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Rapid purification of pig heart NAD$^+$–isocitrate dehydrogenase

Studies on the regulation of activity by Ca^{2+}, adenine nucleotides, Mg^{2+} and other metal ions

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1. A new procedure for purifying pig heart NAD$^+$–isocitrate dehydrogenase from mitochondrial extracts has been developed. This relies on the use of f.p.l.c. techniques and exploits the hydrophobic properties of the gel-filtration medium Superose 6 at high ionic strength. A 300-fold purification to apparent homogeneity is achieved within 5 h and with a yield of > 20%. 2. The enzyme had an apparent native molecular mass on gel filtration of 320 kDa. In agreement with previous studies [Ramachandran & Colman (1980) J. Biol. Chem. **255**, 8859–8864], three subunits (all close to 38 kDa) were separable by isoelectric focusing. 3. This preparation was used to investigate the effects of adenine nucleotides, KCl and the required bivalent metal ions, Mg^{2+} and Mn^{2+}, on the regulation of the enzyme by Ca^{2+}. 4. In the presence of 1.5 mM-ADP, increasing the concentration of Mg^{2+} from 20 μM to 6.0 mM raised the concentration of Ca^{2+} required for half-maximal effect ($K_{0.5}$ value) from 1.2 μM to 232 μM. Similarly, in the presence of 2.5 μM-Mn^{2+}, a $K_{0.5}$ value for Ca^{2+} of 3.3 μM was obtained, and this value was increased to 8.9 μM in the presence of 100 μM-Mn^{2+}. In the presence of 1 mM-Mg^{2+} and 1.5 mM-ADP, the $K_{0.5}$ value for Ca^{2+} was raised from 4.7 μM to 10 μM by 75 mM-KCl.

INTRODUCTION

NAD$^+$–isocitrate dehydrogenase (NAD–ICDH; EC 1.1.1.41) is located exclusively within mitochondria in mammalian cells and represents an important control point of the citrate cycle (see Hansford, 1985; Williamson & Cooper, 1980; Plaut & Gabriel, 1983; Gabriel *et al.*, 1986). The activity of the enzyme, which is absolutely dependent on the presence of Mg^{2+}, Mn^{2+} or Co^{2+} ions (Plaut & Sung, 1954; Plaut, 1970), is exquisitely sensitive to regulation by a number of metabolites (Plaut & Gabriel, 1983), including the allosteric activator ADP (Chen & Plaut, 1963; Goebell & Klingenberg, 1964) and the inhibitors ATP and NADH (Chen & Plaut, 1963; Plaut & Aogaichi, 1968).

At sub-saturating isocitrate concentrations, Ca^{2+} ions (in the presence of an adenine nucleotide) enhance the activity of NAD–ICDH up to 10-fold (Denton *et al.*, 1978; Aogaichi *et al.*, 1980; Gabriel *et al.*, 1985; Rutter & Denton, 1988). The stimulation by Ca^{2+} of this enzyme, and also of two other mitochondrial dehydrogenases, 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979) and the pyruvate dehydrogenase complex (Denton *et al.*, 1972), may allow hormones and other extracellular stimuli to directly influence mitochondrial oxidative metabolism (for reviews see Denton & McCormack, 1985; Hansford, 1985; Denton *et al.*, 1987; McCormack *et al.*, 1989).

Recent studies have indicated that the Ca^{2+}-sensitivity of NAD–ICDH may be substantially less than that of the other mitochondrial Ca^{2+}-sensitive dehydrogenases when the enzymes are assayed under identical conditions in permeabilized mitochondria and mitochondrial extracts (Rutter & Denton, 1988). Furthermore, these studies suggested that the sensitivity of the enzyme to Ca^{2+} is strongly influenced by the ADP/ATP ratio. It therefore seemed important to investigate the effects of adenine nucleotides and Mg^{2+} or Mn^{2+} on the regulation by Ca^{2+} of purified NAD–ICDH and, in particular, to compare the Ca^{2+}-binding properties of the purified enzyme with those of 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase complex.

However, although satisfactory methods exist for purifying these last two enzymes (McCormack & Denton, 1979; Cooper *et al.*, 1974), those for NAD–ICDH (Plaut, 1969; Giorgio *et al.*, 1970; Shen *et al.*, 1974; Ehrlich *et al.*, 1981) are lengthy and can make the isolation of this unstable enzyme difficult.

In the present paper a more rapid and convenient means of purifying pig heart NAD–ICDH from mitochondrial extracts is described. This is based on the use of f.p.l.c. and exploits the hydrophobic properties of the gel-filtration medium, Superose 6, which are apparent at high ionic strength. By this approach a 50–100-fold purification of the enzyme to near homogeneity can be achieved from a 35–65%-satd.-$(NH_4)_2SO_4$ fraction in a single step. Further purification and concentration by anion-exchange (Mono Q) chromatography yields electrophoretically pure NAD–ICDH.

The regulation of this preparation is described, including the recognition that the Ca^{2+}-sensitivity of the enzyme is critically dependent on the concentration of Mg^{2+}. In the accompanying paper (Rutter & Denton, 1989) the preparation is used to study Ca^{2+} binding.

Abbreviations used: NAD–ICDH, NAD$^+$–isocitrate dehydrogenase; D_s-IC, *threo*-D_s-isocitrate; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetate; PMSF, phenylmethanesulphonyl fluoride. Throughout this paper, [Ca^{2+}] and [Mg^{2+}] represent the concentrations of the free unbound species of these metal ions.

