A comparative study of the regulation by Ca^{2+} of the activities of the 2-oxoglutarate dehydrogenase complex and NAD^+ –isocitrate dehydrogenase from a variety of sources

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Ca^{2+} was shown to activate oxoglutarate dehydrogenase and NAD^+ –isocitrate dehydrogenase from heart and other rat tissues by markedly decreasing the K_m values of the enzymes for their respective substrates [see Denton & McCormack (1980) *FEBS Lett.* 119, 1–8]. Similar effects of Ca^{2+} were observed in the present study with both enzymes from other vertebrate sources (pigeon, trout, frog and human heart), but not with the enzymes from blowfly or locust flight muscle, or potato or *Escherichia coli*. In contrast, the K_m values of the oxoglutarate dehydrogenases were affected by ADP, ATP and H^+ to a similar extent in every case, except for the enzyme from *E. coli*, which was not sensitive to regulation by these agents.

There are three, exclusively intramitochondrial, enzymes that are activated by Ca^{2+} with a $k_{0.5}$ value (concentration required for half-maximal effect) of about $1\ \mu\text{M}$ in rat and pig heart. These are pyruvate dehydrogenase phosphate phosphatase (Denton *et al.*, 1972), NAD –ICDH (Denton *et al.*, 1978) and OGDH (McCormack & Denton, 1979). Activation of the phosphatase by Ca^{2+} results in an increased amount of active, non-phosphorylated, pyruvate dehydrogenase (Denton *et al.*, 1972), and possibly also branched-chain oxo acid dehydrogenase, which may be activated by the same phosphatase (Parker & Randle, 1980; Hughes & Halestrap, 1981). The effects of Ca^{2+} on NAD –ICDH and OGDH are more direct and lead to greatly decreased K_m values for their substrates (*threo*- D_5 -isocitrate and 2-oxoglutarate respectively) (Denton *et al.*, 1978; McCormack & Denton, 1979).

Sensitivity to Ca^{2+} of all three enzymes has also been shown in mitochondrial extracts from several rat tissues other than heart (see Denton & McCormack, 1980).

The enzymes have been shown to retain their sensitivity to Ca^{2+} within intact incoupled mitochondria (McCormack & Denton, 1980). In studies with intact coupled mitochondria from rat heart, both pyruvate dehydrogenase and OGDH activities were found to be stimulated by increases in the extra-mitochondrial concentration of Ca^{2+} within the

expected physiological range (Denton *et al.*, 1980). These observations led to the proposal that Ca^{2+} may be an important regulator of intramitochondrial oxidative metabolism, at least in mammalian tissues (Denton *et al.*, 1980; Denton & McCormack, 1980). Since we have suggested that Ca^{2+} could act as a means whereby extrinsic agents, such as hormones, could regulate intramitochondrial metabolism, it seemed important to determine how widespread this regulation by Ca^{2+} is in living organisms. We have therefore investigated the sensitivity to Ca^{2+} of NAD –ICDH and OGDH activities from a spectrum of sources ranging from *Escherichia coli* to human heart. The activities of both these enzymes from a number of mammalian sources are also affected by ADP, ATP, H^+ , NADH and NAD^+ ; it has been suggested that these properties may be important means of intrinsic regulation of the citrate cycle (see Denton & McCormack, 1980; Williamson & Cooper, 1980; Hansford, 1980). We have therefore taken the opportunity to compare the occurrence of sensitivity of OGDH to adenine nucleotides and H^+ with that of OGDH to Ca^{2+} .

Experimental

Sources of materials used and details of the use of EGTA–Ca buffers, the calculation of free $[\text{Ca}^{2+}]$ and of enzyme-kinetic parameters, are as given in Denton *et al.* (1978) and McCormack & Denton

Abbreviations used: OGDH, the 2-oxoglutarate dehydrogenase complex; NAD –ICDH, NAD^+ –linked isocitrate dehydrogenase (EC 1.1.1.41).

(1979). It should be noted that our calculated values of $[Ca^{2+}]$ may be underestimated by a factor of 1.8, as discussed by Tsien & Rink (1980).

