Regulation of NAD⁺-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase by Ca²⁺ ions within toluene-permeabilized rat heart mitochondria

Interactions with regulation by adenine nucleotides and NADH/NAD⁺ ratios

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1. Toluene-permeabilized rat heart mitochondria have been used to study the regulation of NAD⁺-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase by Ca²⁺, adenine and nicotinamide nucleotides, and to compare the properties of the enzymes *in situ*, with those in mitochondrial extracts. 2. Although $K_{0.5}$ values (concn. giving half-maximal effect) for Ca²⁺ of 2-oxoglutarate dehydrogenase were around 1 μ M under all conditions, corresponding values for NAD⁺-linked isocitrate dehydrogenase were in the range 5–43 μ M. 3. For both enzymes, $K_{0.5}$ values for Ca²⁺ observed in the presence of ATP were 3–10-fold higher than those in the presence of ADP, with values increasing over the ADP/ATP range 0.0–1.0. 4. 2-Oxoglutarate dehydrogenase was less sensitive to inhibition by NADH when assayed in permeabilized mitochondria than in mitochondrial extracts. Similarly, the K_m of NAD⁺-linked isocitrate dehydrogenase for *threo*-D₅-isocitrate was lower in permeabilized mitochondria than in extracts under all the conditions investigated. 5. It is concluded that in the intact heart Ca²⁺ activation of NAD⁺-linked isocitrate dehydrogenase may not necessarily occur in parallel with that of the other mitochondrial Ca²⁺-sensitive enzymes, 2-oxoglutarate dehydrogenase and the pyruvate dehydrogenase system.

INTRODUCTION

Three dehydrogenases within mammalian mitochondria have been shown to be regulated by Ca²⁺ ions. NAD⁺-linked isocitrate dehydrogenase (NAD-ICDH; Denton *et al.*, 1978; Aogaichi *et al.*, 1980) and 2-oxoglutarate dehydrogenase (OGDH; McCormack & Denton, 1979; Lawlis & Roche, 1980) are activated by a lowering of K_m values for substrates (*threo*-D₅-isocitrate and 2-oxoglutarate respectively), with little effect of Ca²⁺ at saturating concentrations of substrates. The pyruvate dehydrogenase (PDH) complex, on the other hand, is activated through increases in the active, dephosphorylated, form of the enzyme, largely through stimulation of PDHP phosphatase (Denton *et al.*, 1972). Each enzyme thus represents a site at which hormones and other stimuli which raise cytosolic Ca²⁺ concentrations may, via a parallel increase in mitochondrial concentration of Ca²⁺, stimulate citrate-cycle flux and mitochondrial ATP synthesis (for reviews see Denton & McCormack, 1980, 1985; Hansford, 1985; Brand & Murphy, 1987).

Studies with purified PDHP phosphatase and OGDH have given $K_{0.5}$ values for Ca²⁺ in the range 0.2–1.0 μ M, and parallel activation of PDH and OGDH by Ca²⁺ occurs in isolated mitochondria (Denton *et al.*, 1980; McCormack & Denton, 1980, 1981; Marshall *et al.*, 1984; McCormack, 1985; Hansford, 1985). However, the position with NAD-ICDH is less clear. In the presence of ADP, the extracted or purified enzyme has been reported to exhibit $K_{0.5}$ values for Ca²⁺ close to 1 μ M

(Denton *et al.*, 1978; Aogaichi *et al.*, 1980), but under other conditions, for example in the presence of ATP, much higher values have been observed (Gabriel & Plaut, 1984; Gabriel *et al.*, 1985, 1986). Moreover, it is difficult to measure flux through NAD-ICDH in intact mitochondria unambiguously.

In this study, we have used rat heart mitochondria, permeabilized with toluene to all low- M_r compounds (Matlib *et al.*, 1977; Thomas & Denton, 1986) to allow the unambiguous study of the kinetic properties of NAD-ICDH and OGDH *in situ* and under essentially identical conditions. In a previous study, we demonstrated that the kinetic properties with respect to Ca²⁺ of PDHP phosphatase within permeabilized mitochondria were markedly different from those apparent in extracts (Thomas *et al.*, 1986; Midgley *et al.*, 1987).

EXPERIMENTAL

Materials

All chemicals and biochemicals were supplied by Boehringer, Lewes, East Sussex, U.K., or BDH, Poole, Dorset, U.K., or Sigma, Poole, Dorset, U.K., except rotenone and HEDTA [*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (Aldrich Chemical Co., Gillingham, Dorset, U.K.)], Sagatal (May and Baker, Dagenham, Essex, U.K.) and Nagarse (Teikoku Chemical Industry Co., Osaka, Japan). Arsenazo III (Ca²⁺-free) was kindly

Abbreviations used: NAD-ICDH, NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.41); OGDH, 2-oxoglutarate dehydrogenase; PDH, PDHP, pyruvate dehydrogenase and pyruvate dehydrogenase phosphate respectively; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; PEG, poly(ethylene glycol) 6000. Throughout this paper, Ca²⁺ and Mg²⁺ refer to the free unbound species of these bivalent metal ions.

given by Dr. A. P. Halestrap (this Department). Anti-serum was raised in chickens to OGDH purified from pig hearts as described by McCormack & Denton (1979).