EXPERIMENTAL

Materials

Sources of chemicals and biochemicals were given by Rutter & Denton (1988). In addition, pepstatin, antipain and leupeptin were from Cambridge Research Biochemicals, Harston, Cambs., U.K., and ultra-pure urea was from Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD, U.S.A. Chelex resin (50–100 mesh) was from Sigma Chemical Co., Poole, Dorset, U.K., and ampholytes were from Pharmacia LKB Biotechnology, Milton Keynes, U.K. Chromatography media were from either Pharmacia LKB Biotechnology or Sigma. Acetyl-CoA carboxylase was prepared as described by Borthwick *et al.* (1987), and the pig heart pyruvate dehydrogenase complex as described by Cooper *et al.* (1974).

Methods

Fast protein liquid chromatography (f.p.l.c). Automated f.p.l.c. systems and columns as supplied by Pharmacia LKB Biotechnology were used and maintained according to the manufacturers' instructions. Elution of proteins was continuously monitored at 280 nm. All buffers were passed through a 0.2 μm-pore-size filter before use.

Analytical gel chromatography. Native-molecular-mass determinations were made at 4 °C on an analytical Superose 6 column (20 ml bed volume) equilibrated in 50 mM-Mops/K^+ (pH 7.2)/0.2 M-KCl/1 mM-EGTA/1 mM-HEDTA/0.1 mM-dithiothreitol/0.02% NaN_3, plus Ca^{2+} as indicated. If necessary, samples were concentrated by $(NH_4)_2SO_4$ precipitation, and were transferred into the chromatography buffer by centrifugation (2 min, 1000 *g*) through a 2 ml column of Sephadex G-25 (fine grade), previously equilibrated in chromatography buffer (McCarthy & Hardie, 1982).

SDS/polyacrylamide-gel electrophoresis and isoelectric-focusing gels. SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), with gels containing 10% (w/v) acrylamide, was performed as described by Belsham *et al.* (1980), except that a Bio-Rad mini-gel system (Bio-Rad Laboratories, Watford, Herts., U.K.) was used. Slab isoelectric-focusing gels, containing 9.3 M-urea and the ampholytes indicated, were prepared and run as described by Wong *et al.* (1982).

A Joyce–Loebl Chromoscan was used for densitometric scanning of Coomassie-Blue-stained gels at 700 nm. Areas under each peak were determined after transmission of data to a Hewlett–Packard 9000/300 computer (Brownsey *et al.*, 1984).

Assay of NAD–ICDH activity, measurement of protein and handling of kinetic data. During the isolation procedure NAD–ICDH activity was measured by monitoring the production of NADH at 340 nm and 30 °C in 50 mM-Mops/35.5 mM-triethanolamine (pH 7.2)/2 mM-NAD^+/1 mM-ADP/2 mM-$MgCl_2$/5 mM-DL-isocitrate.

Buffers used for kinetic studies were as indicated. In all cases, sodium DL-isocitrate, containing 50% *threo*-D_s-isocitrate (D_s-IC) was used. Assays, with a Pye–Unicam PU-8800 spectrophotometer, were carried out in a total volume of 1 ml, and NAD–ICDH (3–10 munits; see below) was added to initiate reactions. Rates of NADH production were linear for at least 2 min.

Ca^{2+}-free (< 0.5 μM-Ca^{2+}) medium was prepared by passing 20 mM-Mops/triethanolamine, pH 7.2, 2 mM-NAD^+ and 1.5 mM-ADP over a 60 cm × 1 cm column of Chelex ion-exchange resin. Assays of activity in this medium were made as described above, with additions as indicated.

Free concentrations of metal ions and of metal–ligand complexes at pH 7.2 were calculated as described previously (Denton *et al.*, 1978; Midgley *et al.*, 1987; Rutter & Denton, 1988). Kinetic constants were calculated by non-linear regression as described by Rutter & Denton (1988). Data are given as parameter value ± S.E.M. for the number of degrees of freedom in parentheses. One unit of activity is defined as the amount catalysing the conversion of 1 μmol of substrate/min at 30 °C.

Protein was measured as described by Bradford (1976), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Purification of pig heart NAD–ICDH

All procedures were carried out at 0–4 °C.

(1) Preparation of mitochondrial extracts. These were prepared from 6–12 pig hearts essentially by the method of Cooper *et al.* (1974). Hearts were obtained fresh from the carcass and immediately packed on ice. The muscle was cut into 1–2 cm cubes and dispersed with a Waring blender into 30 mM-KH_2PO_4 (pH 7.6)/250 mM-sucrose/1 mM-EDTA (400 ml/heart). Care was taken to ensure that the pH did not fall below 6.5, and adjustment was made with 10 M-KOH if necessary. The homogenate was centrifuged at 2075 *g* for 25 min in a Mistral 6L centrifuge. The supernatant was filtered through muslin and retained; the pellet was re-dispersed with the blender (300 ml of dispersal buffer/heart) and re-centrifuged. The combined supernatants were adjusted to pH 5.4 with 40% (v/v) acetic acid (over 10 min with continuous stirring). Precipitated mitochondria were separated by centrifuging at 15000 *g* for 20 min, washed once by resuspension in water and collected by centrifugation.

The mitochondrial pellet was resuspended in 20 mM-KH_2PO_4, pH 7.2, containing 1 mM-EDTA, 0.1 mM-ADP, 2 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride (PMSF) and 1 mM-dithiothreitol (40 ml/heart). The suspension was shell-frozen and thawed three times, alternating between liquid N_2 and a water bath (30–40 °C). The slurry was centrifuged for 2 h at 18000 *g* and the supernatant retained.

