The binding of Ca^{2+} ions to pig heart NAD^+ -isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex

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1. The binding of Ca^{2+} ions to purified pig heart NAD^+ -isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, freed of contaminating Ca^{2+} by parvalbumin/polyacrylamide chromatography, has been studied by flow dialysis and by the use of fura-2. 2. For the 2-oxoglutarate dehydrogenase complex, 3.5 mol of Ca^{2+} -binding sites/mol of complex were apparent, with an apparent dissociation constant (K_d value) for Ca^{2+} of $2.0 \mu\text{M}$. These values were little affected by Mg^{2+} ions, ADP or 2-oxoglutarate. 3. By contrast, binding of Ca^{2+} to NAD^+ -isocitrate dehydrogenase ($K_d = 14 \mu\text{M}$) required ADP, isocitrate and Mg^{2+} ions. The number of Ca^{2+} -binding sites associated with NAD^+ -isocitrate dehydrogenase was then 0.9 mol/mol of tetrameric enzyme. 4. The 2-oxoglutarate dehydrogenase complex bound ADP (as ADP^{3-}) to a group of tight-binding sites ($K_d = 3.1 \mu\text{M}$) with a stoichiometry, 3.3 mol/mol of complex, similar to that for the binding of Ca^{2+} ; a variable number of much weaker sites ($K_d = 100 \mu\text{M}$) for ADP^{3-} was also apparent.

INTRODUCTION

Although the regulation by Ca^{2+} ions of three mitochondrial enzymes, NAD^+ -isocitrate dehydrogenase (NAD-ICDH) and the 2-oxoglutarate dehydrogenase (OGDH) and pyruvate dehydrogenase (PDH) complexes, has been recognized for some time (see Denton & McCormack, 1980, 1985; Hansford, 1985), the molecular nature of the interaction between these enzymes and Ca^{2+} remains largely obscure. Indeed, only for the PDH complex has an attempt been made to study the binding of Ca^{2+} to the enzyme (Teague *et al.*, 1982).

Both NAD-ICDH and OGDH are multi-subunit enzymes. The OGDH complex probably consists of 12 decarboxylase (E1), 24 succinyltransferase (E2) and 12 lipoamide dehydrogenase (E3) subunits (Koike & Koike, 1976). NAD-ICDH also possesses three types of subunit (Ramachandran & Colman, 1980; Rutter & Denton, 1989), of poorly defined function (Ehrlich & Colman, 1983, 1984) and stoichiometry (see Rutter & Denton, 1989). However, for neither enzyme is it clear which subunits are involved in binding Ca^{2+} or whether any common Ca^{2+} -binding domain exists.

The recent observations (Rutter & Denton, 1988) indicating that the sensitivity to Ca^{2+} ions of NAD-ICDH may be considerably lower than that of both OGDH and the PDH complex, and that Mg^{2+} ions have markedly different effects on the Ca^{2+} -sensitivity of each of these enzymes (see Rutter & Denton, 1989), have further emphasized the need to study the binding of Ca^{2+} to the purified enzymes directly.

In the present paper we have used the Ca^{2+} indicator fura-2 (Gryniewicz *et al.*, 1985) and flow dialysis (Colowick & Womack, 1969; Haiech *et al.*, 1981) to compare the Ca^{2+} -binding properties of purified OGDH with those of purified NAD-ICDH and calmodulin (Klee & Vanaman, 1982). The stoichiometry of binding of the activator ADP to OGDH was also examined and compared with that of Ca^{2+} .

EXPERIMENTAL

Materials

Aristar KCl, KOH and KH_2PO_4 , AnalaR CaCl_2 , MgCl_2 , EDTA and Tris, and Spectrosol LaCl_3 , were obtained from BDH, Poole, Dorset, U.K. $^{45}\text{CaCl}_2$ (15 Ci/g), $[2\text{-}^3\text{H}]\text{ADP}$ (18 Ci/mmol) and 2-oxo[1- ^{14}C]glutarate (59 Ci/mol) were from Amersham International, Amersham, Bucks., U.K. Xylene-based scintillant (Unisolve E) was from Koch-Light, Haverhill, Suffolk, U.K. Fura-2 (pentapotassium salt) was obtained from Molecular Probes, Eugene, OR, U.S.A., and Chelex (100–200 mesh) was from Bio-Rad Laboratories, Watford, Herts., U.K. Chelex was regenerated with HCl and converted into the Tris form before use as described by the manufacturers. Visking Dialysis tubing (14000 Da cut-off) was from Medicell International, London N.1, U.K., and Centricon filters were from Amicon, Stonehouse, Glos., U.K. All solutions used for Ca^{2+} -binding experiments were made up in ultra-pure water (Milli-Q instrument from Millipore Corp.) All other chemicals and biochemicals were obtained from the sources given previously (Rutter & Denton, 1988, 1989).

Purification of proteins

Pig heart NAD-ICDH was purified as described previously (Rutter & Denton, 1989).

Pig heart OGDH was isolated by a modification of the method of McCormack & Denton (1979) which included an additional anion-exchange step. This was necessary to eliminate trace contamination with PDH activity, and to remove an unidentified protein contaminant with subunit molecular mass 40 kDa. A 35%-(NH_4) $_2\text{SO}_4$ fraction of pig heart mitochondrial extracts, obtained as described in the preparation of NAD-ICDH (Rutter & Denton, 1989), was dispersed in 20 mM- KH_2PO_4 , pH 7.2, containing 1 mM-EDTA, 0.1 mM-phenylmethane-

Abbreviations used: NAD-ICDH , NAD^+ -isocitrate dehydrogenase; OGDH , 2-oxoglutarate dehydrogenase; PDH , the pyruvate dehydrogenase complex. $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ refer to the free unbound species of these metal ions.