Preparation and toluene permeabilization of mitochondria

Rat heart mitochondria were prepared essentially as described by Halestrap (1987), involving Nagarse digestion (Chappell & Hansford, 1972) and purification on a Percoll gradient (Belsham *et al.*, 1980) in 'isolation medium' containing 250 mM-sucrose, 20 mM-Tris/HCl (pH 7.4) and 2 mM-EGTA. The final mitochondrial pellet was either resuspended at 20–30 mg/ml in isolation medium plus 8.5% (w/v) poly(ethylene glycol) 6000 (PEG) and 0.05% (w/v) essentially-fatty-acid-free bovine serum albumin for permeabilization, or frozen at the temperature of liquid N₂ for subsequent extraction of enzymes (see below).

Toluene permeabilization of mitochondria was as described by Thomas & Denton (1986). Mitochondrial protein concentration was determined by the method of Gornall *et al.* (1949); recovery through the permeabilization procedure was $\geq 90\%$.

Preparation of mitochondrial extracts

Unless otherwise indicated, these were prepared as described by McCormack & Denton (1981). In some experiments (e.g. Fig. 5) extracts of permeabilized mitochondria were prepared in the cuvette by incubation with 0.2% (v/v) Triton X-100 in PEG-free medium.

Assay of enzymes within permeabilized mitochondria and mitochondrial extracts

All assays were carried out at 30 °C in 1 ml of a 'basic incubation medium' consisting of 50 mM-Mops, 35.5 mM-triethanolamine (pH 7.2 unless otherwise stated), 75 mM-KCl, 60 mM-sucrose, 2 mM-KH₂PO₄, 1 mM-EGTA, 1 mM-HEDTA, rotenone (1 µg/ml), and sufficient CaCl₂ and MgCl₂ to give the free concentrations of Ca²⁺ indicated and 1 mM free Mg²⁺ unless otherwise stated. For studies involving Sr²⁺, HEDTA was omitted, and the concentration of EGTA was 5 mM. PEG (8.5%, w/v) was included for all assays of enzymes within permeabilized mitochondria, and oligomycin (1 µg/ml) for all assays in which ATP was present. Malate dehydrogenase (MDH) was assayed by the addition of 0.2 mM-oxaloacetate plus 0.0–0.4 mM-NADH, NAD-ICDH with 2 mM-NAD⁺ and varying DL-isocitric acid (trisodium salt; 50% *threo*-D_s-isocitrate), and OGDH with 2 mM-NAD⁺, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA, plus varying 2-oxoglutarate. Glutamate dehydrogenase (GDH) was assayed as described by Thomas & Denton (1986). A Pye-Unicam PU-8800 spectrophotometer, equipped to enable positioning of cuvettes close to the photomultiplier (necessary to minimize light-scattering) was used and time-course data were analysed with a Hewlett-Packard 9000/300 computer. Permeabilized mitochondria (25–200 µg of protein/ml) were preincubated for up to 1 min before initiation of reactions with appropriate substrate. With mitochondrial extracts, assays were initiated with either substrate, as described above, or extract. All measurements of activity were based on initial rates, except for NAD-ICDH assayed in permeabilized mitochondria, where linear rates were achieved after an initial short lag period (approx. 30 s).

ATPase activity of mitochondrial preparations was determined in 'basic incubation medium' by measuring the production of ADP from ATP by linked enzymic assay (Scharschmidt *et al.*, 1979).

Control of free concentrations of Ca²⁺ and Mg²⁺ ions

Concentrations of free and bound ligands were calculated as described by Thomas & Denton (1986) and Midgley *et al.* (1987).

Owing to the variations in the purity of commercially available EGTA (Miller & Smith, 1984), stock EGTA solutions were standardized by the following methods: (i) titration with NaOH of protons released on adding excess CaCl₂; (ii) direct measurement of proton release with a pH-meter, on adding CaCl₂; (iii) titration with CaCl₂ in the presence of ammonium oxalate. The results of these three methods agreed to within 1.0%.

Arsenazo III was standardized by the method of Kendrick (1976). Under standard assay conditions a Ca–arsenazo III dissociation constant (K_d) of 43.2 ± 1.4 µM was determined spectrophotometrically. On the basis of this value, free Ca²⁺ concentrations used in all assays were checked after adding 35 µM–arsenazo III to the medium, and were found to be in good agreement with calculated values. Further checks of free Ca²⁺ concentrations were also made with a Ca²⁺-specific electrode (Ammann *et al.*, 1975). Again, this technique indicated close correspondence between calculated and measured free Ca²⁺ concentrations, when based on the extrapolation of measured free Ca²⁺ at high concentrations of added CaCl₂ (0.01–1.0 mM), where $> 99\%$ of added Ca²⁺ was free, to measurements at low buffered Ca²⁺ concentrations (0.1–10 µM). No difference in free Ca²⁺ was detected by this technique in the presence and the absence of 8.5% PEG.

Handling of data and calculation of kinetic constants

Kinetic constants were calculated by fitting data to equations of the type:

$$v - V_{\min.} = V_{\max.} / \{1 + (K_{0.5}/[S])^h\}$$

where [S] represents the concentration of substrate or metal ion, $K_{0.5}$ the concentration of substrate or metal ion giving half-maximal effects (equivalent to the Michaelis constant, K_m , for the substrate) and h is the Hill coefficient. Data were fitted with a BBC Master computer by using a non-linear least-squares regression program written by Dr. P. J. England (Smith, Kline and French Research, Welwyn, Herts., U.K.). Results are given as parameter value $\pm$ s.d. for the degrees of freedom shown in parentheses, usually based on observations with one preparation. However, all these results were typical of between two and five experiments on separate preparations. One unit of enzyme activity is defined as the amount catalysing the conversion of 1 µmol of substrate/min at 30 °C.