(2) Preparation of a 35–65%-satd.-$(NH_4)_2SO_4$ fraction. After the gradual addition of solid $(NH_4)_2SO_4$ to 35% saturation, maintaining a constant pH of 7.2, the extract was stirred for 20 min and then centrifuged (20000 *g*, 15 min). The supernatant was adjusted to 65% saturation with $(NH_4)_2SO_4$, stirred and centrifuged as described above. The resulting pellet was dissolved in 5 mM-KH_2PO_4, pH 7.1, containing 1 mM-ADP, 0.1 mM-EDTA, 5% (w/v) glycerol, 2 mM-benzamidine, 0.1 mM-PMSF, 0.1 mM-dithiothreitol and 0.02% NaN_3 (buffer A) to give 50–100 mg of protein/ml (equivalent to 1–4 ml/original pig heart).

(3) Gel filtration on Superose 6. After centrifuging at 180000 *g* for 20 min, the redissolved $(NH_4)_2SO_4$ pellet (10–20 ml) was loaded on a column (2.6 cm × 70 cm) of

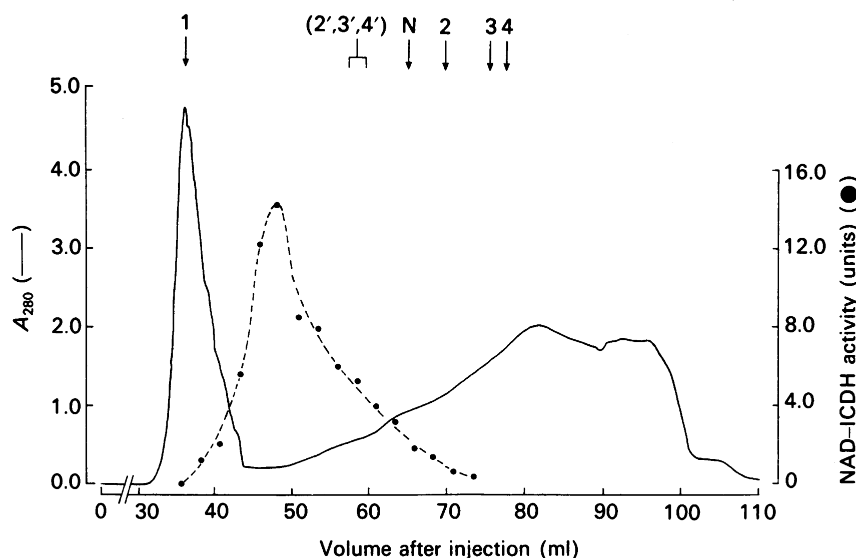

Fig. 1. Separation of protein by gel filtration on Superose 6

Redissolved pellets (3.5 ml) from step 2 were chromatographed in the presence of 0.8 M-$(NH_4)_2SO_4$ on Superose 6 (1.6 cm × 60 cm column). Marked are the positions of elution of: 1, acetyl-CoA carboxylase (polymeric form; 4×10^7 Da; Borthwick *et al.*, 1987); 2, ferritin (460 kDa); 3, β-galactosidase (540 kDa); 4, acetyl-CoA carboxylase (dimeric form; 460 kDa). The position marked 2′,3′,4′ indicates the corresponding region of elution of the above markers when the column was equilibrated in 20 mM-Mops/K^+, pH 7.2, containing 10 mM-sodium citrate, 10 mM-$MgCl_2$, 2 mM-dithiothreitol and 5 % glycerol. 'N' indicates the position of elution of NAD–ICDH when the column was equilibrated in buffer B (see the text).

Superose 6 (prep grade) equilibrated in buffer A plus 0.8 M-$(NH_4)_2SO_4$, and running at 90 ml/h. Alternatively, 3–4 ml of redissolved pellet was loaded on a 1.6 cm × 60 cm column of Superose 6, running at 30 ml/h. Under these conditions NAD–ICDH activity migrated as a single band between two other well-separated protein bands, which together contained more than 95 % of the loaded protein (Fig. 1).

Separation of proteins is achieved at this high ionic strength not merely on the basis of molecular mass. Hence, when fractions from step 2 were run on the column in the absence of added $(NH_4)_2SO_4$, most of the protein was eluted as a single rather broad and complex band (results not shown). The effect of $(NH_4)_2SO_4$ on the column profile would seem to be the result of enhanced hydrophobic interactions with the column matrix. Thus β-galactosidase, ferritin and the dimeric form of acetyl-CoA carboxylase (Borthwick *et al.*, 1987), which each have molecular masses close to 5×10^5 and closely co-migrated on the column in lower-ionic-strength buffer, migrated at different rates in the $(NH_4)_2SO_4$-containing buffer (Fig. 1). Moreover, the rate of migration of each of the proteins was lower in the higher-ionic-strength buffer.

In contrast with the proteins mentioned above, the rate of migration of NAD–ICDH activity was higher in buffers containing 0.8 M-$(NH_4)_2SO_4$ than in low-ionic-strength buffer (Fig. 1). A likely explanation of this is that the protein undergoes dimerization under these conditions, as observed by Giorgio *et al.* (1970).

Peak fractions of NAD–ICDH activity obtained after Superose 6 chromatography were pooled and rapidly (< 15 min) de-salted on a 5 cm × 17 cm column of Sephadex G-25 (coarse grade) equilibrated in 20 mM-Bistris, pH 6.5, containing 0.1 mM-ADP, 20 mM-KCl, 5 % glycerol, 2 mM-benzamidine, 0.1 mM-PMSF, 0.1 mM-dithiothreitol and 0.002 % NaN_3 (buffer B), and running at approx. 10 ml/min.