sulphonyl fluoride, 2 mM-benzamidine and 1 mM-dithiothreitol (buffer A), and dialysed against this buffer overnight at 5 °C. The dialysed pellet was diluted with buffer A to give approx. 20 units of OGDH/ml, adjusted to 10 mM-MgCl₂ (to cause dephosphorylation and activation of PDH) and allowed to warm to 18 °C. The pH of the mixture was lowered to 6.5 with acetic acid, and poly(ethylene glycol) 6000 was added to 0.5% (w/v). After centrifugation (30000 g, 15 min, 18 °C), the supernatant was adjusted to 3% (w/v) poly(ethylene glycol), and re-centrifuged as above. The resultant pellet was resuspended in 10 mM-triethanolamine/HCl (pH 7.5 with KOH)/1 mM-dithiothreitol (5 °C; buffer B) at approx. 50 units/ml, and re-centrifuged (30000 g, 15 min, 5 °C). The supernatant was loaded on to a 10 ml Mono Q column (HR 10/10, Pharmacia) equilibrated in buffer B, and connected to a Pharmacia f.p.l.c. system. A gradient was developed into buffer B plus 1.0 M-KCl up to 420 mM-KCl, and OGDH was then eluted isocratically. Peak fractions were combined and dialysed for 2 h against 50 mM-Mops/K⁺ (pH 7.2)/0.1 mM-phenylmethanesulphonyl fluoride/2 mM-benzamidine/1 mM-dithiothreitol (5 °C), before centrifuging at 290000 g for 2 h, 3 °C. The sedimented OGDH was carefully redissolved at 20–50 mg of protein/ml in 10 mM-Mops/Tris (pH 7.2)/0.1 M-KCl/1 mM-dithiothreitol, plus 1 µg each of pepstatin, antipain and leupeptin/ml, and frozen in small samples at –70 °C.

OGDH prepared in this way had specific activity of 8–10 units/mg (30 °C), and contamination by PDH activity was < 3%. Three bands, of 113, 55 and 48 kDa, corresponding to the E1, E3 and E2 subunits of OGDH (Koike & Koike, 1976), were resolved by SDS/polyacrylamide-gel electrophoresis and represented > 90% of the Coomassie-Blue-stained protein. The intensity of Coomassie Blue staining of these proteins (E1:E3:E2) was in the proportion 1.64:1.0:1.42, which is consistent with subunit proportions of 12 E1:12 E3:24 E2 (Koike & Koike, 1976).

Bovine brain calmodulin was prepared by the method of Gopalakrishna & Anderson (1982) and was dialysed extensively against Chelex-treated 10 mM-Mops/Tris (pH 7.2)/0.1 mM-dithiothreitol before use.

Assay of enzymes and protein

NAD-ICDH activity was assayed as described by Rutter & Denton (1989). OGDH activity was assayed as for NAD-ICDH, except that isocitrate was replaced by 5 mM-2-oxoglutarate, and the buffer was supplemented with 0.1 mM-CoA plus 1 mM-thiamin pyrophosphate. Calmodulin activity was determined by measuring the stimulation of cyclic AMP phosphodiesterase (Klee, 1977).

One unit of activity is defined as that amount which catalyses the conversion of 1 µmol of substrate/min at 30 °C. For NAD-ICDH and OGDH, protein concentration was determined as described by Bradford (1976), with bovine serum albumin as standard. With calmodulin, protein was assayed as described by Lowry *et al.* (1951) (standardized as above), or spectrophotometrically at 277 nm, by assuming $A_{1\text{ mg/ml}} = 0.18$ (Klee, 1977; Olwin & Storm, 1985).

Removal of Ca²⁺ from buffers and protein solutions

Untreated solutions of 10 mM-Mops/Tris (pH 7.2)/0.1 mM-dithiothreitol, with or without 0.1 M-KCl

(Aristar), contained approx. 5 µM-Ca²⁺, measured as described below. This Ca²⁺ concentration could be lowered to ≤ 200 nM by passing the solutions (< 500 ml) over a 60 cm × 1 cm column of freshly regenerated Chelex after equilibrating the column with buffer.

Stock solutions of ADP, isocitrate and 2-oxoglutarate (each 100 mM; 10 ml) were treated to remove Ca²⁺ by passing them over a 4 ml column of Chelex, pre-equilibrated with an equal volume of stock solution.

Dialysis of protein solutions against Chelex-treated buffers was performed at 4 °C. For the dialysis of NAD-ICDH, 1 mM-ADP was always present to stabilize the enzyme.

Buffer and protein solutions were treated with parvalbumin/polyacrylamide (Lehky *et al.*, 1977; Keller *et al.*, 1982) by passage over a 200 mg (2–3 ml bed vol.) column containing 8 mg of immobilized parvalbumin. Protein solutions (< 200 µl) were loaded on the column equilibrated in Chelex-treated 10 mM-Mops/Tris (pH 7.2)/0.1 mM-dithiothreitol, plus Aristar KCl as indicated, and eluted after 30 min. The column was regenerated with 0.1 M-NaEDTA, pH 7.0, and washed through with 100 ml of ultra-pure water before use to ensure the complete removal of EDTA.

Disposable plastic-ware, rinsed extensively in ultra-pure water, was used for handling all Chelex- and parvalbumin/polyacrylamide-treated solutions.

Assay of total [Ca²⁺]

(i) **Atomic absorption.** This was carried out on a Pye-Unicam SP-9 spectrophotometer, with a nitrous oxide/acetylene flame. Protein solutions were supplemented with 0.1% LaCl₃ and 2 mM-EDTA to suppress background absorption.

(ii) **Fura-2.** This was used to assess the Ca²⁺ concentration of buffers by recording the 450–320 nm spectrum of dye (usually between 0.5 and 10.0 µM). This allowed measurements of A_{352} (the isosbestic point of the dye under the conditions used) and A_{380} (decreased on the binding of Ca²⁺ by fura-2) to be corrected for any changes in background absorbance (measured at 440 nm). Spectra at saturating Ca²⁺ and < 1 nM-Ca²⁺ were then taken after the sequential addition of 10 µM-CaCl₂ and 1 mM-EGTA. A Pye-Unicam PU-8800 spectrophotometer was used, and all measurements were made in ultra-pure-water-rinsed disposable plastic cuvettes. Particular care was taken to prevent the re-contamination of solutions with Ca²⁺, through the use of disposable plastic pipette tips and stirrers.