RESULTS

Preparation and assessment of permeabilized mitochondria

In the present study, toluene treatment of heart mitochondria by the method of Thomas & Denton (1986) gave a stable preparation of permeabilized mitochondria in which the activities of NAD-ICDH, OGDH, GDH and MDH were fully expressed while the

Fig. 1. Accessibility of OGDH to anti-ODGH antiserum in permeabilized mitochondria

Permeabilized mitochondria ($\sim 25 \mu\text{g/ml}$) were incubated as described in the Experimental section. In each case, 'basic incubation medium' was supplemented with 2 mM-NAD⁺, 10 mM-oxoglutarate and 1 mM-thiamin pyrophosphate plus the following additions: (i) 20 μl of anti-ODGH antiserum, 0.2% Triton X-100; (ii) 20 μl of anti-ODGH antiserum; (iii) 20 μl of control serum. At (a) 0.25 mM-CoA was added to initiate OGDH activity in all cuvettes, and at (b) 0.2% Triton X-100 was added to (ii) and (iii) only.

enzymes were retained within the mitochondrial matrix. Under standard assay conditions (see the Experimental section) in the presence of 8.5% PEG, 80–90% of total NAD-ICDH activity could be sedimented (10000 *g*, 5 min), whereas after incubation with 0.2% Triton X-100 less than 20% of the activity was sedimentable.

In contrast, disruption of mitochondria in the presence of PEG had little apparent effect on the sedimentability of OGDH, with approx. 75% of the total activity remaining sedimentable after incubation with up to 1% Triton X-100. This is not surprising, as use is made of the precipitation of OGDH at low PEG concentrations in its purification (Linn *et al.*, 1972; McCormack & Denton, 1979). However, essentially all OGDH activity could be shown to be located within the permeabilized mitochondria by the fact that it was inaccessible to an antiserum to the enzyme which inhibited catalytic activity until Triton X-100 was added (Fig. 1).

Effects of Ca²⁺ and adenine nucleotides on the sensitivity of NAD-ICDH to *threo*-D_s-isocitrate

Fig. 2 illustrates the effects of Ca²⁺ ions and adenine nucleotides on the activity of NAD-ICDH at various concentrations of *threo*-D_s-isocitrate in permeabilized mitochondria and mitochondrial extracts. Corresponding kinetic data are given in Table 1.

The response of NAD-ICDH to Ca²⁺, ADP and ATP when assayed within permeabilized mitochondria was qualitatively the same as that observed in extracts, with essentially additive effects of Ca²⁺ and adenine nucleotides on the *K_m* for *threo*-D_s-isocitrate in both cases. However, Ca²⁺ ions had no effect in the absence of adenine nucleotides, as previously observed with the extracted enzyme (Denton *et al.*, 1978; Aogaichi *et al.*, 1980). In all cases *K_m* values for *threo*-D_s-isocitrate were higher in the presence of ATP than with ADP alone or ADP plus ATP, indicating that *in vivo* the enzyme may become increasingly activated with increases in the mitochondrial ratio of ADP/ATP in the range 0:1–1:1.

As shown in Table 1, the maximal activity of the enzyme was essentially the same in permeabilized mitochondria and extracts. Disruption of permeabilized mitochondria with Triton X-100 had little effect on this parameter, with the activity in the absence of the detergent being $\geq 90\%$ of that in the presence of 0.2% Triton X-100. However, *K_m* values for *threo*-D_s-isocitrate were consistently 2–4-fold higher in extracts under all the conditions studied.

Effects of Ca²⁺ and adenine nucleotides on the sensitivity of OGDH to 2-oxoglutarate

The effects of Ca²⁺ ions on OGDH activity assayed in permeabilized mitochondria and mitochondrial extracts in the presence and absence of ADP, ATP, or both are given in Table 2. Again, maximal activities were essentially the same in permeabilized mitochondria, in both the presence and the absence of Triton X-100, and in mitochondrial extracts. The difference in *K_m* values for substrate between permeabilized mitochondria and extracts was quite small for this enzyme. As with NAD-ICDH, *K_m* values for substrate were greater in the presence of ATP than with ATP/ADP (1:1) or ADP alone, at least in the absence ($< 1 \text{ nM}$) of Ca²⁺. However, in the presence of saturating Ca²⁺, *K_m* values for 2-oxoglutarate were similar in the presence of either ADP or ATP, indicating that under these conditions the activating effect of high ADP/ATP ratios may be by-passed. Activities with ATP were similar to those observed in the absence of any added adenine nucleotide. This is in contrast with previous studies with the purified pig heart enzyme (McCormack & Denton, 1979), where inhibition by additions of ATP was observed at sub-saturating concentrations of 2-oxoglutarate. These differences may be the result of species differences between pig heart and rat heart OGDH. Alternatively, the Mg-ATP chelate (the form of 90–95% of total ATP at the concentration of free Mg²⁺ (1 mM) used in the present studies) may be a rather weaker inhibitor of the enzyme than free ATP. Hydrolysis of ATP may be excluded, since ATPase activity of mitochondrial preparations, measured under standard assay conditions (1.5 mM-ATP, 1 mM-Mg²⁺) was less than 30 munits/mg of mitochondrial protein, so that, after a 2 min incubation at 200 μg of mitochondrial protein/ml, less than 2% of total ATP was hydrolysed.

Sensitivity of NAD-ICDH and OGDH to Ca²⁺ ions; effects of adenine nucleotides

K_{0.5} values for Ca²⁺ and Sr²⁺ of NAD-ICDH and OGDH under a number of different conditions are given in Table 3, and the response to Ca²⁺ of the two enzymes in the presence of either ADP or ATP shown in Fig. 3.