(4) Anion-exchange chromatography on Mono Q. Desalted fractions were loaded on a 10 ml (HR 10/10) (or 1 ml; HR 5/5) column of Mono Q, equilibrated in buffer B and running at 60 ml/h (30 ml/h for the 1 ml column). The column was developed with a gradient of buffer B plus 0–250 mM-KCl, and NAD–ICDH activity was eluted isocratically at about 150 mM-KCl (Fig. 2). Peak fractions were pooled, giving 1–3 mg of NAD–ICDH/ml, supplemented with 1 μg each of pepstatin, antipain and leupeptin/ml, and stored in small samples at −70 °C. The enzyme was stable under these conditions for at least 6 months.

The above procedure gives approx. 300-fold purification of NAD–ICDH from mitochondrial extracts within hours. A typical example is detailed in Table 1, and an SDS/polyacrylamide gel of fractions obtained at each stage of the purification is shown in Fig. 3.

The specific activity of NAD–ICDH prepared by this method was in the range 25–35 units/mg, based on an assay at 30 °C and in the presence of Mg^{2+} (see the Experimental section). This range is identical with that reported for the purified pig heart enzyme (Ehrlich & Colman, 1981) and for the purified bovine heart enzyme (Plaut, 1969; Giorgio *et al.*, 1970).

Subunit composition and native molecular mass

On SDS/polyacrylamide-gel-electrophoretic analysis of purified NAD–ICDH (see Fig. 3), two or three closely spaced bands with molecular masses of approx. 38 kDa were apparent, as previously observed for the pig heart enzyme by Ramachandran & Colman (1978).

Three bands, however, were clearly resolved by isoelectric focusing in the presence of 9.3 M-urea (Fig. 4) with pI

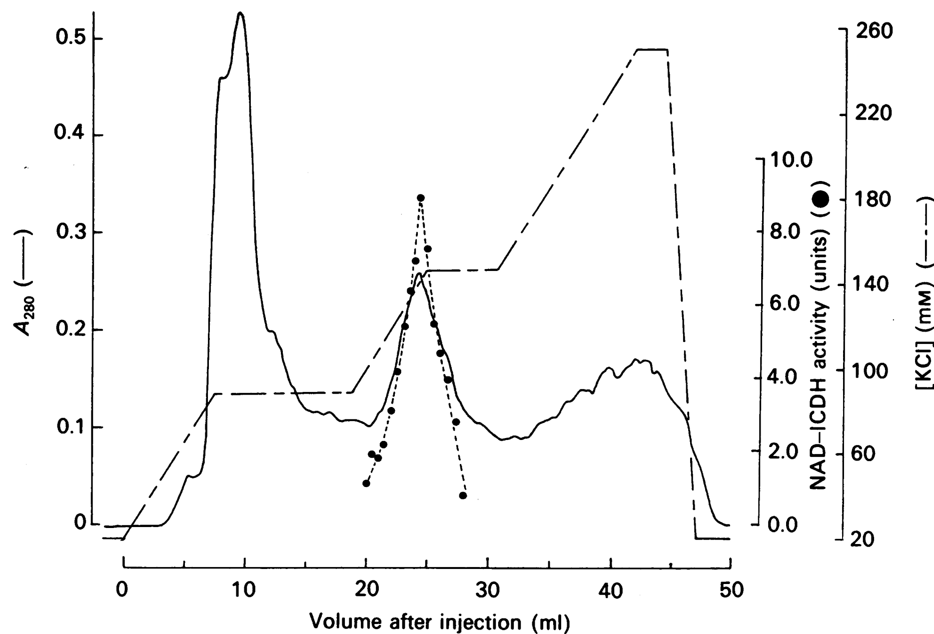

Fig. 2. Anion exchange on Mono Q

Desalted fractions (30 ml, 15 mg of protein) after Superose 6 chromatography were loaded on the 1 ml (HR 5/5) Mono Q column, and NAD–ICDH activity was eluted with the KCl gradient shown.

Table 1. Purification of pig heart NAD$^+$–ICDH

Values are taken for a preparation from 10 hearts. Details of the assay of activity and protein are given in the Experimental section.

	Protein (mg)	NAD–ICDH activity (units)	Specific activity (units/mg)	Recovery (%)
Mitochondrial extract	11050	885	0.08	100
35–65%-satd.-$(NH_4)_2SO_4$ fraction	3100	380	0.12	43
Superose 6 chromatography	32	261	8.3	30
Mono Q	8	210	26.2	24

values of 6.1, 6.3 and 7.05. These represented 32%, 25% and 17% respectively of the Coomassie-Blue-stained protein present on the gel. A further band (pI ~ 6.8) was also apparent, representing about 10% of the stained protein.

The resolution of three sharp bands in the present work is in contrast with earlier attempts to separate the subunits of pig heart NAD–ICDH by isoelectric focusing (Ramachandran & Colman, 1980). In those earlier studies, three groups of three to six bands were apparent, with estimated pI values of 5.7, 6.6 and 7.2, representing 51%, 28% and 21% of the Coomassie-Blue-stained protein. These bands were taken to represent three subunits of NAD–ICDH, termed α, β and γ, with a probable stoichiometry of $\alpha_2\beta\gamma$. However, the current data suggest that a stoichiometry of $\alpha_2\beta_2\gamma$ is also possible.