The total [fura-2] was determined from the value of $(A_{352} - A_{440})/\epsilon_1$, and the free [fura-2] (before the addition of CaCl₂ or EGTA) from:

$$[\text{fura-2}]_{\text{free}} = [(A_{380} - A_{440}) - (A_{380} - A_{440})_{\text{Ca}}]/\epsilon_2$$

where $(A_{380} - A_{440})_{\text{Ca}}$ represents this value in the presence of 10 µM-CaCl₂, and ϵ_2 the difference in absorption coefficient at 380 nm between the free and Ca-bound forms of fura-2. Calculated values of ϵ_1 and ϵ_2 were 23.3 mM⁻¹·cm⁻¹ and 19.1 mM⁻¹·cm⁻¹ respectively. Suitable concentrations of fura-2 were chosen such that $[\text{Ca-fura-2}] < 0.5 \times [\text{fura-2}]_{\text{total}}$. Since the binding of fura-2 to Ca²⁺ is tight ($K_d < 50$ nM in the buffers tested; see Fig. 2), under these conditions $[\text{Ca-fura-2}] \approx [\text{Ca}^{2+}]_{\text{total}}$.

Ca²⁺-binding assays

(i) **Fura-2 spectra.** These (or measurements of absorbance at individual wavelengths) were recorded as described above in 0.5 ml of protein solution [in 10 mM-Mops/Tris (pH 7.2)/0.1 M-KCl/0.1 mM-dithiothreitol unless otherwise indicated], with an identical protein solution (minus fura-2) in the reference cell. Concentrations of free and bound dye were determined from the values of A_{352} , A_{380} and A_{440} , as described above. The value of $(A_{380} - A_{440})_{Ca}/(A_{352} - A_{440})$ was measured and found to equal 0.08. The concentration of free Ca²⁺ was calculated from the ratio of bound:free fura-2, after the determination of the K_d for the Ca-fura-2 complex by titration with EGTA-Ca buffers (Rutter & Denton, 1988).

(ii) **Flow dialysis.** This was performed at $25 \pm 1^\circ\text{C}$ essentially as described by Haiech *et al.* (1981), by using the flow cell designed by Feldman (1978) and built by Mr. D. Clements at the School of Medical Sciences Mechanical Workshop, University of Bristol.

The basic buffer composition was 10 mM-Mops/Tris (pH 7.2)/0.1 M-KCl/0.1 mM-dithiothreitol, plus additions as indicated, and experiments were performed with 200 μl of parvalbumin/polyacrylamide-treated protein solutions (or parvalbumin/polyacrylamide-treated buffer as control). The flow cell was perfused at a constant flow rate of 1.6 ml/min with Chelex-treated buffer, by using a Pharmacia P1 peristaltic pump. Each run was initiated with ⁴⁵CaCl₂ (0.1–0.2 μCi), followed at 5 min intervals by additions of increasing concentrations of ⁴⁰CaCl₂ up to a final pulse of 25 mM. Radioactivity in the collected fractions (800 μl) was determined after addition of 10 ml of scintillant.

Possible metabolism of additions made during runs was investigated by preparing neutralized HClO₄ extracts (Davidson & Halestrap, 1987) and then employing enzymic assays: Jaworek *et al.* (1974) for ADP and AMP; Lamprecht & Trautschold (1974) for ATP; and Moellering & Gruber (1966) for citrate. Isocitrate was assayed by using aconitase-free NADP⁺-isocitrate dehydrogenase (Sigma Chemical Co., Poole, Dorset, U.K.). Rates of decarboxylation of 2-oxoglutarate were measured with 2-oxo[1-¹⁴C]glutarate (McCormack, 1985).

Membranes (boiled twice in 1 mM-EDTA/NaHCO₃, pH 7.0, and twice in ultra-pure water) were replaced between runs, and the cell was washed sequentially with ultra-pure water, 0.2% (w/v) Triton X-100, 20% (v/v) ethanol, 1 mM-EGTA (pH 7.6), and then copiously with ultra-pure water.

ADP binding

ADP binding was studied by flow-dialysis in 10 mM-Mops/Tris (pH 7.2)/0.1 M-KCl/0.2 mM-EDTA/0.1 mM-dithiothreitol, essentially as for Ca²⁺ binding. Runs were initiated by adding 2 μCi of [2-³H]ADP, followed by additions of unlabelled ADP, up to a final pulse of 5 mM.

H.p.l.c. analysis of [2-³H]ADP in protein solutions used in binding experiments was performed on a Nucleosil 5SB column (Hopkirk & Denton, 1986) after removal of protein by Centricon filtration. Under the conditions used, > 95% of the label remained associated with ADP during binding assays.

Analysis of binding data

In order to correct for the loss of radioactivity from the cell during flow-dialysis experiments, the rate of efflux of radioactivity at each steady state (proportional to the concentration of free ligand) was first defined in terms of a quotient, Q , obtained from the final four fractions at each steady state, such that:

$$Q = \Sigma \left(\frac{\text{radioactivity per fraction}}{\text{radioactivity remaining in cell}} \right) / 4$$

This value was then related to an analogous one, Q_{max} , obtained at the end of the run and in the presence of sufficiently high [ligand] to ensure that all the radioisotope was free. Thus, at each steady state:

$$[\text{ligand}]_{\text{free}} = (Q/Q_{\text{max}}) \times [\text{ligand}]_{\text{total}}$$

Values of Q_{max} were in the range 3×10^{-3} – 4×10^{-3} for Ca²⁺ binding and 0.9×10^{-3} – 1.1×10^{-3} for ADP binding.