Preparations of mitochondria from fresh pigeon, trout and frog hearts were by the method given by Denton *et al.* (1980) for rat hearts. Flight-muscle mitochondria from mature locusts and blowflies were prepared as described by Chappell & Hansford (1972) and potato mitochondria as described by Laties (1974).

Mitochondrial extracts (1–5 mg of protein/ml) were prepared by freezing and thawing three times in 100 mM- KH_2PO_4 buffer (pH 7) containing 1 mM-dithiothreitol and rat serum (50 μ l/ml), and in addition, when NAD-ICDH was to be assayed, 1 mM-ADP (see McCormack & Denton, 1980). Human heart tissue (10 g) was obtained approx. 4 h *post mortem* from a 45-year-old man who died of a cerebro-vascular accident. Approval and permission of the Bristol Royal Infirmary ethical committee was given to Dr. D. Stansbie (see the acknowledgements). The tissue was stored at $-70^\circ C$ and then samples of the frozen tissue were powdered and extracted in the above buffer (with additions) by

homogenization with a Polytron PT20 (30 s at Mark 5) at $0^\circ C$ (250 mg wet wt./ml). *E. coli* was grown aerobically on nutrient broth at $37^\circ C$, washed in buffer [20 mM-Tris/HCl (pH 8) containing 1 mM- $MgCl_2$] and then extracted by sonication: the extract was centrifuged at 20 000 g for 20 min and the supernatant stored at $-20^\circ C$. All extracts were spun for 1 min in an Eppendorf 3200 microfuge just before use and enzyme activities assayed in samples (5–25 μ l) of the supernatant.

NAD-ICDH and OGDH were assayed at $30^\circ C$ by following the production of NADH at 340 nm, essentially as described by Denton *et al.* (1978) and McCormack & Denton (1979) respectively, in 50 mM-Mops (4-morpholinepropanesulphonic acid) buffer (pH 7.0 or 7.3, as indicated) containing 2 μ g of rotenone/ml, 1 mM-dithiothreitol and 1 mM- $MgCl_2$, with the additional presence of 1 mM-ADP for NAD-ICDH assay. Other additions are as indicated. Rates were linear with time for at least 2 min and also varied linearly with changes in extract volume assayed. A unit of enzyme activity is defined as the amount of enzyme which transforms 1 μ mol of substrate/min at $30^\circ C$.

Table 1. Kinetic parameters for OGDH and NAD-ICDH from a variety of sources

The enzymes were assayed at pH 7 as described in the Experimental section in the presence of either 5 mM-EGTA ($Ca^{2+} < 1$ nM) or 5 mM-EGTA plus 5 mM- $CaCl_2$ (Ca^{2+} about 30 μ M). Parameter values were calculated from the equation $v = V_{max}/\{1 + (K_m/[S])^n\}$ and are given as parameter value $\pm$ s.d. for the numbers of degrees of freedom in parentheses. V_{max} values, measured in the presence of 5 mM-EGTA, are given as munits/mg of mitochondrial protein, except for human heart (munits/g wet wt.) and *E. coli* (munits/mg of protein in extract); V_{max} values measured in the presence of 5 mM-EGTA plus 5 mM- $CaCl_2$ (Ca^{2+} about 30 μ M) did not differ significantly from these values, except with NAD-ICDH from both locust and blowfly, where the V_{max} was 20–30% less than that determined in the presence of < 1 nM- Ca^{2+} . In all cases, n values did not differ from 1 for OGDH and ranged from 1.7 to 2.5 for NAD-ICDH (at < 1 nM or 30 μ M- Ca^{2+}). Abbreviation: N.D., not detectable.

