated. The numbers in parentheses are estimates of K_D for $NADP^+$ obtained from protein-fluorescence titrations.

Buffer	Additions	<i>I</i>	K_D (μM)	
			NADPH	NADP
0.05 M-Triethanolamine chloride/0.1 mM-EDTA	None	0.05	~ 0.001	~ 0.4 (0.2–1.0)
	NaCl	0.125	0.008	—
	NaCl	0.15	0.015	4 (3)
	NaCl	0.25	0.04	7 (4)
	NaCl	1.04	> 10	—
	5 mM- $MgCl_2$	0.065	0.01	3 (0.6)
	NaCl, 5 mM- $MgCl_2$	0.165	0.075	17 (9)
20 mM-Sodium phosphate (pH 7.2)/1 mM-EDTA	NaCl, 5 mM- $MgCl_2$	0.265	0.18	25 (36)
	None	0.05	~ 0.002	—
	2.5 mM- $MgCl_2$	0.055	0.015	—
26 mM-Sodium phosphate/0.1 mM-EDTA	NaCl	0.15	0.02	6 (9)
	NaCl, 5 mM- $MgCl_2$	0.165	0.06	7
0.1 M-Sodium phosphate/1 mM-EDTA	None	0.222	0.20	17
	2.5 mM- $MgCl_2$	0.23	0.26	13

parentheses in Table 1, provide general support for the validity of the indirect estimates.

Langan (1960) found that in the presence of Mn^{2+} , isocitrate decreased the fluorescence of the NADPH compound of isocitrate dehydrogenase from pig heart, and concluded that isocitrate, like $NADP^+$, displaces NADPH from the enzyme. The same conclusion was reached by Ehrlich & Colman (1975). Qualitatively similar observations were made by Londesborough (1969) with partially purified enzyme from bovine heart, but he also showed that with increasing concentrations of isocitrate and Mg^{2+} the NADPH-fluorescence intensity became constant at a value significantly greater than that expected for free NADPH. He concluded that isocitrate and Mg^{2+} form a complex with enzyme and NADPH, and we confirmed his findings. The enzyme was titrated with NADPH in the presence of 5 mM- $MgCl_2$ and several concentrations of DL-isocitrate, and the apparent dissociation constant for NADPH ($K_{app.}$) was calculated. The results (Table 2) show that $K_{app.}$ increases with increase in the isocitrate concentration. The apparent dissociation constant for *threo*-D₅-isocitrate from its complex with the enzyme (in the presence of Mg^{2+}) was calculated from $K_{app.}$ and the true dissociation constant for NADPH (Table 1) in two ways. First, eqn. (3) was used, assuming that isocitrate binds at the same site as NADPH and displaces it from the enzyme. Secondly, eqn. (4) was used, on the assumptions that an abortive enzyme-NADPH- Mg^{2+} -isocitrate complex is formed, and that the value of 20 μM for $K_{app.}$ obtained in the presence of the highest concentration of isocitrate used is the dissociation constant for NADPH from this complex. Because this dissociation constant (20 μM) is so much larger than that for the binary

complex of NADPH with the enzyme (75 nm), the difference between the two hypotheses is one of degree, and similar estimates for the dissociation constant of the complex of enzyme and isocitrate are therefore obtained by eqn. (3) and eqn. (4) from titration data with the two smallest isocitrate concentrations (Table 2). It is therefore clear that, in the presence of 5 mM- $MgCl_2$ and absence of NADPH, the enzyme binds *threo*-D₅-isocitrate very firmly, with a dissociation constant of about 14 nm. The larger value of 110 nm, obtained by applying eqn. (3) to the titration data with the highest isocitrate concentration, indicates that isocitrate does not completely displace NADPH from the enzyme. This was confirmed by the further addition of 270 μM -DL-isocitrate at the end of this titration; the fluorescence intensity was unchanged. The calculations using eqn. (4) involve the assumption that the fluorescence-enhancement factor for NADPH is the same in the abortive complex as in its binary complex, and this is difficult to establish quantitatively. Supporting evidence is given by the Scatchard *et al.* (1957) plot (Fig. 3) of data calculated with this assumption from a titration in the presence of 67 μM -DL-isocitrate, from which the value of 20 μM for $K_{app.}$ in Table 2 was obtained. The admittedly long extrapolation is consistent with an unchanged fluorescence enhancement in the abortive complex if it is formed at both subunits. These results therefore indicate the formation of an abortive complex with a dissociation constant for