The above calculations were performed with a program written for an Apple II microcomputer, with on-line data from the scintillation counter.

For Ca²⁺-binding experiments in the presence of diffusible Ca²⁺-binding ligands (L) (such as ADP and isocitrate), free [Ca²⁺] was determined from the values ($[\text{Ca}^{2+}]_{\text{app}}$) obtained as above, after correction for the contribution of the efflux of ligand-bound Ca²⁺ (CaL). This was done after measuring the rates of efflux of each CaL as a fraction (F_{CaL}) of the rate of efflux of free Ca²⁺ (by the method of Haiech *et al.*, 1980). The free Ca²⁺ concentration could then be obtained, since

$$[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{app}} - \Sigma F_{\text{CaL}} \cdot [\text{CaL}]$$

which, by substituting for [CaL], gives expressions of the form:

$$[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{app}} - \Sigma \left(\frac{F_{\text{CaL}} \cdot [\text{Ca}^{2+}] \cdot [\text{L}]_{\text{tot}}}{[\text{Ca}^{2+}] + K_d^{\text{CaL}}} \right)$$

These were solved by standard numerical methods (Gerald & Wheatley, 1984). Values of K_d^{CaL} were calculated from published stability constants (Grzybowski *et al.*, 1970; Martell & Smith, 1974) after compensating for the binding of H⁺ and Mg²⁺ ions (Denton *et al.*, 1978; Thomas *et al.*, 1986). Under the conditions chosen, values of F_{CaL} for Ca-ADP and Ca-isocitrate were 0.4 and 0.42 respectively, giving $[\text{Ca}^{2+}] \geq 0.72 \times [\text{Ca}^{2+}]_{\text{app}}$.

Ca²⁺-binding isotherms were obtained by non-linear regression analysis (Rutter & Denton, 1988) of flow-dialysis data with a Hewlett-Packard 9000/300 microcomputer. Data were fitted to either

$$(i) [\text{ligand}]_{\text{bound}} - B \cdot [\text{ligand}]_{\text{free}} =$$

$$\text{or} \quad N / \{1 + (K_d / [\text{ligand}]_{\text{free}})^h\}$$

$$(ii) [\text{ligand}]_{\text{bound}} - B \cdot [\text{ligand}]_{\text{free}} =$$

$$N_1 / (1 + K_{d1} / [\text{ligand}]_{\text{free}}) + N_2 / (1 + K_{d2} / [\text{ligand}]_{\text{free}})$$

where the constant B , identical in control and experimental runs, is included to account for weak binding to elements of the dialysis cell, including slowly diffusing molecules such as ADP etc., N , N_1 and N_2 represent the concentrations of binding sites with dissociation constants K_d , K_{d1} and K_{d2} respectively, and h is the Hill coefficient. Data obtained from Ca²⁺-binding experiments by using fura-2, and from ADP-binding experiments, were fitted to the above equations, but with $B = 0$.

Expression of results

Data are given as means $\pm$ S.E.M. for the numbers of degrees of freedom in parentheses.

Stoichiometries of binding were calculated by assuming minimum molecular masses of 3.2×10^6 Da for the OGDH complex (Koike & Koike, 1976), and 16000 Da for calmodulin (Klee & Vanaman, 1982). A value of 160000 Da was assumed for NAD-ICDH, based on the enzyme existing in the tetrameric form $\alpha_2\beta_2\gamma$ (Ramachandran & Colman, 1980; Ehrlich *et al.*, 1981); however, it should be recognized that this stoichiometry has not been firmly established (Rutter & Denton, 1989).

RESULTS

Preliminary experiments: use of parvalbumin/polyacrylamide chromatography and fura-2

As a first approach to investigate the binding of Ca^{2+} to OGDH and NAD-ICDH, flow-dialysis experiments were performed on proteins dialysed against Chelex-treated buffers to remove bound Ca^{2+} (Haiech *et al.*, 1981). Two observations, which implied that a fundamental difference existed in the binding properties of NAD-ICDH and OGDH, were made during these experiments.

(a) With OGDH solutions (10–20 mg/ml), dialysis for 3–4 days with three changes of buffer was necessary to achieve $> 95\%$ loss of added $^{45}\text{CaCl}_2$ (1 $\mu\text{Ci}/\text{ml}$). By contrast, essentially all the radioactivity associated with NAD-ICDH, dialysed under identical conditions except for the presence of 1 mM-ADP, was lost after 12 h.

(b) Essentially no Ca^{2+} binding was detectable to NAD-ICDH (assayed in the presence of 1 mM-ADP and 1 mM- MgCl_2), whereas binding to OGDH was apparent under identical conditions, and also in the absence of ADP and/or MgCl_2 .

Analysis of the binding data for OGDH (as described in the Experimental section) gave variable results, with the apparent stoichiometry ranging between 1 and 5 binding sites per molecule of OGDH complex, and K_d values (for this single class of sites) from 0.2 to 1.6 μM in different experiments.

Buffers treated with Chelex contained approx. 200 nM- Ca^{2+} as assayed with fura-2. The possibility was therefore considered that the variable results obtained with OGDH, and perhaps also the absence of detectable binding to NAD-ICDH, might simply represent a failure to strip the proteins completely of Ca^{2+} . For this reason, and to avoid the need for lengthy dialysis, an alternative means of removing Ca^{2+} from protein and buffer solutions, involving parvalbumin/polyacrylamide chromatography (Lehky *et al.*, 1977; Keller *et al.*, 1982), was employed. The level of Ca^{2+} contamination of solutions treated in this way, as assayed by fura-2, was found to be ≤ 10 nM. That this value represented the total (bound+free) concentration of Ca^{2+} in protein solutions was demonstrated by the fact that the concentration of Ca-fura-2 remained unchanged as the total [fura-2] was increased from 2 to 50 μM (results not shown). Consistent with this, atomic-absorption measurements also indicated that parvalbumin/polyacrylamide-treated solutions of OGDH and NAD-ICDH were essentially free of Ca^{2+} contamination.