The native molecular mass of the purified enzyme was estimated by gel filtration on Superose 6 at an ionic strength where the migration rate was related directly to this parameter for a number of standards. This gave an apparent value of 329 ± 13 kDa (mean ± S.E.M for three observations), which was essentially unchanged by the presence of 100 μM-Ca^{2+}.

This value is comparable with those obtained by Giorgio *et al.* (1970) for the bovine heart enzyme and by Cohen & Colman (1971) for the pig heart enzyme under similar conditions, and would appear to be most consistent with an octameric subunit composition $(\alpha_2\beta\gamma)_2$. However, Ehrlich *et al.* (1981) have suggested that this apparent molecular mass obtained by gel filtration represents an overestimate of the true value, as a result of the anomalously high Stokes radius of NAD–ICDH. Thus, through equilibrium ultracentrifugation and light-scattering studies, those authors obtained a value for the native molecular mass of the enzyme of around 224 kDa, and suggested that a rapid equilibrium exists between tetramaric, $\alpha_2\beta\gamma$ (160 kDa), and octameric, $(\alpha_2\beta\gamma)_2$ (320 kDa), forms. An alternative explanation for this apparent native molecular mass value of close to 200 kDa is that the stoichiometry of the individual subunits of the

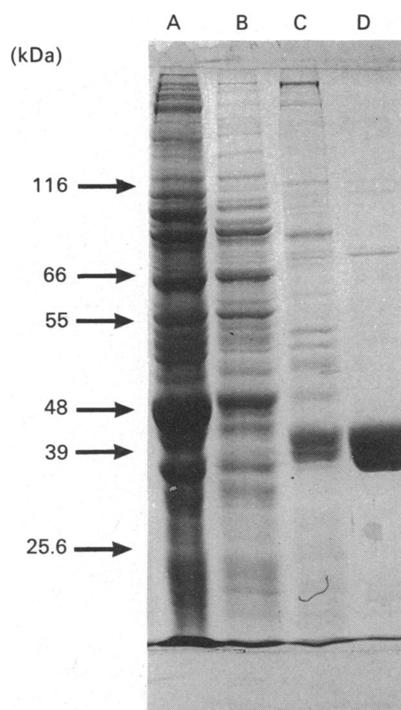

Fig. 3. SDS/polyacrylamide-gel-electrophoretic analysis of fractions during the purification of NAD–ICDH

Electrophoresis was performed as described in the Experimental section. Samples were obtained after: A, extraction of mitochondria (step 1); B, $(NH_4)_2SO_4$ fractionation (step 2); C, Superose 6 chromatography (step 3); D, anion exchange on Mono Q (step 4).

enzyme (each with molecular masses close to 40 kDa) is $\alpha_2\beta_2\gamma$, rather than $\alpha_2\beta\gamma$. It is evident that further studies are required to establish firmly the subunit stoichiometry.

Regulation of activity by Ca^{2+}: effects of adenine nucleotides, Mg^{2+}, Mn^{2+} and KCl

The sensitivity of the enzyme to Ca^{2+} ions was first determined under conditions similar to those used to study rat heart NAD–ICDH in permeabilized mitochondria and extracts (Rutter & Denton, 1988), namely at close to physiological ionic strength and in the presence of 1 mM-Mg^{2+}. Kinetic parameters are given in Table 2 and are similar to the values obtained with the rat heart enzyme. In particular, Ca^{2+} lowered apparent K_m values for added D_s-IC in the presence of either ADP or ATP. As found in earlier studies using mitochondrial extracts (Denton *et al.*, 1987), no effect of Ca^{2+} was seen in the absence of an adenine nucleotide (results not shown). The apparent K_m values for D_s-IC were lower in the presence of ADP than of ATP in both the presence and the absence of Ca^{2+}, but the effect of the change of adenine nucleotide was more marked in the presence of Ca^{2+}. Finally, the concentrations of Ca^{2+} required for half-maximal effects ($K_{0.5}$ values) were 3–6-fold higher in the presence of ATP than of ADP.

KCl inhibited the enzyme uncompetitively with respect to D_s-IC under a wide variety of conditions (Table 2). Furthermore, 75 mM-KCl also increased $K_{0.5}$ values for Ca^{2+} approx. 2-fold in the presence of either ADP or ATP.

The effect of [Mg^{2+}] on the sensitivity of NAD–ICDH activity to Ca^{2+} ions was investigated in the experiments shown in Figs. 5 and 6. Previous studies (Cohen &