Table 2. *Effects of isocitrate and MgCl₂ on the affinity of isocitrate dehydrogenase for NADPH*

The apparent dissociation constant for NADPH ($K_{app.}$) was estimated by coenzyme-fluorescence titrations of the enzyme in 0.05 M-triethanolamine chloride buffer, pH 7.0, containing 0.1 M-NaCl, 5 mM- $MgCl_2$ and the stated concentrations of DL-isocitrate. The dissociation constant for *threo*-D₅-isocitrate from its compound with the enzyme (K_D) was calculated in two ways: (1) by assuming that isocitrate displaces NADPH, from eqn. (3) in the text, and (2) by assuming that a complex of enzyme-NADPH- Mg^{2+} -isocitrate is formed, from eqn. (4) in the text.

Enzyme (nm)	DL-Isocitrate (μM)	$K_{app.}$ (μM)	K_D (μM)	
			(1)	(2)
25	0	0.075	—	—
25	0.98	2.5	0.014	0.012
25	4.5	7.1	0.021	0.014
260	67.0	20	0.11	—

Fig. 3. *Equilibrium binding of NADPH in the presence of isocitrate and MgCl₂*
Results are from a fluorescence titration of 0.26 μM -enzyme, 67 μM -isocitrate and 5 mM- $MgCl_2$ with NADPH. Other details are given in the legend to Fig. 2.

NADPH 270 times larger than that of the enzyme-Mg²⁺-NADPH complex. The dissociation constant for isocitrate must also be increased in the abortive complex by this factor, and is therefore 4 μ M. If the enzyme binds the Mg²⁺ chelate of isocitrate, rather than free isocitrate (Londesborough & Dalziel, 1970; Colman, 1972a), its dissociation constant will be half that calculated for free isocitrate, namely 2 μ M for the abortive complex and 7 nM for the binary complex, calculated from the stability constant of 195 M⁻¹ for the chelate under the conditions of these experiments (Londesborough & Dalziel, 1971).

These conclusions differ from those reached by Ehrlich & Colman (1975) from similar experiments with the enzyme from pig heart, not only with regard to whether isocitrate completely displaces NADPH or forms an abortive complex, but also, more seriously, with regard to the dissociation constant for isocitrate from the complex of enzyme, bivalent metal ion and isocitrate, for which these workers obtained the much greater value of 1.3 μ M. This may be due to the higher pH of 7.4, the larger ionic strength or the use of 2 mM-MnSO₄ in their experiments. Unlike Mg²⁺, Mn²⁺ in concentrations greater than 0.2 mM inhibits the oxidative decarboxylation of isocitrate competitively (Londesborough & Dalziel, 1970), and might therefore compete with isocitrate or its metal complex in equilibrium-binding experiments.