In marked contrast, with untreated solutions of OGDH (which had been exposed during isolation to

Fig. 1. Use of fura-2 to estimate $[\text{Ca}^{2+}]$ associated with OGDH as isolated

Solutions (0.1 ml) of isolation buffer (see the Experimental section) containing OGDH were diluted to a final volume of 0.5 ml with 10 mM-Mops/Tris (pH 7.2)/0.1 M-KCl/0.1 mM-dithiothreitol (Chelex-treated) to give OGDH concns. of 4 mg/ml (●), 2 mg/ml (■), or zero (○). $[\text{Ca-fura-2}]$ was determined at each total [fura-2] as described in the Experimental section.

buffers containing $> 5 \mu\text{M-Ca}^{2+}$), increasing $[\text{fura-2}]_{\text{total}}$ led to an increase in $[\text{Ca-fura-2}]$ up to a limiting value where all the protein-bound Ca^{2+} became associated with the indicator (Fig. 1). This approach demonstrated that the total Ca^{2+} associated with OGDH as isolated was approx. 6 mol/mol of complex.

Ca^{2+} -binding isotherms for parvalbumin/polyacrylamide-treated OGDH, and calmodulin, obtained by adding increasing CaCl_2 concentrations at a fixed [fura-2], are shown in Fig. 2. At the concentration of protein chosen (4 mg/ml), calculation of binding parameters for OGDH [assuming a single class of binding sites; eqn. (i) in the Experimental section] indicated the presence of $4.6 \pm 0.8 \mu\text{M}$ (9) Ca^{2+} -binding sites (or 3.7 ± 0.6 mol of sites/mol of complex) with a dissociation constant of $0.2 \pm 0.1 \mu\text{M}$, and Hill coefficient (h) of 1.0 ± 0.3 . Corresponding values for calmodulin (0.25 mg/ml) were $41.1 \pm 1.2 \mu\text{M}$ (10) sites (or 2.7 ± 0.1 mol/mol), with a K_d value of $0.22 \pm 0.02 \mu\text{M}$ ($h = 1.2 \pm 0.1$). Thus, although the use of fura-2 allowed the rather precise determination of the Ca^{2+} -binding parameters for calmodulin [consistent with the presence of two tight-binding sites/molecule, plus one or two further weaker sites/molecule (Haiech *et al.*, 1981)], for OGDH the precision of the data obtained was limited by the lower number of Ca^{2+} -binding sites associated with this latter protein. Furthermore, this approach was also limited by the difficulty of measuring binding at concentrations of Ca^{2+} above about 1 μM , at which the fura-2 was essentially completely saturated with Ca^{2+} .

Similar experiments to those illustrated in Fig. 2 were also performed with NAD-ICDH. Under the conditions employed in Fig. 2(a), plus 1 mM-ADP and 1 mM- MgCl_2 , we were unable to detect any binding of Ca^{2+} to this

Fig. 2. Ca²⁺-binding isotherms for parvalbumin-treated (a) OGDH and (b) calmodulin, obtained by using fura-2

Data were obtained as described in the Experimental section, with (a) 4 mg of OGDH/ml, 1.2 μ M-fura-2, 10 mM-Mops/Tris, pH 7.2, 0.1 M-KCl, 0.1 mM-dithiothreitol ($K_d^{\text{Ca-fura-2}} = 47 \pm 7.0$ nM) and (b) 0.25 mg of calmodulin/ml, 3.0 μ M-fura-2, 10 mM-Mops/Tris, pH 7.2, 0.1 mM-dithiothreitol ($K_d^{\text{Ca-fura-2}} = 19 \pm 1.2$ nM).

enzyme (results not shown). However, with this enzyme the above approach was of even more limited value, as a result of light-scattering artefacts [which may reflect changes in the polymerization state of the enzyme (Giorgio *et al.*, 1970; Ehrlich *et al.*, 1981)].

Ca²⁺ binding to OGDH studied by flow dialysis: comparison with calmodulin

In order to obtain more precise binding parameters for OGDH, and to examine further the Ca²⁺ binding of NAD-ICDH, the use of flow dialysis (Colowick & Womack, 1969) was adopted.

Fig. 3 shows the pattern of efflux of radioactivity from the flow cell when the Ca²⁺ binding to OGDH and calmodulin was compared by flow dialysis after parvalbumin/polyacrylamide treatment. Binding isotherms for the two proteins, calculated after correction for the loss of radioactivity from the cell during the experiment, and for background binding to components of the flow cell (see the Experimental section) are shown in Fig. 4. Binding parameters for OGDH, under a variety of conditions, and calmodulin, are given in Table 1. The parameters given were obtained by analysis of the data assuming a single class of sites and co-operative binding [eqn. (i); see the Experimental section]. The apparent binding parameters for calmodulin are closely comparable with those obtained by others (Dedman *et al.*, 1977; Haiech *et al.*, 1981) under similar conditions, and suggest that flow dialysis was sufficiently sensitive to study the binding of Ca²⁺ to sites at the concentrations (≈ 10 μ M) apparent in the experiments with OGDH.

The number of binding sites apparent on OGDH, between 2.5 and 5 mol/mol of complex under a number of different conditions, is broadly compatible with that estimated by the use of fura-2. When analysed by a single-site model as above, binding of Ca²⁺ to OGDH displayed apparent negative co-operativity (Table 1), under all the conditions tested. However, it should be

mentioned that analysis assuming binding to two classes of (non-co-operative) site [eqn. (ii)] produced an equally good fit to the data. For example, for the experiment shown in Fig. 4 this analysis gave 0.8 mol of high-affinity sites ($K_d = 63$ nM)/mol and 2.3 mol of lower-affinity sites ($K_d = 2.9$ μ M)/mol of complex.