Fig. 2. Effects of Ca^{2+} , ADP and ATP on the activity of NAD^{+} -ICDH dehydrogenase in (a) permeabilized mitochondria and (b) mitochondrial extracts

Measurements of NAD-ICDH activity were made in 'basic incubation medium' (see the Experimental section) supplemented with 2 mM- NAD^{+} and appropriate additions of DL-isocitrate together with sufficient CaCl_2 to give 0.1 mM- Ca^{2+} (●, ■, ▲) or no added CaCl_2 (i.e. $\text{Ca}^{2+} < 0.1$ nM), plus no further additions (△, ▲), 1.5 mM-ADP (○, ●) or 1.5 mM-ATP (□, ■). The calculated $[\text{Mg}^{2+}]$ was 1 mM in the absence of added DL-isocitrate, falling to 0.5 mM at the highest concentration of DL-isocitrate added. Calculated kinetic constants are given in Table 1.

Table 1. Kinetic parameters of NAD-ICDH in permeabilized mitochondria and mitochondrial extracts

Details of incubation of mitochondria and mitochondrial extracts, measurements of activity and calculation of constants are given in the Experimental section and in the legend to Fig. 2. V_{max} values (in munits/mg of mitochondrial protein) were 51.0 ± 5.7 (16) for permeabilized mitochondria and 59.5 ± 7.0 (16) for mitochondrial extracts and were essentially unaffected by Ca^{2+} and adenine nucleotides.

Additions		Parameter value for NAD-ICDH activity measured in:			
		Permeabilized mitochondria		Extracts	
Ca^{2+}	Adenine nucleotides	K_m for <i>threo</i> -D ₃ -isocitrate (μM)	h	K_m for <i>threo</i> -D ₃ -isocitrate (μM)	h
< 1 nM	—	557 ± 11 (11)	2.7	1082 ± 22 (10)	3.7
	ADP (1.5 mM)	169 ± 5 (15)	1.9	554 ± 14 (8)	3.8
	ADP (0.75 mM), ATP (0.75 mM)	185 ± 8 (7)	2.4	697 ± 28 (8)	2.8
	ATP (1.5 mM)	460 ± 20 (10)	2.4	1140 ± 45 (14)	2.5
0.1 mM	—	545 ± 17 (11)	3.2	992 ± 30 (10)	4.1
	ADP (1.5 mM)	60 ± 4 (7)	1.8	162 ± 4 (8)	3.0
	ADP (0.75 mM), ATP (0.75 mM)	63 ± 5 (9)	1.6	179 ± 10 (7)	3.6
	ATP (1.5 mM)	196 ± 6 (13)	3.2	339 ± 12 (12)	3.0

In all cases concentrations of *threo*-D₃-isocitrate and 2-oxoglutarate were carefully chosen to ensure that rates observed at saturating Ca^{2+} were always in the range of 50–75% of the maximal activity of the enzymes. Three important points can be made from these data.

Firstly, when assayed under a number of identical conditions, the $K_{0.5}$ for Ca^{2+} of NAD-ICDH is at least an order of magnitude greater than that of OGDH. With

the extracted enzymes this marked difference was somewhat decreased if KCl and sucrose were omitted from the incubation medium. In this case, $K_{0.5}$ values of NAD-ICDH for Ca^{2+} were 7.4–0.6 μM (20) with 1.5 mM-ADP and $20.0 \pm 2.7 \mu\text{M}$ (19) with 1.5 mM-ATP. Similarly, with permeabilized mitochondria, lowering the Mg^{2+} concentration to 0.1 mM decreased the $K_{0.5}$ values of NAD-ICDH for Ca^{2+} , giving values of

Table 2. Kinetic parameters of OGDH in permeabilized mitochondria and mitochondrial extracts

Details of incubations and calculation of constants are given in the Experimental section. $V_{\max}$ values (in munits/mg of mitochondrial protein) were 169 ± 19 (12) for permeabilized mitochondria and 133 ± 14 (12) for mitochondrial extracts and were essentially unchanged by Ca²⁺ and adenine nucleotides. Hill coefficients (h) were in the range 0.9–1.6 and are not given.

Additions		K_m (μ M) for 2-oxoglutarate of OGDH measured in:	
Ca ²⁺	Adenine nucleotides	Permeabilized mitochondria	Extracts
< 1 nM	–	1335 $\pm$ 88 (8)	1653 $\pm$ 121 (11)
	ADP (1.5 mM)	153 $\pm$ 12 (12)	221 $\pm$ 21 (12)
	ADP (0.75 mM), ATP (0.75 mM)	176 $\pm$ 13 (10)	356 $\pm$ 29 (12)
	ATP (1.5 mM)	856 $\pm$ 70 (8)	1280 $\pm$ 170 (10)
0.1 mM	–	348 $\pm$ 69 (12)	359 $\pm$ 22 (13)
	ADP (1.5 mM)	69 $\pm$ 6 (11)	71 $\pm$ 6 (12)
	ADP (0.75 mM), ATP (0.75 mM)	108 $\pm$ 27 (11)	87 $\pm$ 7 (12)
	ATP (1.5 mM)	87 $\pm$ 19 (9)	105 $\pm$ 10 (11)

Table 3. $K_{0.5}$ values for Ca²⁺ and Sr²⁺ of NAD-ICDH and OGDH in permeabilized mitochondria and mitochondrial extracts

Measurements of activity and calculation of constants were as described in the Experimental section and in the legend to Fig. 2. Concentrations of *threo*-D₃-isocitrate were: for permeabilized mitochondria, 0.1 mM (ADP alone, ADP plus ATP) or 0.2 mM (ATP alone); for extracts, 0.1 mM (ADP alone, ADP plus ATP) or 1.0 mM (ATP alone). Concentrations of 2-oxoglutarate (identical for permeabilized mitochondria and extracts) were: 0.05 mM (ADP alone); 0.1 mM (ADP plus ATP, ATP alone); 0.2 mM (no nucleotide). Hill coefficients (h) were in the range 0.9–1.9 and are not given.