The estimated affinities of the bovine heart enzyme for its coenzymes and isocitrate have implications for the kinetic properties of the enzyme *in vitro* and *in vivo*. Since the affinity for NADPH is about 100-fold greater than that for NADP⁺ under a variety of conditions, which probably embrace the ionic strength and phosphate and Mg²⁺ concentrations in the mitochondrion (Table 1), severe product inhibition of the oxidative decarboxylation reaction by NADPH might be expected. Although there is disagreement about the relative concentrations of free NADP⁺ and NADPH in the mitochondrion, all the available data (Krebs & Veech, 1969; Sies *et al.*, 1977) indicate that competition between NADP⁺ and NADPH for the formation of a binary enzyme-coenzyme complex *in vivo* would also be in favour of the reduced coenzyme. The findings that the enzyme binds isocitrate very firmly in the presence of Mg²⁺, and also forms an abortive enzyme-Mg²⁺-NADPH-isocitrate complex, must be taken into account, however. The dissociation constant for isocitrate from the latter complex (4 μ M) is smaller than the *K_m* value for isocitrate under comparable conditions (Londesborough & Dalziel, 1970), and this complex will be formed, therefore, in initial-rate experiments on the oxidative decarboxylation reaction. The relatively large dissociation constant for NADPH of 20 μ M from this complex accounts for the absence of severe product inhibition

by NADPH, and it is likely that the dissociation of NADPH from this complex is the rate-limiting step in the oxidative decarboxylation reaction under maximum-rate conditions (Dalziel *et al.*, 1978).

From the dissociation constant of 4 μ M for isocitrate from the abortive complex and the available estimates of the cellular concentration of isocitrate, it is likely that this complex will also be found in the mitochondrion. In rat heart, the cellular concentration of isocitrate is about 50 μ M (Bowman, 1966; Kraup *et al.*, 1967; Neely *et al.*, 1972). The distribution of isocitrate between mitochondrion and cytoplasm in the heart cell is not known, but in rat liver reported values for isocitrate in the whole cell are in the range 20–50 μ M (Veech *et al.*, 1969; Sies *et al.*, 1977), and the concentration in the mitochondrion (142 μ M) is much larger than that in the cytoplasm (Sies *et al.*, 1977). Thus the concentration of isocitrate appears to be large enough to influence the rate of oxidative decarboxylation not only as the substrate, but also by facilitating the release of the product NADPH through the formation of the abortive complex.

Formation of the abortive complex requires the presence of Mg²⁺ or Mn²⁺. The total concentration of Mg²⁺ in rat heart and other tissues is about 10 mM (Watchorn & McCance, 1937; Widdowson & Dickerson, 1964; Wacker & Vallee, 1964), but the concentration of free Mg²⁺ is unknown. The amount available for complex-formation with isocitrate may be small, as was suggested by England *et al.* (1967) from the observed citrate/isocitrate concentration in rat heart, the effect of Mg²⁺ on the aconitase equilibrium, and the greater affinity of Mg²⁺ for citrate (*K_s* = 2000 M⁻¹; Sillen & Martell, 1964) than for isocitrate (*K_s* = 195 M⁻¹; Londesborough & Dalziel, 1971). The concentration of free Mg²⁺ in rat liver, brain and kidney cortex has been estimated by Veloso *et al.* (1973) to be about 1 mM, from which it can be calculated that about 20% of the isocitrate will be present as the Mg²⁺ complex. The NADP⁺-dependent isocitrate dehydrogenase appears to play a significant role in the oxidative decarboxylation of isocitrate in the mitochondrion, and its activity under assay conditions, with the optimum concentrations of substrates and activating metal ion, is greater in rat liver and heart than that of the NAD⁺-dependent enzyme (Stein *et al.*, 1967; Kaplan *et al.*, 1956; Sies *et al.*, 1977). Moreover, the *K_m* value for isocitrate, and especially for its Mg²⁺ complex, is smaller for the NADP⁺-linked enzyme than for the NAD⁺-linked enzyme (Londesborough & Dalziel, 1970; Colman, 1975). Need for control of the activity of the former enzyme, which would otherwise nullify the effects of allosteric control of the latter, has therefore been envisaged (Stein *et al.*, 1967). The availability of Mg²⁺ for chelation by isocitrate might provide such control, and would be affected by the ATP/ADP concentration ratio (Londesborough &

Dalziel, 1970). There is disagreement, however, as to whether the NADP⁺-linked isocitrate dehydrogenase reaction in the mitochondrion is close to equilibrium (Sies *et al.*, 1977) in which case such control would be ineffective, or is essentially irreversible (Veech *et al.*, 1969; Krebs & Veech, 1969).