	Kinetic parameters for						
	OGDH				NAD-ICDH		
	K_m for oxoglutarate (μ M)				K_m for <i>threo</i> -D ₅ -isocitrate (μ M)		
	V_{max} (at < 1 nM- Ca^{2+})	at < 1 nM- Ca^{2+}	at ≈ 30 μ M- Ca^{2+}	V_{max} (at < 1 nM- Ca^{2+})	at < 1 nM- Ca^{2+}	at ≈ 30 μ M- Ca^{2+}	
Extracts from							
Rat heart mitochondria	125 $\pm$ 4 (16)	1741 $\pm$ 88 (16)	95.6 $\pm$ 4.4 (16)	98 $\pm$ 3 (15)	466 $\pm$ 34 (15)	74.1 $\pm$ 3.9 (15)	
Human heart tissue	1515 $\pm$ 83 (27)	1308 $\pm$ 93 (27)	64.8 $\pm$ 2.7 (28)	N.D.	N.D.	N.D.	
Pigeon heart mitochondria	113 $\pm$ 6 (30)	1108 $\pm$ 98 (30)	50.4 $\pm$ 3.6 (27)	123 $\pm$ 9 (8)	522 $\pm$ 61 (8)	119 $\pm$ 13 (8)	
Trout heart mitochondria	123 $\pm$ 10 (13)	733 $\pm$ 77 (13)	196 $\pm$ 20 (13)	103 $\pm$ 6 (16)	387 $\pm$ 19 (16)	80.3 $\pm$ 6.5 (15)	
Frog heart mitochondria	154 $\pm$ 12 (12)	78.9 $\pm$ 2.9 (12)	15.2 $\pm$ 1.6 (11)	122 $\pm$ 8 (11)	303 $\pm$ 21 (11)	43.4 $\pm$ 1.5 (10)	
Locust flight-muscle mitochondria	266 $\pm$ 11 (49)	326 $\pm$ 17 (49)	184 $\pm$ 10 (47)	340 $\pm$ 16 (22)	1220 $\pm$ 70 (22)	1750 $\pm$ 136 (21)	
Blowfly flight-muscle mitochondria	294 $\pm$ 8 (21)	25.7 $\pm$ 3.7 (21)	27.9 $\pm$ 2.3 (19)	287 $\pm$ 12 (11)	364 $\pm$ 20 (11)	595 $\pm$ 50 (11)	
Potato mitochondria	219 $\pm$ 14 (16)	78.5 $\pm$ 6.3 (16)	81.2 $\pm$ 8.1 (16)	193 $\pm$ 15 (8)	135 $\pm$ 22 (9)	140 $\pm$ 11 (8)	
<i>E. coli</i>	218 $\pm$ 7 (32)	9.2 $\pm$ 1.4 (29)	10.5 $\pm$ 1.4 (29)	N.D.	N.D.	N.D.	

Protein was measured by the method of Gornall *et al.* (1949).

Results

The values given in Tables 1 and 2 are derived from results obtained from at least three separate extracts in each instance; for mitochondria this involved separate preparations. In all cases, results on the separate extracts were very similar and were therefore pooled.

The effects of Ca²⁺ on the K_m values of OGDH and NAD-ICDH for their respective substrates from the various sources investigated are shown in Table 1. Also included are values for the two enzymes from rat heart mitochondria; these values are close to those reported previously for the rat heart enzymes (Denton *et al.*, 1978, 1980) and are included to facilitate comparisons. It should be noted that the values found for rat heart are typical for the two enzymes from all other rat tissues so far studied, which include skeletal muscle, brain, liver, kidney and white and brown adipose tissue [see Denton & McCormack, 1980; and unpublished work by J. G. McCormack, R. M. Denton and 2nd-year students reading biochemistry (1979) at the University of Bristol]. No values are given for NAD-ICDH from human heart, as even freshly prepared extracts were found to contain little or no activity. It is possible that this enzyme was largely inactivated during the 4 h period between death of the donor and the freezing of tissue samples. *E. coli* contains only NADP-linked isocitrate dehydrogenase (Rogland *et al.*, 1966; Burke *et al.*, 1973).

The results in Table 1 show that 30 μ M-Ca²⁺ greatly decreased the K_m for oxoglutarate of OGDH from human, pigeon, trout and frog heart. The kinetic constants for the human and pigeon enzymes were very close to those found for the rat enzyme and, in other studies, for the pig heart enzyme (McCormack & Denton, 1979). The effect of Ca²⁺ on the trout and frog enzymes may be a little smaller, and the activity of OGDH from frog heart exhibited much lower K_m values for oxoglutarate, in both the presence and absence of Ca²⁺, under the assay conditions employed. In marked contrast, Ca²⁺ had no effect on OGDH from blowfly, potato and *E. coli*, and only decreased the K_m of the locust enzyme by a very modest amount. Also, no effects of Ca²⁺ were found on the activity of OGDH in extracts of mitochondria prepared from the spadix of *Arum maculatum*, but a full kinetic analysis was not carried out and thus results are not shown in Table 1. No significant effects of Ca²⁺ were found on the maximum activities of OGDH from any of the sources studied, and thus only the values obtained in the presence of 5 mM-EGTA (i.e. <1 nM-Ca²⁺) are shown in Table 1.