ADP binding to OGDH

ADP binding to OGDH was examined by flow dialysis at two different protein concentrations (Fig. 5a). In both cases, binding to two classes of sites was apparent. Since binding parameters for the tighter-binding site were similar in these two experiments, giving values of 2.89 ± 0.74 mol/mol (6), $K_d = 2.69 \pm 1.69$ μ M (13 mg of OGDH/ml) and 3.73 ± 0.87 mol/mol (6), $K_d = 3.69 \pm 1.59$ μ M (4 mg of OGDH/ml), these data were combined (Fig. 5b) after correction for binding to the weaker sites (see below). Analysis of the combined data according to a single-site model [eqn. (i)] gave a value for the number of tight-binding sites of 3.31 ± 0.135 mol/mol (18) and a K_d of 3.09 ± 0.86 μ M.

Although the apparent stoichiometry and K_d values for tight-binding were similar at the two protein concentrations chosen, binding parameters for the weaker sites differed markedly between these two experiments. Thus a value of 8.91 ± 0.70 mol/mol ($K_d = 195 \pm 78$ μ M) was obtained at the higher concentration of protein, and 22.2 ± 1.93 mol/mol ($K_d = 167 \pm 47$ μ M) at the lower. This discrepancy would seem to reflect the general difficulty of obtaining precise binding parameters to rather weak sites, where the concentration of these is considerably below the K_d value for binding.

Ca²⁺ binding to NAD-ICDH studied by flow dialysis

Data obtained during flow-dialysis experiments with parvalbumin/polyacrylamide-treated NAD-ICDH are shown in Fig. 6. Consistent with preliminary experiments using enzyme dialysed against Chelex-treated buffers, we

Fig. 3. Ca^{2+} binding to (a) OGDH and (b) calmodulin, studied by flow dialysis

Radioactivity was measured in fractions collected from the flow cell in experiments with parvalbumin/polyacrylamide-treated protein solutions (black symbols) or control buffer only (white symbols), as detailed in the Experimental section. The data points shown represent the means of the radioactivity in two successive fractions. Each run was initiated with $0.8 \mu\text{M}$ - $^{45}\text{CaCl}_2$ (arrow A). Compositions of the solutions, and additions made during the experiments, were as follows. (a) (i) 8.3 mg of OGDH/ml; a-n, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 15.0, 20.0, 25.0, 30.0, 50.0 and $100.0 \mu\text{M}$ - $^{40}\text{CaCl}_2$; D, 25 mM- $^{40}\text{CaCl}_2$; (ii) control buffer; B, $2.0 \mu\text{M}$ - $^{40}\text{CaCl}_2$; C, 25 mM- $^{40}\text{CaCl}_2$. (b) (i) 1.0 mg of calmodulin/ml; a-n, 2.0, 4.0, 6.0, 10.0, 15.0, 20.0, 30.0, 50.0, 75.0, 100.0, 125.0, 150.0, 200.0 and $300.0 \mu\text{M}$ - $^{40}\text{CaCl}_2$; C, 25 mM- $^{40}\text{CaCl}_2$; (ii) 0.07 mg of calmodulin/ml; a-n, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 15.0, 20.0, 25.0, 30.0, 50.0 and $100 \mu\text{M}$ - $^{40}\text{CaCl}_2$; C, 25 mM- $^{40}\text{CaCl}_2$. (iii) control buffer; B, 25 mM- $^{40}\text{CaCl}_2$.

Fig. 4. Ca^{2+} -binding isotherms for OGDH (●) and calmodulin (■) obtained by flow dialysis

Data were calculated as described in the Experimental section for the experiments shown in Fig. 3 with 8.3 mg of OGDH/ml and 0.07 mg of calmodulin/ml. Binding parameters are given in Table 1.

were unable to detect any binding of Ca^{2+} to the enzyme in flow-dialysis buffer (see Fig. 3) supplemented only with 1 mM-ADP and 1 mM- MgCl_2 (Fig. 6a). However, the further addition of 1 mM-DL-isocitrate after the initial addition of $^{45}\text{CaCl}_2$ caused an immediate fall in the rate of efflux of radioactivity from the cell (Fig. 6b). This fall was considerably greater than that caused by the chelation of $^{45}\text{CaCl}_2$ by isocitrate (apparent in the absence of NAD-ICDH), and can thus be attributed to the binding of Ca^{2+} to the enzyme. This conclusion is supported by the fact that the bound $^{45}\text{Ca}^{2+}$ could be titrated off the enzyme by additions of $^{40}\text{CaCl}_2$.

The experiment shown in Fig. 6(c) illustrates that the effect of isocitrate was only apparent in the presence of MgCl_2 ; essentially no binding of Ca^{2+} to the enzyme was apparent in the presence of isocitrate alone. Fig. 6(d) demonstrates that the combined effect of isocitrate and MgCl_2 was also entirely dependent on the presence of ADP.

Combined data from the three experiments shown in Figs 6(b), 6(c) and 6(d) and one further experiment (identical with that illustrated in Fig. 6b) indicated the presence of a single class of sites, with a stoichiometry of 0.92 ± 0.21 mol/mol of tetrameric enzyme (see the Experimental section), and a K_d value of $14.3 \pm 5.3 \mu\text{M}$ (41) with $h = 0.72 \pm 0.20$. The calculated $[\text{Mg}^{2+}]$ in these experiments was 0.5 mM.

Table 1. Ca²⁺-binding parameters for OGDH, NAD-ICDH and calmodulin, obtained by flow dialysis

Details of measurements and calculation of binding parameters are given in the Experimental section.