Parameter	Adenine nucleotides	Parameter values for enzymes assayed in:			
		Permeabilized mitochondria		Extracts	
		NAD-ICDH	OGDH	NAD-ICDH	OGDH
$K_{0.5}$ (μ M) for Ca ²⁺	–	–	0.24 $\pm$ 0.026 (11)	–	1.2 $\pm$ 0.13 (12)
	ADP (1.5 mM)	5.4 $\pm$ 0.7 (28)	0.28 $\pm$ 0.04 (18)	15.8 $\pm$ 0.7 (27)	0.21 $\pm$ 0.3 (10)
	ADP (0.75 mM) + ATP (0.75 mM)	10.5 $\pm$ 1.8 (25)	0.19 $\pm$ 0.04 (15)	13.0 $\pm$ 1.7 (14)	0.41 $\pm$ 0.05 (8)
	ATP (1.5 mM)	41.0 $\pm$ 8.2 (30)	0.81 $\pm$ 0.08 (22)	43.2 $\pm$ 2.5 (31)	2.12 $\pm$ 0.19 (12)
$K_{0.5}$ (μ M) for Sr ²⁺	ADP (1.5 mM)	21.8 $\pm$ 2.4 (21)	2.3 $\pm$ 0.02 (9)	> 100	2.43 $\pm$ 0.4 (13)
	ATP (1.5 mM)	31.5 $\pm$ 7.0 (11)	17.6 $\pm$ 0.8 (11)	> 100	35.5 $\pm$ 2.6 (11)

Fig. 3. Sensitivity to Ca²⁺ of NAD-ICDH (Δ , $\blacktriangle$) and OGDH ($\circ$, $\bullet$) in permeabilized mitochondria

NAD-ICDH and OGDH activities were measured as described in the Experimental section in the presence of either 1.5 mM-ADP (Δ , $\circ$) or 1.5 mM-ATP ($\bullet$, $\blacktriangle$). Calculated kinetic constants are given in Table 3, where further details of conditions can be found.

3.2 $\pm$ 0.4 μ M (14) and 20.0 $\pm$ 2.0 μ M (12) in the presence of 1.5 mM-ADP and 1.5 mM-ATP respectively. Neither of the above alterations in incubation media had a significant effect on the $K_{0.5}$ for Ca²⁺ of OGDH (results not shown).

Secondly, $K_{0.5}$ values for Ca²⁺ of both enzymes are 2–10-fold higher when assayed in the presence of ATP than in the presence of ADP. Evidence for this with purified bovine heart NAD-ICDH has been reported (Gabriel *et al.*, 1986). With OGDH these effects were also matched by changes in sensitivity to Sr²⁺ ions.

Finally, under certain conditions, sensitivities of both enzymes to Ca²⁺ (and Sr²⁺) were greater within permeabilized mitochondria than for extracts. This would appear to be due to real differences between the properties of the enzymes within permeabilized mitochondria and those in extracts rather than to any gradient of Ca²⁺, since addition of the bivalent-metal ionophore A23187 (5 μ M), the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 1 μ M) and the K⁺ ionophore valinomycin (0.5 μ M) did not alter the sensi-

Fig. 4. Effects of changes in ADP/ATP ratio on (a) NAD-ICDH and (b) OGDH activities in permeabilized mitochondria in the presence of (●) 1 nM-, (■) 1 μ M- and (▲) 0.1 mM- Ca^{2+} .

For NAD-ICDH assays the concentration of *threo*-D₅-isocitrate was 0.15 mM, and in OGDH assays the 2-oxoglutarate concentration was 0.05 mM.

tivity of OGDH within permeabilized mitochondria to Ca^{2+} .

Effects of ADP/ATP ratio on NAD-ICDH and OGDH located within permeabilized mitochondria: modulation by Ca^{2+}

Previous studies of purified OGDH (McCormack & Denton, 1979) and NAD-ICDH (Gabriel *et al.*, 1986) have shown that increases in ADP/ATP ratio result in a decrease in K_m values for the respective substrates. Fig. 4 shows the response of NAD-ICDH and OGDH to changes in ADP/ATP ratio, when assayed within permeabilized mitochondria and at sub-saturating concentrations of substrates. Both enzymes showed a response to changes in the ratio of ADP/ATP, which was altered by Ca^{2+} . However, whereas OGDH was most sensitive to a change in the ratio in the absence (< 1 nM) of Ca^{2+} , with NAD-ICDH the response was greatest at high concentrations (0.1 mM) of Ca^{2+} .

In addition, the results shown in Fig. 4 also illustrate the range over which changes in ADP/ATP ratio may affect the sensitivity of the two enzymes to Ca^{2+} . In both cases a steady increase in the stimulation by 1 μ M- Ca^{2+} as a fraction of the total stimulation achieved at saturating Ca^{2+} occurred up to an ADP/ATP ratio of around 1.0, indicating a decrease in $K_{0.5}$ values for Ca^{2+} . At ADP/ATP ratios greater than this, stimulation by 1 μ M- Ca^{2+} as a function of maximal stimulation remained relatively constant.