The effects of Ca²⁺ on the K_m of NAD-ICDH for *threo*-D₅-isocitrate follow much the same pattern as that found for the effects of Ca²⁺ on OGDH. Thus Ca²⁺ caused marked decreases in the K_m of the dehydrogenase from pigeon, frog and trout heart, but did not decrease the K_m of NAD-ICDH from locust, blowfly and potato (Table 1), or the K_m of the enzyme from *Arum maculatum* (results not shown). Indeed, Ca²⁺ appeared actually to inhibit NAD-ICDH activity from both locust and blowfly flight muscle by a combination of a modest decrease in V_{max} and a modest increase in K_m . Vaughan & Newsholme (1969) and Zammit & Newsholme (1976) have also studied the sensitivity to Ca²⁺ (using EGTA-Ca buffers) of NAD-ICDH from a variety of vertebrate and invertebrate muscles, and have reported that micromolar concentrations of Ca²⁺ increase the K_m of the enzyme for isocitrate. However, as pointed out previously (Denton *et al.*, 1978), those authors did not allow for the binding of Mn²⁺ to EGTA, which meant that they may have been comparing the effects of Ca²⁺ at about 50 μ M with that of about 1 mM-Ca²⁺ rather than the 1 nM and 10 μ M stated. Under the conditions of the present study, in which activity of NAD-ICDH is assayed in the presence of Mg²⁺ rather than Mn²⁺, we found little or no evidence for effects of 1 mM-Ca²⁺ over and above those observed at 30 μ M-Ca²⁺.

Table 2. $k_{0.5}$ values for activation by Ca²⁺ of OGDH and NAD-ICDH from a variety of sources

The enzymes were assayed at pH 7 as described in the Experimental section in the presence of 5 mM-EGTA and additions of CaCl₂ to give a suitable range of [Ca²⁺]. A suitable substrate concentration was chosen in each case such that maximal stimulation by Ca²⁺ achieved a rate about 80% of the V_{max} measured at saturating substrate concentration; $k_{0.5}$ values were calculated from the equation $v = V_0 + (V_{max}/\{1 + (k_{0.5}/[Ca^{2+}])^n\})$ (where V_0 is the activity in the presence of 5 mM-EGTA) and are given as parameter values $\pm$ s.d. for the number of degrees of freedom in parentheses. Abbreviation: N.D., not detectable.

Extracts from	$k_{0.5}$ values (μ M) for activation by Ca ²⁺ of	
	OGDH	NAD-ICDH
Rat heart mitochondria	0.95 $\pm$ 0.05 (20)	1.12 $\pm$ 0.07 (20)
Human heart tissue	1.07 $\pm$ 0.07 (16)	N.D.
Pigeon heart mitochondria	0.63 $\pm$ 0.06 (23)	1.19 $\pm$ 0.09 (9)
Trout heart mitochondria	4.95 $\pm$ 0.7 (12)	4.21 $\pm$ 0.59 (15)
Frog heart mitochondria	0.31 $\pm$ 0.03 (10)	0.56 $\pm$ 0.04 (9)

Previously we have reported that the effects of Ca^{2+} on the activities of both pig heart OGDH and rat heart NAD-ICDH are mimicked by Sr^{2+} , but that 10-fold higher concentrations are required (Denton *et al.*, 1978; McCormack & Denton, 1979). Where an effect of $30\text{ }\mu\text{M-Ca}^{2+}$ was apparent in the present study, it was also found that a similar effect was apparent in the presence of a Sr-EGTA buffer giving $500\text{ }\mu\text{M-Sr}^{2+}$ (results not shown).