Protein	Concn. (mg/ml)	Additions	Binding sites		K_d (μM)	h
			Apparent concn. (μM)	(mol/mol)		
OGDH	8.3	—	8.8 ± 1.0	3.3 ± 0.4	1.5 ± 0.7 (12)	0.7
	8.0	—	7.5 ± 0.4	3.1 ± 0.2	2.6 ± 0.5 (9)	1.1
	8.0	1 mM-MgCl ₂	8.8 ± 0.6	3.3 ± 0.2	3.9 ± 0.8 (12)	0.8
	8.1	1 mM-ADP	12.3 ± 1.2	4.7 ± 0.5	3.2 ± 1.1 (11)	0.8
	12.0	1 mM-2-Oxoglutarate	13.5 ± 3.1	3.5 ± 0.8	7.5 ± 6.0 (12)	0.7
NAD-ICDH*	0.75–1.5	1 mM-DL-isocitrate, 1 mM-MgCl ₂ , 1 mM-ADP	—	0.92 ± 0.2	14.4 ± 5.3 (41)	0.7
Calmodulin	1.0	—	119 ± 2.9	2.0 ± 0.05	2.3 ± 0.2 (11)	1.0
	0.07	—	12.4 ± 0.6	2.9 ± 0.1	4.9 ± 0.5 (12)	0.9

* Data were combined from four individual experiments under identical conditions. The apparent concentration of binding sites was in the range 6–10 μM in each case.

Fig. 5. ADP binding to OGDH

(a) Original binding data obtained at 2 mg/ml (■) and 4 mg/ml (●) concentrations of OGDH (see the Experimental section for details). (b) Combined and corrected data from (a) showing binding to a class of tight-binding sites. Binding parameters are given in the text.

DISCUSSION

Ca²⁺ and ADP binding to OGDH

Studied by flow dialysis, the purified OGDH complex has been shown to bind Ca²⁺ ions with high affinity (K_d 1.5–7.5 μM), and with a stoichiometry of between 3 and 5 mol of Ca²⁺/mol of complex. Although a less precise assay, broadly similar data were obtained by using the Ca²⁺ indicator fura-2.

This range of apparent dissociation constants is largely compatible with the concentration of Ca²⁺ required for half-maximal effects ($K_{0.5}$ values) on the activity of the purified or partially purified enzyme (0.2–2.0 μM ; McCormack & Denton, 1979; Denton *et al.*, 1980; Lawlis & Roche, 1980, 1981a; Rutter & Denton, 1988). These $K_{0.5}$ values were obtained by using the Ca²⁺ chelators

EGTA and *N*-(2-hydroxyethyl)ethylenediaminetriacetate (HEDTA). Since no correction was made for the activity of H⁺ in the calculation of Ca²⁺-binding constants for these chelators, the apparent $K_{0.5}$ values may represent underestimates of the true values by a factor of about 2 (see Tsien & Rink, 1980).

The apparent stoichiometry of binding, 3.5 ± 0.07 mol of Ca²⁺/mol of complex (combined data from five experiments), is clearly lower than that which would be expected for a site on each individual molecule of any of the three types of subunit of the complex (Koike & Koike, 1976). This would require the binding of 12 mol of Ca²⁺/mol of complex (for a site on the E1 or E3 subunits) or 24 mol of Ca²⁺/mol of complex (site on the E2 subunit). However, both isolated E1 and E3 subunits have been shown to exist as dimers (Koike & Koike,

Fig. 6. Binding of Ca^{2+} ions to NAD-ICDH, studied by flow dialysis

Radioactivity was measured in fractions from the flow cell in experiments with either parvalbumin/polyacrylamide-treated NAD-ICDH (■) or control buffer (□). The data shown are the means of the radioactivity in two successive fractions. Each run was initiated with $0.8 \mu\text{M}$ - $^{45}\text{CaCl}_2$ (arrow A). Concentrations of NAD-ICDH, initial additions to the buffer, and additions made during the run, were as follows. (a), (i) NAD-ICDH (10 mg/ml) plus 1.0 mM-ADP, 1.0 mM- MgCl_2 ; a-l, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 15.0, 30.0, 50.0, 100.0 and $200 \mu\text{M}$ - $^{40}\text{CaCl}_2$; B, 25 mM- $^{40}\text{CaCl}_2$; (ii) control buffer, plus 1.0 mM-ADP, 1.0 mM- MgCl_2 ; p, q, r, 2.0, 10.0 and $100.0 \mu\text{M}$ - $^{40}\text{CaCl}_2$; C, 25 mM- $^{40}\text{CaCl}_2$. (b), (i) NAD-ICDH (1.0 mg/ml) plus 1.0 mM-ADP, 1.0 mM- MgCl_2 ; B, 1 mM-DL-isocitrate; a-h, 2.0, 3.0, 5.0, 10.0, 20.0, 50.0, 100.0 and $200 \mu\text{M}$ - $^{40}\text{CaCl}_2$; D, 25 mM- $^{40}\text{CaCl}_2$; (ii) control buffer plus 1.0 mM-ADP and 1.0 mM- MgCl_2 ; B, 1 mM-DL-isocitrate; C, 25 mM- $^{40}\text{CaCl}_2$. (c), (i) NAD-ICDH (1.5 mg/ml) plus 1.0 mM-ADP only; B, 1.0 mM-DL-isocitrate; C, 1.0 mM- MgCl_2 ; a-k, 2.0, 3.0, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, 50.0, 100.0 and $200.0 \mu\text{M}$ - $^{40}\text{CaCl}_2$; D, 25 mM- $^{40}\text{CaCl}_2$; (ii) control buffer plus 1.0 mM-ADP only; B and C as for (i); E, 25 mM- $^{40}\text{CaCl}_2$. (d), (i) NAD-ICDH (1.3 mg/ml) plus 1.0 mM-DL-isocitrate, 1.0 mM- MgCl_2 ; B, 1.0 mM-ADP; a-k, as for (c); l, $300 \mu\text{M}$ - $^{40}\text{CaCl}_2$; D, 25 mM- $^{40}\text{CaCl}_2$; (ii) control buffer, plus 1.0 mM-DL-isocitrate, 1.0 mM- MgCl_2 ; B, 1.0 mM-ADP; C, 25 mM- $^{40}\text{CaCl}_2$.