Effects of ligands on the fluorescence of the enzyme

The intensity of fluorescence of the enzyme protein, excited at 300nm and emitted at 350nm, varied with the ionic strength of the medium. After a sample of an enzyme solution in 0.05M-triethanolamine chloride buffer containing 0.1M-NaCl (pH 7.0, *I* 0.15) had been diluted with 0.05M-triethanolamine chloride buffer (*I* 0.05), the fluorescence intensity increased slowly. The constant value reached after about 1 min was 30% greater than that obtained at once when the enzyme solution was diluted with the original buffer of *I* 0.15. The effect was reversible: dilution of an enzyme solution in buffer of *I* 0.05 with buffer of *I* 0.15 was followed by a slow decrease in the fluorescence.

The effects of coenzymes and substrates on the protein fluorescence also depended on the ionic strength of the medium, and were most marked at low ionic strength. The addition of isocitrate or 2-oxoglutarate to the enzyme in 0.05M-triethanolamine chloride buffer, pH 7.0, containing 5mM-MgCl₂ quenched the protein fluorescence by 30%, and like the quenching caused by an increase in salt concentration, the change was relatively slow, with a half-time of 10–20s. The addition of NADPH caused a more rapid fluorescence quenching of similar magnitude, whereas NADP⁺ caused a 30% increase in the protein fluorescence, again developing with a half-time of 10–20s.

The slowness of these fluorescence changes suggests that conformational changes in the protein molecule, or even dissociation of the dimer into subunits, are involved. Evidence for such dissociation of isocitrate dehydrogenase from ox liver in dilute solution, and its prevention by Mn²⁺ and isocitrate, has been reported (Carlier & Pantaloni, 1973).

Accordingly, three sedimentation-equilibrium experiments were carried out with the bovine heart enzyme at the same concentration as that used in the protein-fluorescence measurements just described, namely 0.34mg/ml, in 0.05M-triethanolamine chloride buffer, pH 7.0. With or without the addition of 50mM-NADP⁺ or 0.1mM-DL-isocitrate and 5mM-MgCl₂, we obtained no evidence of dissociation. The apparent mol.wt. was 93000–96000 in all these experiments, in reasonably good agreement with earlier estimates (MacFarlane *et al.*, 1977a). We conclude that a slow structural transition of the dimeric enzyme molecule follows changes of ionic

strength or ligand combination. Evidence for a slow structural change that affects the activity of the enzyme, induced by combination of isocitrate and Mg²⁺, has been obtained from kinetic studies in the pre-steady state (Dalziel *et al.*, 1978).

Attempts to study the equilibrium binding of coenzymes and substrates to the enzyme by measurements of these protein-fluorescence changes were made, even though their small amplitude and slowness obviously limits the precision of the measurements. Approximate estimates of the dissociation constants for NADP⁺ in various buffer media obtained in this way (see Table 1) are of the same order as those obtained by competition with NADPH. Attempts to determine the stoichiometry of NADP⁺ binding by protein-fluorescence titrations with high concentrations of enzyme gave surprising results, however. The results of a titration of 6.0μM-enzyme with NADP⁺ in 0.05M-triethanolamine chloride buffer, pH 7.0, are shown in Fig. 4(a). A satisfactory estimate of the maximum fluorescence increment ($\Delta F_{\max.} = 3.18$) is obtained. After the addition of 1 equivalent of NADP⁺ the increment is 0.70 $\Delta F_{\max.}$. A value greater than 0.5 $\Delta F_{\max.}$ is obviously inconsistent with a binding capacity of 2mol of NADP⁺/mol of enzyme, if the assumption that the fluorescence increment is proportional to the fractional saturation of the ligand-binding site is valid. Extrapolation of the initial slope and final asymptote of the titration curve suggests a stoichiometry of 1mol of NADP⁺/mol of enzyme. An analogous titration of 0.105μM-enzyme with NADPH, which quenches the protein fluorescence and binds much more firmly than NADP⁺ (Table 1), is shown in Fig. 4(b). The end-point is at 0.20μM-NADPH, and confirms the conclusion from measurements of coenzyme-fluorescence enhancement (Fig. 2) that the enzyme molecule has two binding sites for NADPH.