Table 2 compares the concentrations of Ca^{2+} giving half-maximal stimulation ($k_{0.5}$ values) of OGDH and NAD-ICDH from rat, human, pigeon, trout and frog hearts. In each case, except human heart, where NAD-ICDH could not be investigated satisfactorily, the values of $k_{0.5}$ for Ca^{2+} activation were broadly similar for both OGDH and NAD-ICDH. Highest values (4.2 and $5\text{ }\mu\text{M}$) were found for the two dehydrogenases from trout heart, and the lowest values (0.3 and $0.6\text{ }\mu\text{M}$) were found for the frog heart enzymes. The $k_{0.5}$ value for the small activation by Ca^{2+} of the locust OGDH was $1.65 \pm 0.41\text{ }\mu\text{M}$ (value $\pm$ S.D. for 12 degrees of freedom).

The effects of the presence of ADP or ATP or a modest change in H^+ concentration on the activity of OGDH from the various sources studied are given in Table 3. It was found previously, for OGDH from pig heart, that the K_m for oxoglutarate was decreased by the presence of ADP or changing the pH from pH 7.3 to 7, and increased in the presence of ATP; these effects were noted in either the presence or absence of Ca^{2+} (McCormack & Denton, 1979). In the present study we found that

the enzyme from all the sources studied except *E. coli* was similarly affected by ADP, ATP and changing the pH from 7.0 to 7.3. Thus OGDH from insect flight muscle and potato appeared to be just as susceptible to regulation by H^+ concentration and adenine nucleotides as the enzyme from the hearts of vertebrates, while exhibiting little or no activation by Ca^{2+} . Stimulation of the activity of OGDH by ADP from pigeon breast muscle has been reported previously by Gomazkova & Krasovskaya (1979) and from blowfly flight muscle by Hansford (1972).

Discussion

It should be noted that the present studies only used a fairly restricted selection of sources, and thus caution must be taken in making general conclusions. Nevertheless, it has been found that micromolar concentrations of Ca^{2+} activate both OGDH and NAD-ICDH from all vertebrate sources investigated (namely rat, man, frog, pigeon and trout heart); in contrast, Ca^{2+} had little or no effect on the activity of these same dehydrogenases extracted from the mitochondria of insect and plant sources or on OGDH activity from *E. coli*. On the other hand, the activity of OGDH from all the sources studied was found to be sensitive to regulation by ATP, ADP and H^+ , with the single exception of the enzyme from *E. coli*. The lack of regulatory properties of OGDH from *E. coli* fits in with the idea that intrinsic regulation of the citrate cycle in this and many other Gram-negative bacteria may largely be exerted at the level of citrate synthase

Table 3. Influence of pH and the effects of ADP and ATP on the activity of OGDH from a number of sources. OGDH was assayed at either pH 7.0 or 7.3 (as shown) in the presence of 5 mM-EGTA with the concentration of oxoglutarate and additions of 1.5 mM-ADP or 1.5 mM-ATP as indicated. The rate obtained for each condition is expressed as a percentage of the V_{max} rate measured under the same conditions except for a saturating concentration of oxoglutarate (1–25 mM); V_{max} rates were not affected significantly by any of the conditions indicated, and were similar in every case to values given in Table 1. Rates are given as means $\pm$ S.E.M. for the numbers of independent observations on separate extracts shown in parentheses. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus the appropriate control value by Student's *t* test.

Extracts from	[Oxoglutarate] (μM)	OGDH activity (% of V_{max}) in the presence of			
		pH 7.0 (control)	pH 7.3	1.5 mM-ADP (pH 7.0)	1.5 mM-ATP (pH 7.0)
Rat heart mitochondria	200	15.6 ± 1.1 (4)	5.5 ± 0.4 (3)***	41.5 ± 5.0 (3)**	6.6 ± 0.9 (3)**
Human heart tissue	200	20.1 ± 2.2 (6)	8.8 ± 1.0 (3)*	51.6 ± 4.2 (3)***	6.8 ± 0.6 (3)**
Pigeon heart mitochondria	200	21.6 ± 1.9 (8)	12.8 ± 0.9 (3)*	67.4 ± 3.7 (4)***	7.4 ± 0.7 (4)***
Trout heart mitochondria	200	28.4 ± 2.6 (3)	9.0 ± 1.1 (3)**	92.6 ± 5.1 (3)***	12.5 ± 1.3 (3)**
Frog heart mitochondria	20	35.3 ± 3.7 (3)	20.8 ± 2.2 (3)*	56.5 ± 5.4 (3)*	12.4 ± 1.9 (3)**
Locust flight-muscle mitochondria	100	21.7 ± 1.3 (12)	6.2 ± 0.5 (8)***	74.8 ± 3.1 (4)***	7.4 ± 0.6 (3)***
Blowfly flight-muscle mitochondria	10	25.1 ± 3.5 (3)	11.1 ± 1.0 (3)*	51.4 ± 3.8 (3)**	4.4 ± 0.4 (3)*
Potato mitochondria	50	40.7 ± 1.9 (6)	24.9 ± 2.3 (3)**	82.0 ± 9.2 (3)*	15.8 ± 2.3 (3)***
<i>E. coli</i>	10	54.4 ± 1.7 (7)	52.8 ± 3.6 (3)	56.8 ± 1.3 (4)	53.0 ± 3.3 (4)