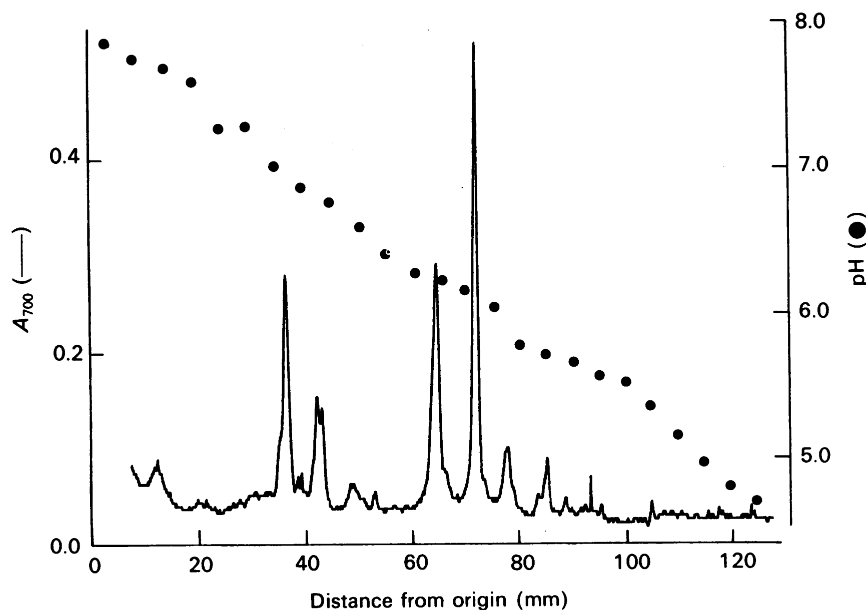

Fig. 4. Isoelectric focusing of NAD–ICDH

Subunits were resolved in a gel containing 9.3 M-urea and 6% (w/v) acrylamide, with 3% (v/v) ampholytes pH 5–8 and pH 3.5–10 in the ratio 4:1.

Table 2. Effects of ADP and ATP on the regulation of NAD–ICDH by Ca^{2+} ions

Measurement of enzyme activity and calculation of kinetic constants were as described in the Experimental section. The assay buffer was 50 mM-Mops/triethanolamine, pH 7.2, with 2 mM-NAD^+, 1 mM-EGTA, 1 mM-HEDTA, plus other additions as indicated. [Mg^{2+}] was 1.0 mM in all assays. When added, [KCl] was 75 mM. $K_{0.5}$ values for Ca^{2+} were determined in the presence of 0.6 mM-D_s-IC (in the presence of ADP) or 1.0 mM-D_s-IC (in the presence of ATP).

	1.5 mM-ADP		1.5 mM-ATP	
Parameter and condition	No KCl	+KCl	No KCl	+KCl
< 1 nM-Ca^{2+}				
K_m for D_s-IC (μM)	751 ± 28 (9)	643 ± 25 (9)	1196 ± 23 (10)	983 ± 93 (9)
h	2.5	3.1	2.7	3.3
$V_{max.}$ (munits/mg)	34.2 ± 0.7	22.7 ± 0.5	24.6 ± 0.3	19.2 ± 0.5
100 μM-Ca^{2+}				
K_m for D_s-IC (μM)	261 ± 13 (10)	155 ± 8.0 (11)	710 ± 27 (9)	499 ± 27 (9)
h	2.3	2.9	2.2	2.3
$V_{max.}$ (munits/mg)	32.5 ± 0.6	26.1 ± 0.5	32.0 ± 0.6	23.6 ± 0.5
$K_{0.5}$ for Ca^{2+} (μM)	4.7 ± 0.3 (12)	10.8 ± 1.5 (13)	24.8 ± 4.9 (12)	69.9 ± 11.0 (12)

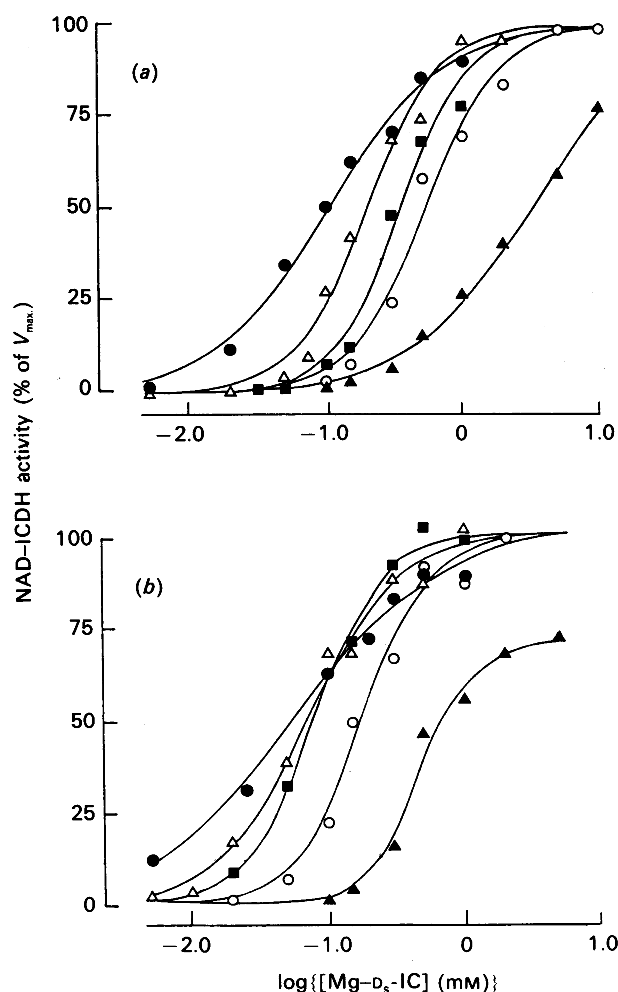

Fig. 5. Effect of [Mg^{2+}] on the sensitivity of NAD–ICDH to Mg–D_s-IC concentration at (a) < 1 nM-Ca^{2+} and (b) 100 μM- (●, △, ■, ○) or 1.0 mM- (▲) Ca^{2+}

Activity was measured in 50 mM-Mops/Tris, pH 7.2, with 2 mM-NAD^+, 1.5 mM-ADP, 1 mM-EGTA, 1 mM-HEDTA, supplemented with DL-isocitrate, $MgCl_2$ and $CaCl_2$ to give the required concentrations of Mg–D_s-IC and free metal ions (see the Experimental section). Concentrations (mM) of Mg^{2+} were 0.02 (●), 0.2 (△), 2.0 (□), 6.0 (○) and 20.0 (▲). The continuous lines are those obtained by fitting the data by non-linear least squares regression analysis to the following equation:

$$v = V_{max.}/\{1+(K_m^{Mg\text{-}D_s\text{-}IC}/[Mg\text{-}D_s\text{-}IC])^h\}$$