For isocitrate dehydrogenase from ox liver, Carlier & Pantaloni (1976) have also presented evidence that there are two binding sites with similar affinities for NADPH in the molecule, but only one for NADP⁺. For the protein-fluorescence titrations with NADP⁺ described here, however, there is an alternative interpretation, namely that there are two binding sites in the molecule for NADP⁺, but binding of a molecule of NADP⁺ to one subunit of the enzyme causes a change in the protein fluorescence of both subunits, whereas combination of the second NADP⁺ molecule causes a much smaller change in protein fluorescence. The fluorescence increment would not then be proportional to the fractional saturation of the ligand-binding sites. This hypothesis has been substantiated in two ways. First, titration curves such as that in Fig. 4(a) and Scatchard *et al.* (1957) plots obtained from them, cannot be described satisfactorily on the assumption that there is one binding site in the molecule with a

Fig. 4. Protein-fluorescence titrations of enzyme with coenzymes

The medium was 0.05 M-triethanolamine chloride buffer, pH 7.0. The fluorescence was excited at 300 nm, and measured at 340 nm. ΔF is the observed change in protein-fluorescence intensity resulting from addition of coenzyme. For (a), 6.0 μM -enzyme was titrated with NADP⁺, and the apparent equivalence point obtained by extrapolation of the initial slope and final asymptote is at 6 μM -NADP⁺. For (b), 0.105 μM -enzyme was titrated with NADPH, and the equivalence point is at 0.20 μM -NADPH.

single dissociation constant. The Scatchard plots showed small but reproducible deviations from linearity of the kind associated with positively co-operative binding, and apparent dissociation constants obtained from the slopes of such plots in the range 25–75% saturation varied significantly with the enzyme concentration used. For example, from titrations of 1.5, 2.5 and 6 μM -enzyme in 0.05 M-triethanolamine chloride buffer, the apparent dissociation constants were 0.2, 0.3 and 1.0 μM respec-

Table 3. Measurements of the binding capacity of isocitrate dehydrogenase for NADP⁺ by ultrafiltration

Solutions of enzyme and NADP⁺ in 0.05 M-triethanolamine chloride buffer, pH 7.0, were subjected to ultrafiltration at room temperature. Samples of the filtrate were analysed for NADP⁺ enzymically. The enzyme concentration was 7.3 μM in all the experiments.

Concn. of NADP ⁺ (μM)		NADP ⁺ bound (mol/mol of enzyme)
Initial	Filtrate	
15.7	3.7	1.7
15.7	4.0	1.6
23.6	7.4	2.2
23.6	10.9	1.7
31.5	13.5	2.5
31.5	16.3	2.1
13.1*	12.9*	—
13.1*	13.1*	—

* Control, without enzyme.

tively. It was shown theoretically that the alternative model suggested, with two binding sites with the same dissociation constant of 0.5 μM in the molecule and by assuming that only binding at the first site causes fluorescence quenching, accounted for these discrepancies.

Secondly, and more convincingly, the maximum binding capacity for NADP⁺, under the conditions of the protein-fluorescence titration in Fig. 4(a), was determined directly by ultrafiltration (Paulus, 1966). The results obtained with 7.3 μM -enzyme and NADP⁺ concentrations of 15.7, 23.6 and 31.5 μM (Table 3) show that the enzyme does bind 2 mol of NADP⁺/mol at the end of the titration in Fig. 4(a). It is concluded that the protein-fluorescence measurements indicate that a concerted structural transition of the dimeric enzyme molecule occurs when one molecule of NADP⁺ combines.