Effects of NADH/NAD⁺ ratio on OGDH in permeabilized mitochondria and mitochondrial extracts

During the course of the kinetic studies described above, it was noticed that the extent of non-linearity in assays of OGDH was much less when the enzyme was assayed in permeabilized mitochondria than in extracts (Fig. 5a). Such differences were not apparent for NAD-ICDH activity.

A decline in OGDH activity with time, attributed to feedback inhibition by increasing concentrations of NADH and succinyl-CoA, has previously been observed in studies of the pure enzyme (Garland, 1964; Smith *et al.*, 1974; McCormack & Denton, 1979). In the present studies, succinyl-CoA alone was found to have little inhibitory effect on the enzyme, assayed either in permeabilized mitochondria or in extracts (results not

shown). Hence feedback inhibition appeared to be largely due to increasing concentrations of NADH. That the difference in non-linearity between permeabilized mitochondria and mitochondrial extracts corresponds to a lower sensitivity to increasing NADH/NAD⁺ ratios is further suggested by the observations shown in Fig. 5(b) in which initial rates of activity were determined at various NADH/NAD⁺ ratios. The apparently lower sensitivity to inhibition by increasing NADH/NAD⁺ ratios in permeabilized mitochondria would appear not to be the result of any gradient of NADH across the mitochondrial inner membrane, since the K_m of malate dehydrogenase for NADH was unchanged between extracts and permeabilized mitochondria (results not shown).

As first described by Lawlis & Roche (1980, 1981), Ca^{2+} was found to reverse partially the inhibitory effects of high NADH/NAD⁺ ratios. However, this effect was again less marked with permeabilized mitochondria than with extracts (Fig. 5b). These results indicate that inhibition of OGDH *in vivo* by an increase in the redox state of the mitochondrial NAD⁺ pool may be quantitatively less significant than has previously been thought (Smith *et al.*, 1974; Hansford, 1980; Williamson & Cooper, 1980). However, the data are consistent with the suggestion by Lawlis & Roche (1980, 1981) that Ca^{2+} ions may be important in suppressing feedback inhibition of OGDH by high NADH/NAD⁺ ratios.

DISCUSSION

Kinetic properties of NAD-ICDH and OGDH when located within permeabilized mitochondria compared with those evident in extracts

Although the enzymes exhibited broadly similar properties when assayed within toluene-permeabilized mitochondria to those found under the same assay conditions in extracts of mitochondria, a number of notable differences have been observed. Firstly, the K_m of NAD-ICDH for its substrate, *threo*-D₅-isocitrate, was markedly lower when the enzyme was located within permeabilized mitochondria under a wide variety of assay conditions. The K_m of OGDH for its substrate, 2-oxoglutarate, also exhibited a tendency to be lower, but the differences here were much less marked than for

Fig. 5. Feedback inhibition of OGDH by NADH/NAD⁺ ratios in permeabilized mitochondria and mitochondrial extracts, in the presence and the absence of 25 μM -Ca²⁺

(a) Time courses of NADH production by OGDH within intact permeabilized mitochondria (trace i) and in permeabilized mitochondria disrupted by incubation in the absence of PEG and in the presence of 0.2% Triton X-100 (trace ii). Broken lines show the changes in OGDH activity with time obtained by differentiation of the time courses of NADH production (Midgley *et al.*, 1987). Assay of OGDH was as given in the Experimental section, and the concentration of 2-oxoglutarate was 10 mM in each case. (b) Sensitivity of initial rates of OGDH activity to changes in NADH/NAD⁺ ratio within intact permeabilized mitochondria (●, ▲), or in disrupted mitochondria (○, △) assayed as for (a). Ca²⁺ concentration was either 1 mM (○, ●) or 0.1 mM (△, ▲) and 2-oxoglutarate concentration was 10.0 mM (○, ●) or 0.375 mM (□, ■). Assays were carried out at pH 7.4, with 0.35 mM-Mg²⁺ and 0.4 mM total nicotinamide nucleotide.

NAD-ICDH, and under a number of conditions none were evident. Secondly, the end-product inhibition of OGDH by NADH was found to be much less when the enzyme was within intact mitochondria, whereas there was little or no difference in the inhibition of NAD-ICDH by NADH in permeabilized mitochondria and in extracts. Finally, under certain circumstances, the $K_{0.5}$ for Ca²⁺ activation of both NAD-ICDH and OGDH was appreciably lower in mitochondria than in extracts. For example, the values for the activation of OGDH in the absence of adenine nucleotide were 1.2 μM and 0.24 μM in extracts and in permeabilized mitochondria respectively.

We have considered the possibility that these differences may simply reflect gradients of substrates and effectors across the mitochondrial inner membrane even

after permeabilization by toluene treatment. However, as described in the Results section, we could find no evidence for any such gradients, which, in any case, would not explain why the difference seen with one dehydrogenase was greater than the other in every individual case.