via changes in the NADH/NAD⁺ ratio (Weitzman & Danson, 1976).

The processes concerned with the transfer of Ca²⁺ across the inner membrane of mitochondria from plants and insect flight muscles have not been fully characterized. Indeed, there do not appear to have been any reports of investigations specifically concerned with identifying systems which might allow Ca²⁺ to be transferred out of these mitochondria, despite the membrane potential, analogous to the Ca²⁺/Na⁺ antiport system found in many types of mammalian mitochondria (see Carafoli, 1979; Nicholls & Crompton, 1980). On the other hand, the electrogenic inward transfer of Ca²⁺ appears only to occur at a slow rate even at high extramitochondrial Ca²⁺ concentrations when compared with rates observed with mitochondria from vertebrate sources (Carafoli & Lehninger, 1971; Carafoli *et al.*, 1971; Wiskich, 1977; Moore & Bonner, 1977). The rate with mitochondria from plant and insect sources may simply represent non-carrier-mediated transfer. It would appear on present evidence that sensitivity of intramitochondrial enzymes to Ca²⁺ may be restricted to species where the mitochondria contain the specific transporter involved in the electrogenic uptake of Ca²⁺. One exception, clearly worthy of further study, may be mitochondria of blowfly flight muscle in the first few hours after emergence (Bygrave *et al.*, 1975). In any case, the large increase in the rate of the citrate cycle which occurs in flight muscles of mature blowflies or locusts during flight would not seem to involve parallel activation of NAD-ICDH and OGDH by Ca²⁺. This is in agreement with the view of Hansford (1972, 1980) that a decrease in the intramitochondrial ATP/ADP concentration ratio is the important means whereby the activities of these two hydrogenases are increased during flight.

It appears that OGDH and NAD-ICDH in the mitochondria of vertebrate tissues have acquired the additional property of being sensitive to regulation by Ca²⁺. It should also be noted that all reports of the activation of pyruvate dehydrogenase phosphate phosphatase by Ca²⁺ refer to vertebrate sources (see Denton & McCormack, 1980; Hansford, 1980), and include the enzyme from human heart (Stansbie, 1976) as well as from a number of different tissues of rat and pig (Denton *et al.*, 1972; Kerbey *et al.*, 1976; Roche & Cate, 1977). Presumably, the principal advantage of the acquisition of sensitivity of these key intramitochondrial dehydrogenases to Ca²⁺ together with appropriate systems for transferring Ca²⁺ into and out of mitochondria in vertebrate cells is that it allows extrinsic agents such as hormones to regulate the rate of the citrate cycle without the need for large changes in the concentration ratios of ATP/ADP and NADH/NAD⁺ to occur in mitochondria

(Denton *et al.*, 1980; Denton & McCormack, 1980).

A comparative study of the detailed structures of the OGDH complexes from, e.g., *E. coli*, an insect flight muscle, and mammalian heart, may shed light on the molecular basis of the apparently independent acquisition of sensitivity to adenine nucleotides and Ca²⁺. In particular, it may help to establish whether the sensitivity to Ca²⁺ of the intramitochondrial dehydrogenases in cells of vertebrates involves a common calcium-binding protein, perhaps related to calmodulin (McCormack & Denton, 1979).

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