Comparison of the effects of Ca^{2+} , adenine nucleotides and pH on the kinetic properties of mitochondrial NAD^+ –isocitrate dehydrogenase and oxoglutarate dehydrogenase from the yeast *Saccharomyces cerevisiae* and rat heart

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The regulatory properties of NAD^+ –isocitrate dehydrogenase and oxoglutarate dehydrogenase in extracts of yeast and rat heart mitochondria were studied under identical conditions. Yeast NAD^+ –isocitrate dehydrogenase exhibits a low $K_{0.5}$ for isocitrate and is activated by AMP and ADP, but is insensitive to ATP and Ca^{2+} . In contrast, the rat heart NAD^+ –isocitrate dehydrogenase was insensitive to AMP, but was activated by ADP and by Ca^{2+} in the presence of ADP or ATP. Both yeast

and rat heart oxoglutarate dehydrogenase were stimulated by ADP, but only the heart enzyme was activated by Ca^{2+} . All the enzymes studied were activated by decreases in pH, but to differing extents. The effects of Ca^{2+} , adenine nucleotides and pH were through $K_{0.5}$ for isocitrate or 2-oxoglutarate. These observations are discussed with reference to the deduced amino acid sequences of the constituent subunits of the enzymes, where they are available.

INTRODUCTION

In mammalian cells, Ca^{2+} acts as an intracellular second messenger for several hormones and other extracellular stimuli. Increases in cytoplasmic Ca^{2+} cause changes in cellular processes which commonly lead to an increase in energy demand. This demand has to be met by enhancement of ATP production, usually within mitochondria. One mechanism underlying the increase in mitochondrial ATP synthesis involves activation by Ca^{2+} of three citrate-cycle enzymes: pyruvate dehydrogenase, isocitrate dehydrogenase and oxoglutarate dehydrogenase (reviewed by Denton and McCormack, 1980, 1990; McCormack et al., 1990). NAD^+ –dependent isocitrate dehydrogenase (NAD^+ –ICDH; Denton et al., 1978; Aogaichi et al., 1980) and 2-oxoglutarate dehydrogenase (OGDH; McCormack and Denton, 1979; Lawlis and Roche, 1980) are activated by a lowering of $K_{0.5}$ values for their respective substrates. Pyruvate dehydrogenase is regulated by reversible phosphorylation. Ca^{2+} stimulates this enzyme by activating pyruvate dehydrogenase phosphatase, thus favouring the more active, dephosphorylated, form of pyruvate dehydrogenase (Denton et al., 1972). All three dehydrogenases can also be activated by increases in the mitochondrial ADP/ATP ratio, and this provides another mechanism for matching mitochondrial NADH production to changes in cellular ATP demand (McCormack and Denton, 1979; Lawlis and Roche, 1980; Aogaichi et al., 1980; Denton and McCormack, 1980, 1990; Rutter and Denton, 1988; McCormack et al., 1990).

Mammalian OGDH is a multi-subunit complex consisting of three different enzymes: oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) (Koike and Koike, 1976). Experiments using E1 purified away from the rest of the complex following partial cleavage with papain (Lawlis and Roche, 1981) and using electron acceptors which allow E1 activity to be assayed separately from that of the whole complex (M. Leake and R. M. Denton, un-

published work) show that the step catalysed by E1 is sensitive to changes in Ca^{2+} and adenine nucleotides. Mammalian NAD^+ –ICDH is also a multi-subunit enzyme. It possesses three types of subunit, possibly in the ratio $\alpha_2\beta\gamma$ (Ramachandran and Colman, 1980). The role of the individual subunits of NAD^+ –ICDH is unknown.

The kinetic properties of NAD^+ –ICDH and OGDH from mammalian mitochondria have been extensively studied by using the purified enzymes, toluene-permeabilized mitochondria and mitochondrial extracts (Denton et al., 1978; McCormack and Denton, 1979, 1981; Rutter and Denton, 1988). Less information is available about the properties of these enzymes in non-mammalian organisms. In a number of plant and invertebrate species they are activated by ADP, but not by Ca^{2+} (McCormack and Denton, 1981).

Amino acid sequence deduced from cloned cDNAs is available for all the subunits of OGDH from mammalian sources (Koike et al., 1992; Nakano et al., 1991; Otulakouski and Robinson, 1987). In contrast, only the γ -subunit of NAD^+ –ICDH has been cloned (Nichols et al., 1993). All subunits of OGDH and NAD^+ –ICDH have been cloned from the yeast *Saccharomyces cerevisiae* (Stephens et al., 1983; Repetto and Tzagoloff, 1989, 1990; Cupp and McAlister-Henn, 1991, 1992). The yeast OGDH has three subunits, all of which are similar to their mammalian counterparts. NAD^+ –ICDH from yeast has only two subunits (Barnes et al., 1971). Cupp and McAlister-Henn (1993) have recently suggested that one of these subunits, designated IDH1, has a regulatory function, whereas the other, IDH2, is responsible for catalysis. One approach for increasing our understanding of the molecular basis for the effects of Ca^{2+} and adenine nucleotides on mammalian NAD^+ –ICDH and OGDH is through comparison with their yeast homologues. However, no detailed investigation of the effects of Ca^{2+} and adenine nucleotides on the yeast enzymes has been reported.