Effects of changes in ADP/ATP ratios on the kinetic properties of NAD-ICDH and OGDH

In the course of the present studies, we have confirmed that increases in ADP/ATP ratios result in a marked increase in the activity of both NAD-ICDH and OGDH in mitochondrial extracts and extended this observation to permeabilized mitochondria. We have also shown that increases in this ratio not only decrease the K_m of the two dehydrogenases for their respective substrates, *threo*-D_s-isocitrate and 2-oxoglutarate, but also decrease $K_{0.5}$ values for Ca²⁺. In all cases, it was only changes in the ADP/ATP ratio below 1.0 which resulted in changes in the activities of the two dehydrogenases. Measurements of mitochondrial ADP/ATP ratios within the intact heart (Soboll & Bunge, 1981), as well as in isolated liver cells (Siess *et al.*, 1977; Akerboom *et al.*, 1978; Soboll *et al.*, 1980) and freeze-clamped livers (Soboll *et al.*, 1984) have each given values for this ratio in the range 0.3–1.2, suggesting that changes in the mitochondrial ADP/ATP ratio may be important in determining the activity of the two enzymes *in vivo*, at least under some conditions. For example, in hearts perfused in medium containing pyruvate, there is evidence based on n.m.r. determinations of ATP, P_i and phosphocreatine concentrations that increased work is associated with increases in the cytoplasmic ADP/ATP ratio (From *et al.*, 1986). It seems reasonable to conclude that under these conditions there may be increases in the intramitochondrial ADP/ATP ratio, leading to the stimulation not only of oxidative phosphorylation but also of the supply of NADH for the respiratory chain through the activation of NAD-ICDH and OGDH. However, in direct contrast, the same authors (From *et al.*, 1986) found that, in hearts perfused with medium containing glucose, increased work appeared to have no effect on the tissue ADP/ATP ratio. Similar observations to these have been made by others (Neely *et al.*, 1972; Balaban *et al.*, 1986; Katz *et al.*, 1987). Under these more physiological circumstances, it seems likely that increases in respiration are secondary to activation of intramitochondrial dehydrogenases by Ca²⁺ (Denton & McCormack, 1980, 1985; Hansford, 1985; McCormack & Denton, 1986). Fluorescence studies (Katz *et al.*, 1987) have indicated that mitochondrial NADH concentrations may be increased under these conditions, which provides further evidence for this view.

Sensitivity of NAD-ICDH and OGDH to Ca²⁺

Previous studies (Denton *et al.*, 1978, 1980; McCormack & Denton, 1981) using extracts of rat heart mitochondria have indicated that the $K_{0.5}$ for Ca²⁺ of NAD-ICDH in the presence of ADP may be greater than that of OGDH, but the apparent differences were much smaller than those found in the present studies. There appear to be two reasons for this. Firstly, our earlier studies were carried out in buffers with ionic strengths lower than those likely to be found within mitochondria. As noted in the Results section, addition of KCl (and sucrose) increased observed $K_{0.5}$ values for Ca²⁺ of NAD-

ICDH while having little effect on the values for OGDH. Secondly, EGTA was used as the sole buffer of Ca^{2+} in our earlier studies. Although excellent control of low ($\leq 1 \mu\text{M}$) free Ca^{2+} concentrations is achieved with this buffer, control of high concentrations of Ca^{2+} is rather poor, so that above $1 \mu\text{M}$ - Ca^{2+} a small change (i.e. $\geq 2\%$) in total EGTA or CaCl_2 can have a profound effect on free Ca^{2+} concentration. Variations in the purity of most commercially available EGTA, makes precise standardization essential (Miller & Smith, 1984). The procedure we used in the past for the standardization of CaEGTA buffers (Denton *et al.*, 1978) underestimated the Ca/EGTA ratio, by 1–2%. At pH 7.0–7.4 the errors in calculating Ca^{2+} concentrations below $1 \mu\text{M}$ are negligible, but above $1 \mu\text{M}$ the errors can become progressively greater. In the present work we have not only standardized the EGTA more precisely but also adopted the use of HEDTA (Thomas *et al.*, 1986) in combination with EGTA to ensure the accurate buffering of Ca^{2+} up to much higher concentrations, while at the same time controlling Mg^{2+} concentrations in the physiological range (0.1–2 mM; Corkey *et al.*, 1986; Jung & Brierly, 1986).

Since $K_{0.5}$ values for Ca^{2+} of NAD-ICDH, especially at low ADP/ATP ratios, appear to be somewhat above the concentrations of free Ca^{2+} likely for the mitochondrial matrix *in vivo* (Hansford & Castro, 1982; Crompton *et al.*, 1983; Hansford, 1985), the role of activation of NAD-ICDH by Ca^{2+} is now rather unclear. It follows that the major effects of increases in Ca^{2+} concentration on intramitochondrial metabolism are likely to be the activation of OGDH along with the PDH complex (Denton & McCormack, 1980): $K_{0.5}$ values for Ca^{2+} of PDHP phosphatase, determined as described by Midgley *et al.* (1987), were in the range 0.4–0.7 μM in permeabilized rat heart mitochondria (G. A. Rutter & R. M. Denton, unpublished work).

In apparent conflict with this conclusion, estimates of $K_{0.5}$ values for Ca^{2+} of NAD-ICDH within intact uncoupled brown-adipose-tissue mitochondria (McCormack & Denton, 1980) and coupled mitochondria from white adipose tissue (Marshall *et al.*, 1984) and liver (McCormack, 1985; Johnston & Brand, 1987) have given strikingly similar $K_{0.5}$ values for Ca^{2+} -activation to those found for OGDH and the PDH system. However, it is possible that the rate of isocitrate oxidation in these mitochondria may have been determined by the activity of measuring flux through this enzyme in the absence of observed stimulatory effect of Ca^{2+} could have been largely the result of activation of OGDH. Unequivocal determination of the sensitivity of NAD-ICDH within intact (unpermeabilized) mitochondria requires a means of measuring flux through this enzyme in the absence of OGDH activity. Attempts to block OGDH activity within intact mitochondria with, for example, arsenite, have so far proved unsuccessful (J. G. McCormack, G. A. Rutter & R. M. Denton, unpublished work), possibly owing to the interference of this agent with mitochondrial phosphate transport.