Protein-fluorescence-quenching titrations of the enzyme with isocitrate in the presence of MgCl₂ in 0.05 M-triethanolamine chloride buffer confirmed the tight binding already indicated indirectly by titrations with NADPH (Table 2). The affinity was too large to allow estimates of the dissociation constant. The titrations gave results similar to those obtained with NADP⁺, suggesting again either binding of isocitrate at only one subunit, or that fluorescence quenching was not proportional to fractional saturation. Direct determination of the binding stoichiometry has not been made, however.

Titration of the enzyme with 2-oxoglutarate by protein fluorescence in the same medium indicated that the affinity of the enzyme for this substrate is much smaller than for isocitrate. The results of a titration of 0.12 μM -enzyme are plotted by the method of Scatchard *et al.* (1957) in Fig. 5(a) and as described

Fig. 5. Equilibrium binding of 2-oxoglutarate to isocitrate dehydrogenase

The fractional saturation (α) was calculated from protein-fluorescence titrations and plotted (a) by the method of Scatchard *et al.* (1957) and (b) by the method of Brown & Hill (1922–1923). The enzyme concentration was $0.12 \mu\text{M}$, in 0.05 M -triethanolamine chloride buffer, pH 7.0, containing 5 mM - MgCl_2 .

by Brown & Hill (1922–23) in Fig. 5(b). The results are not consistent with the binding of 2-oxoglutarate, or its Mg^{2+} -ion complex, at two identical and independent sites in the enzyme molecule, nor, of course, at a single site. The slope of the linear plot in Fig. 5(b) is 1.8. The simplest interpretation of these results is positively co-operative binding of two substrate molecules. In view of the results obtained in studies of NADP^+ binding by protein-fluorescence enhancement, however, the possibility of optical, rather than thermodynamic, interactions between the two subunits of the enzyme cannot be ignored. Thus the results might be accounted for by the assumption that binding of a second substrate molecule to the dimeric enzyme causes greater fluorescence quenching than binding of the first substrate molecule. In either

event, interactions between the two subunits are indicated. An apparent dissociation constant for 2-oxoglutarate, defined as the concentration of the latter that causes half the maximum fluorescence quenching, is 1.5 mM (Fig. 5b). The maximal binding capacity of the enzyme for 2-oxoglutarate has not been determined.

Similar titrations of the enzyme with 2-oxoglutarate in the presence of $75 \mu\text{M}$ - NADP^+ as well as 5 mM - MgCl_2 gave a value of 0.22 mM for the apparent dissociation constant of the abortive complex for 2-oxoglutarate. Scatchard *et al.* (1957) plots showed small deviations from linearity, of the kind associated with positive co-operativity, but these were not clearly outside the experimental error. In the absence of MgCl_2 , no significant quenching of the protein fluorescence by 2-oxoglutarate was observed, with or without NADP^+ present.

These results show that 2-oxoglutarate is much less firmly bound by the enzyme than is isocitrate, in the presence of MgCl_2 . Unfortunately the binding of isocitrate is too firm to permit evaluation of the saturation function, to show whether or not there are binding interactions, whereas the binding of 2-oxoglutarate is too weak to allow estimation of the stoichiometry of binding by protein-fluorescence titrations. The results obtained with NADP^+ show that the method is in any case unreliable for this purpose. The interpretation of the titration with 2-oxoglutarate is also complicated by the large concentrations used and the relatively small stability constant for its Mg^{2+} chelate, which is about 60 M^{-1} (Londesborough & Dalziel, 1968). The titrations with NADP^+ , however, in contrast with those with NADPH , show that there are interactions between the two subunits of the enzyme. Further investigations, particularly kinetic studies, are needed to show whether these findings have significance for the reaction mechanism.

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