Colman, 1974; Plaut *et al.*, 1974; Wilson & Tipton, 1981) have suggested that the true substrate of mammalian NAD–ICDH is the Mg^{2+} chelate of D_s-IC (Mg–D_s-IC), whereas free Mg^{2+} ions act as competitive inhibitors against this substrate. The effects of Ca^{2+} on the K_m value for Mg–D_s-IC were therefore first examined over a wide range of Mg^{2+} concentrations (Fig. 5, Table 3). Consistent with the above model, increasing [Mg^{2+}] led to an increase in the K_m of the enzyme for Mg–D_s-IC, in both the presence (100 μM–1 mM) and the absence (< 1 nM) of Ca^{2+}, with essentially no effect on $V_{max.}$ values up to 6.0 mM-Mg^{2+}. However, the relationship between [Mg^{2+}] and the K_m values for Mg–D_s-IC was complex, precluding an estimate of a K_i value for Mg^{2+}. Furthermore, at a higher concentration (20 mM), and in the presence of Ca^{2+} ions, Mg^{2+} also caused an apparent fall in $V_{max.}$, to 70% of the value apparent at lower concentrations of the ion.

At each [Mg^{2+}], Ca^{2+} caused a fall in the K_m value for Mg–D_s-IC of 2–7-fold, with the effect of Ca^{2+} increasing at higher [Mg^{2+}]. However, as shown in Fig. 6 and in Table 3, increasing [Mg^{2+}] caused a marked rise in $K_{0.5}$ values for Ca^{2+}, from 1.4 μM at 20 μM-Mg^{2+} to about 1 mM at 20 mM-Mg^{2+}. Again, the relationship between these parameters was complex, and could not be described through a simple K_i value for Mg^{2+}.

The above studies were performed with the chelators EGTA and HEDTA to allow the precise control of the free concentrations of Ca^{2+} and Mg^{2+} ions. However, these and other nitrogen-containing polycarboxylate Ca^{2+} chelators have been reported to have a direct inhibitory effect on NAD–ICDH (Gabriel & Plaut, 1985). Although in our hands the effects are small (Denton *et al.*, 1978; G. A. Rutter, unpublished work), it seemed

Table 3. Effect of [Mg^{2+}] on the regulation of NAD–ICDH by Ca^{2+} ions

Details of the assay and calculation of kinetic constants are given in the Experimental section. The buffer was 50 mM-Mops/Tris, pH 7.2, with 2 mM-NAD^+, 1 mM-EGTA, 1 mM-HEDTA, 1.5 mM-ADP, plus DL-isocitrate, $MgCl_2$ and $CaCl_2$ to give the required [Mg–D_s-IC], [Mg^{2+}] and [Ca^{2+}]. $K_{0.5}$ values for Ca^{2+} were determined in the presence of 20.0, 50.0, 22.0, 100.0 and 500.0 μM-Mg–D_s-IC at 0.02, 0.2, 2.0, 6.0 and 20.0 mM-Mg^{2+} respectively. Abbreviation: N.D., not determined.

	< 1 nM-Ca^{2+}		100 μM-Ca^{2+}			
[Mg^{2+}] (mM)	$K_m^{Mg\text{-}D_s\text{-}IC}$ (μM)	h	$K_m^{Mg\text{-}D_s\text{-}IC}$ (μM)	h	$K_{0.5}$ for Ca^{2+} (μM)	h
0.02	107±7.7 (6)	1.0±0.1	61.0±5.9 (5)	0.9±0.1	1.24±0.21 (5)	1.8±0.5
0.20	204±14.3 (7)	1.7±0.2	70.6±5.8 (6)	1.3±0.1	5.77±1.20 (10)	1.0±0.2
2.00	360±27.1 (6)	1.8±0.2	80.0±5.0 (6)	1.7±0.1	14.7±2.10 (6)	1.1±0.2
6.00	537±52.0 (6)	1.6±0.2	181±16.0 (6)	1.7±0.3	232.0±48.0 (7)	1.4±0.3
20.0	3300±243.0 (6)	1.0±0.1	434±44.0* (6)	2.6±0.6	> 1000	N.D.

* Calculated assuming mixed (competitive plus non-competitive) inhibition (see the text); [Ca^{2+}] = 1.0 mM.

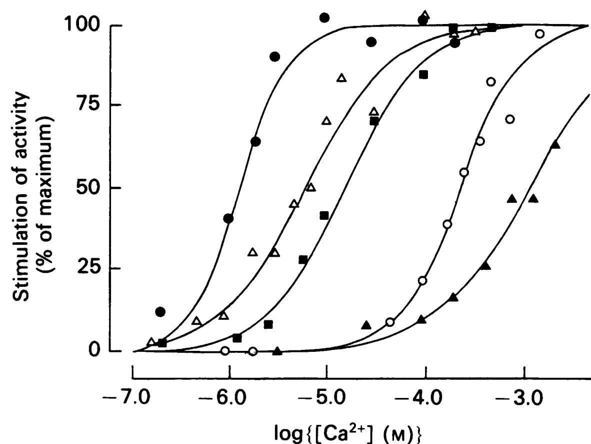

Fig. 6. Effect of [Mg^{2+}] on the sensitivity of NAD–ICDH to Ca^{2+} ions

Details of the assays and concentrations of Mg^{2+} are as given in the legend to Fig. 5. In each case the stimulation of activity by Ca^{2+} was 5–10-fold. The continuous lines are those obtained by fitting the data by non-linear least-squares regression analysis to the following equation:

$$v - V'_{min.} = V'_{max.}/\{1 + (K_{0.5}/[Ca^{2+}])^h\}$$

where $V'_{min.}$ and $V'_{max.}$ represent the activity of the enzyme at zero and saturating [Ca^{2+}] respectively.