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REFERENCES

- Akerboom, T. P. M., Bookelman, H., Zuurendonk, P. F., van der Meer, R. & Tager, J. M. (1978) *Eur. J. Biochem.* **84**, 413–420
- Ammann, D., Guggi, M., Pretsch, E. & Simon, W. (1975) *Anal. Lett.* **8**, 707–720
- Aogaichi, T., Evans, J., Gabriel, J. & Plant, G. W. E. (1980) *Arch. Biochem. Biophys.* **195**, 30–34
- Balaban, R. S., Kantor, H. L., Katz, L. A. & Briggs, R. W. (1986) *Science* **232**, 1121–1122
- Belsham, G. J., Denton, R. M. & Tanner, M. J. A. (1980) *Biochem. J.* **192**, 457–467
- Brand, M. D. & Murphy, M. P. (1987) *Biol. Rev. Cambridge Philos. Soc.* **62**, 141–193
- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation*, 2nd edn. (Birnie, G. D., ed.), pp. 71–79, Butterworths, London
- Corkey, B. A., Duszynski, J., Rich, T. L., Matschinsky, B. & Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 2567–2574
- Crompton, M., Kessar, P. & Al-Nasser, I. (1983) *Biochem. J.* **216**, 333–342
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Denton, R. M. & McCormack, J. G. (1985) *Am. J. Physiol.* **249**, E543–E554
- Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
- Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
- Denton, R. M., McCormack, J. G. & Edgell, W. J. (1980) *Biochem. J.* **190**, 107–117
- From, A. H. L., Petein, M. A., Michurski, S. P., Zimmer, S. D. & Ugurbil, K. (1986) *FEBS Lett.* **206**, 257–261
- Gabriel, J. L. & Plaut, G. W. E. (1984) *Biochemistry* **23**, 2773–2778
- Gabriel, J. L., Milner, R. & Plaut, G. W. E. (1985) *Arch. Biochem. Biophys.* **240**, 128–134
- Gabriel, J. L., Zervos, P. R. & Plaut, G. W. E. (1986) *Metab. Clin. Exp.* **35**, 661–667
- Garland, P. B. (1964) *Biochem. J.* **92**, 10C–12C
- Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Halestrap, A. P. (1987) *Biochem. J.* **244**, 159–164
- Hansford, R. G. (1980) *Curr. Top. Bioenerg.* **10**, 217–278
- Hansford, R. G. (1985) *Rev. Physiol. Biochem. Pharmacol.* **102**, 1–62
- Hansford, R. G. & Castro, F. (1982) *J. Bioenerg. Biomembr.* **14**, 361–376
- Johnston, J. D. & Brand, M. D. (1987) *Biochem. J.* **245**, 217–222
- Jung, D. W. & Brierly, G. P. (1986) *J. Biol. Chem.* **261**, 6408–6415
- Katz, L. A., Koretsky, A. P. & Balaban, R. S. (1987) *FEBS Lett.* **221**, 270–276
- Kendrick, N. C. (1976) *Anal. Biochem.* **76**, 487–501
- Lawlis, T. E. & Roche, V. B. (1980) *Mol. Cell. Biochem.* **32**, 147–152
- Lawlis, T. E. & Roche, V. B. (1981) *Biochemistry* **20**, 2519–2524
- Linn, T. C., Pelley, J. W., Pettit, F. H., Randall, D. D. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 327–342
- Marshall, S. E., McCormack, J. G. & Denton, R. M. (1984) *Biochem. J.* **218**, 249–260
- Matlib, M. A., Shannon, W. A. & Srere, P. A. (1977) *Arch. Biochem. Biophys.* **179**, 396–407
- McCormack, J. G. (1985) *Biochem. J.* **231**, 581–595
- McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544
- McCormack, J. G. & Denton, R. M. (1980) *Biochem. J.* **190**, 95–105

- McCormack, J. G. & Denton, R. M. (1981) *Biochem. J.* **196**, 619–624
- McCormack, J. G. & Denton, R. M. (1986) *Trends Biochem. Sci.* **11**, 258–262
- Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & Denton, R. M. (1987) *Biochem. J.* **241**, 371–377
- Miller, D. J. & Smith, G. L. (1984) *Am. J. Physiol.* **246**, C160–C166
- Neely, J. R., Denton, R. M., England, P. J. & Randle, P. J. (1972) *Biochem. J.* **128**, 147–159
- Scharschmidt, B. F., Keefe, E. B., Blankenship, N. M. & Ockner, R. K. (1979) *J. Lab. Clin. Med.* **93**, 790–799
- Siess, E. A., Brocks, D. G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235
- Smith, C. M., Bryla, J. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 1497–1505
- Soboll, S. & Bunger, R. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 125–132
- Soboll, S., Akerman, T. P. M., Schwenke, W.-D., Haave, R. & Sies, H. (1980) *Biochem. J.* **192**, 951–954
- Soboll, S., Sietz, H. J., Sies, H., Ziegler, B. & Scholz, R. (1984) *Biochem. J.* **220**, 371–376
- Thomas, A. P. & Denton, R. M. (1986) *Biochem. J.* **238**, 93–101
- Thomas, A. P., Diggle, T. A. & Denton, R. M. (1986) *Biochem. J.* **238**, 83–91
- Williamson, J. R. & Cooper, R. H. (1980) *FEBS Lett.* **117**, K73–K83

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