1976). Thus the observed stoichiometry may be most consistent with the presence of a single Ca^{2+} -binding site on each of six dimeric E1 or E3 subcomplexes. Of these, binding to the E1 subcomplex would seem to be the more likely, since (1) evidence obtained from isolated subunit preparations suggests that the activity of E1, but not E3,

may be sensitive to $[\text{Ca}^{2+}]$ (Lawlis & Roche, 1981b); (2) the reaction catalysed by the E1 subunits, decarboxylation of 2-oxoglutarate, probably represents the most important rate-controlling step in overall catalysis (by analogy with the closely similar PDH complex; Walsh *et al.*, 1976). Finally, the direct binding of Ca^{2+} to one of

the subunits of OGDH would seem more likely than that to any low-molecular-mass calmodulin-like Ca²⁺-binding protein, since no such protein was apparent on SDS/polyacrylamide-gel-electrophoretic analysis of OGDH (see the Experimental section), consistent with earlier observations (McCormack & Denton, 1979; Denton & McCormack, 1980). However, it should be noted that the apparent number of Ca²⁺-binding sites is still below that which would be expected for binding to a site on E1 dimers. This may reflect some loss of active E1 from the OGDH complex.

The apparent tight binding of ADP (as ADP³⁻) to OGDH, with a stoichiometry of approx. 3 mol/mol of complex, is perhaps also most compatible with the existence of a single ADP-binding site on each E1 dimer within the complex. Again, evidence exists that the activity of isolated E1 subunits is sensitive to regulation by this nucleotide (Lawlis & Roche, 1981*b*). However, the dissociation constant for binding (about 3 μ M) was appreciably lower than the concentration of ADP required for half-maximal effects on the activity of the enzyme ($67 \pm 19 \mu$ M, assayed in flow-dialysis buffer supplemented with 5 mM-2-oxoglutarate, 2 mM-NAD⁺, 0.1 mM-CoA and 1 mM-thiamin pyrophosphate). One possible explanation of this discrepancy is that NAD⁺ present in the assay competitively inhibited the binding of ADP. However, it is also possible that binding of ADP to the variable number of much weaker sites ($K_d = 150$ – 200μ M) might also be important in regulating the activity of the complex.

It is important to note that each of the above suggestions requires support from binding data for the isolated subunits of the OGDH complex, but we have so far been unable to separate the E1 and E2 subunits.

Ca²⁺ binding to NAD-ICDH

The binding of Ca²⁺ to NAD-ICDH has been shown to require the presence of not only ADP (also required for an effect of Ca²⁺ on the activity of the enzyme; Denton *et al.*, 1978) but also both DL-isocitrate and MgCl₂. This co-requirement for isocitrate and MgCl₂ indicates that the presence of isocitrate is not required merely to form a bridging ligand between the enzyme and Ca²⁺. The most likely explanation would seem to be that isocitrate and MgCl₂ are required together in order to form the actual substrate of NAD-ICDH, Mg²⁺-*threo*-D₅-isocitrate (Cohen & Colman, 1974; Plaut *et al.*, 1974; Wilson & Tipton, 1981; Rutter & Denton, 1989), which then induces Ca²⁺ binding to the enzyme.

The apparent K_d value for Ca²⁺ binding ($14.1 \pm 5.3 \mu$ M) is broadly compatible with a predicted $K_{0.5}$ value for Ca²⁺ activation of 5–10 μ M (Rutter & Denton, 1989) at the concentration of Mg²⁺ (0.5 mM) present in the assays.

The apparent stoichiometry of Ca²⁺ binding, 0.9 mol of Ca²⁺/mol of NAD-ICDH tetramer, is lower than that previously observed for the binding of the activator ADP (in the presence of Mn²⁺ ions) and also for isocitrate (Ehrlich & Colman, 1981), where a stoichiometry of 2 mol/mol of tetrameric enzyme was observed in each case. This is consistent with the binding of these ligands to the α -subunit of NAD-ICDH, where the active site of this enzyme appears to be located (Hayman & Colman, 1982; Ehrlich & Colman, 1984). This leads to the intriguing possibility of a role in Ca²⁺ binding for either the β - or γ -subunits of the enzyme. However, a single binding site on either of the two α -subunits, or at the

interface of the two, clearly cannot be discounted. Again, studies with the isolated subunits will be required to resolve this question.

The observation that Ca²⁺ binding to NAD-ICDH is apparently induced by the substrate of this enzyme, Mg²⁺-*threo*-D₅-isocitrate, suggests that Ca²⁺ may activate this enzyme, but not OGDH, in a rather novel manner. This arises from the fact that Ca²⁺ binding only to the substrate-bound enzyme will in itself tend to cause a lowering of the K_m of the enzyme for its substrate, without (necessarily) inducing any conformational changes at the active site. This is directly analogous to the effects of uncompetitive inhibitors (see Dixon & Webb, 1979; Fersht, 1985); here, however, Ca²⁺ binding is considered to have no effect on catalysis by the enzyme-substrate complex (and therefore on V_{max}).

General conclusions: comparison of the Ca²⁺-binding properties of OGDH and NAD-ICDH

These studies have demonstrated that the Ca²⁺-binding properties of the two citrate-cycle Ca²⁺-sensitive dehydrogenases differ in two important respects under the conditions of this study.

Firstly, and consistent with earlier kinetic data (Rutter & Denton, 1988), the dissociation constant for Ca²⁺ binding to OGDH was 2.0–10-fold lower than that for NAD-ICDH (Table 1). Secondly, the binding of Ca²⁺ to OGDH occurred in the complete absence of other effectors of the enzyme, and was little affected by the activator ADP, by Mg²⁺ ions, or by its substrate, 2-oxoglutarate. This is in marked contrast with the complex requirements of NAD-ICDH for Ca²⁺ binding, described above.

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