important to demonstrate that the above effects of Ca^{2+}, and the interaction between regulation by Ca^{2+} and Mg^{2+} ions, were apparent in the absence of EGTA and HEDTA. Furthermore, the absence of these chelators was also necessary to investigate the effects of Mn^{2+} on the sensitivity of NAD–ICDH to Ca^{2+}. This is because Mn^{2+} ions bind at least as strongly to the chelators as do Ca^{2+} ions (Denton *et al.*, 1978).

These studies were carried out at pH 7.2 in the presence of 1.5 mM-ADP and a total D_s-IC concentration of 0.3 mM. In the presence of added $MgCl_2$ to give 10 μM-Mg^{2+}, $CaCl_2$ stimulated activity 3.5-fold, with a $K_{0.5}$ value for Ca^{2+} of 0.66±0.13 μM (6); this value was raised to 27.0±3.6 μM (3) in the presence of 0.44 mM-Mg^{2+}. Similarly, with added $MnCl_2$ to give 2.6 μM-Mn^{2+}, addition of $CaCl_2$ stimulated the activity of the enzyme 5-fold, with a $K_{0.5}$ value for Ca^{2+} of 3.3±0.7 μM (9); this value was increased to 8.9±3.6 μM (4) at 0.1 mM-Mn^{2+}.

These observations confirm that the enzyme is fully sensitive to Ca^{2+} in the absence of chelators, and in the presence of Mn^{2+} as well as Mg^{2+} ions. Furthermore, increasing the concentration of Mg^{2+} ions raised $K_{0.5}$ values for Ca^{2+} under these conditions, consistent with the results in the presence of chelators, and this effect was also apparent with Mn^{2+} ions. Finally, since activation of the enzyme by Ca^{2+} occurred in the presence of up to 100 μM-Mn^{2+}, this suggests that Mn^{2+} ions are unable to replace Ca^{2+} as activators of the enzyme.

General discussion

The method presented here for purifying pig heart NAD–ICDH represents a marked improvement upon previously published methods (Plaut, 1969; Giorgio *et al.*, 1970; Ehrlich *et al.*, 1981). By this method it is possible to obtain NAD–ICDH of equivalent purity, and with a similar or better yield, but in a matter of hours rather than days. This improvement is due largely to the use of Superose 6 chromatography at high ionic strength as a means of achieving 50–100-fold purification of the enzyme in a single step.

In the presence of a fixed concentration of Mg^{2+} ions (1 mM), the kinetic properties of the enzyme with respect to Ca^{2+}, ADP and ATP are very similar to those described previously for rat heart NAD–ICDH (Denton *et al.*, 1978; Rutter & Denton, 1988). These studies confirm that with the isolated enzyme $K_{0.5}$ values for Ca^{2+}, as well as apparent K_m values for (total) D_s-IC, are higher in the presence of ATP than of ADP (Gabriel *et al.*, 1985; Rutter & Denton, 1988).

In contrast with the other Ca^{2+}-sensitive citrate-cycle enzyme, 2-oxoglutarate dehydrogenase, where Mg^{2+} (up to 1 mM) appears to have essentially no effect on $K_{0.5}$ values for Ca^{2+} (McCormack & Denton, 1979; G. A. Rutter & R. M. Denton, unpublished work), the sensitivity of NAD–ICDH to Ca^{2+} is shown to be critically dependent on the concentration of Mg^{2+} (Fig. 6). Although studied over a narrower concentration range, Mn^{2+} ions also appeared to decrease the sensitivity of the enzyme to Ca^{2+}.

The effects of Mg^{2+} (and Mn^{2+}) would seem to be best explained by the competition of these ions for an activatory Ca^{2+}-binding site on the enzyme. However,

the complex relationship between [Mg^{2+}] and $K_{0.5}$ values for Ca^{2+} may suggest that the binding of Mg^{2+} to another site, possibly the active site of the enzyme, is also involved.

In the present studies, the observed $K_{0.5}$ values for Ca^{2+} of NAD–ICDH in the presence of 1.5 mM-ADP fell to values approaching those for 2-oxoglutarate dehydrogenase (0.2–2.0 μM; McCormack & Denton, 1979; Denton *et al.*, 1980; Lawlis & Roche, 1980; Rutter & Denton, 1988) only at concentrations of Mg^{2+} (20 μM) well below those considered to occur in intact mitochondria (about 0.3 mM; Jung & Brierly, 1986; Corkey *et al.*, 1986).

However, the responses to Ca^{2+} of two further mitochondrial Ca^{2+}-sensitive enzymes, pyruvate dehydrogenase phosphate phosphatase (which catalyses the dephosphorylation and consequent activation of the pyruvate dehydrogenase complex; Midgley *et al.*, 1987; Rutter *et al.*, 1989) and mitochondrial pyrophosphatase (Davidson & Halestrap, 1989) are also diminished with increasing concentrations of Mg^{2+}. It is therefore evident that the intramitochondrial Mg^{2+} concentration may have an important influence on the relative Ca^{2+}-sensitivities of the Ca^{2+}-regulated enzymes within mitochondria.

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