In this study, the effects of potential regulators of OGDH and

NAD-ICDH activity are investigated in extracts of yeast mitochondria, and compared with the properties of the rat heart enzymes assayed under identical conditions.

METHODS

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.

Preparation of mitochondria

Diploid wild-strain yeast (*Saccharomyces cerevisiae*) cells were grown aerobically at 28 °C in a complete medium (pH 4.5) with lactate (20 g/l) as a carbon source. The cells were harvested during exponential growth. Protoplasts were prepared and mitochondria purified as described by Guérin et al. (1979). Mitochondria were then suspended at approx. 40 mg of protein/ml of 0.6 M mannitol/2 mM EGTA/and 10 mM Tris/maleate (pH 6.8). The mitochondrial suspension was immediately frozen in liquid nitrogen and stored at -70 °C.

Fresh hearts from adult male rats (*Rattus norvegicus*) were individually dispersed, by using a Polytron homogenizer, in 2.5 ml of 250 mM sucrose/2 mM EGTA/20 mM Tris/HCl (pH 7.2), at 0 °C. Intact cells, nuclei and cell debris were removed by low-speed centrifugation of the homogenate (1000 g for 3 min at 4 °C). Mitochondria were sedimented by centrifugation at 10000 g for 10 min at 4 °C. They were then washed by re-suspension in the buffer described above and further sedimentation at 10000 g. The resulting mitochondrial pellet was stored at -70 °C.

Preparation of mitochondrial extracts

Extracts containing soluble mitochondrial protein were prepared as described by McCormack and Denton (1981). Mitochondrial pellets from both yeast and rat heart were treated identically, being frozen and thawed three times in 0.1 M KH_2PO_4 (pH 7.2) containing 1 mM dithiothreitol and 30 $\mu\text{l}/\text{ml}$ rat serum. If NAD-ICDH was to be assayed, this extraction buffer was supplemented with 1 mM ADP. The extracts were then centrifuged for 10 min at 10000 g in a micro-centrifuge to remove mitochondrial membranes.

Assay of enzymes within mitochondrial extracts

Activity of NAD-ICDH and OGDH in mitochondrial extracts was assayed by measuring NADH production after addition of the appropriate substrate, in a Pye-Unicam PU-8800 spectrophotometer. All assays were carried out in 1 ml of a buffer containing 50 mM Mops, 75 mM KCl, 1 mM dithiothreitol, 0.2 mM EGTA, 1 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA), 1 $\mu\text{g}/\text{ml}$ oligomycin, 1 $\mu\text{g}/\text{ml}$ rotenone and 1 mM NAD^+ , maintained at 30 °C. Unless otherwise stated, all assays were carried out at pH 7.2. To give stock solutions of 1 mg/ml, oligomycin and rotenone were dissolved in methanol rather than ethanol, as yeast mitochondria have significant ethanol dehydrogenase activity. When OGDH was assayed, 0.2 mM CoA and 1 mM thiamin pyrophosphate were included in the above. MgCl_2 and CaCl_2 were added so as to give 1 mM free Mg^{2+} in all assays and 0.14 mM free Ca^{2+} where appropriate. The amount of each metal ion necessary to do this was calculated as described by Thomas et al. (1986) and Denton and McCormack (1993). ATPase activity of the mitochondrial extracts was determined by measuring the hydrolysis of

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the assay buffer described above; free phosphate was separated from adenine nucleotides by extraction with acid charcoal. In the presence of oligomycin and rotenone, the disappearance of ATP and NADH was less than 2 %/min.

Data from the dehydrogenase assays were processed by using the program Enzfitter, written by R. J. Leatherbarrow (published by Elsevier Biosoft Ltd.). Data from OGDH assays were fitted to the equation:

$$V = V_{\max} [S] / (K_{0.5} + [S])$$

and for NAD-ICDH assays data were fitted to the equation:

$$V = V_{\max} [S]^h / (K_{0.5}^h + [S]^h)$$

Results are given as parameter value $\pm$ S.D. They are derived from data obtained from between one and three mitochondrial extract preparations, each of which was used in 15–25 assays at various substrate concentrations.

RESULTS

Effects of Ca^{2+} and adenine nucleotides on NAD-ICDH

The maximal activity of the yeast NAD-ICDH was essentially unchanged by Ca^{2+} and adenine nucleotides, at 145 ± 30 nmol/min per mg of protein in the mitochondrial extracts. This is about 3 times greater than that observed in comparable extracts of rat heart mitochondria (49 ± 13 nmol/min per mg of protein). Kinetic data illustrating the effects of Ca^{2+} and adenine nucleotides on the activity of yeast NAD-ICDH are given in Table 1, which also includes the equivalent data for the rat heart enzyme, taken in part from Rutter and Denton (1988). The most striking difference between the yeast and rat enzymes is that the yeast enzyme does not appear to be activated by Ca^{2+} , $K_{0.5}$ values for D-isocitrate being approximately the same when measured at < 1 nM Ca^{2+} and at 0.14 mM Ca^{2+} . If anything, values for $K_{0.5}$ are slightly increased by addition of Ca^{2+} , which may reflect the increased ratio of calcium isocitrate to magnesium isocitrate at 0.14 mM Ca^{2+} .

Figure 1(a) shows the effects of AMP, ADP and ATP on the activity of the yeast NAD-ICDH at various concentrations of D-isocitrate. ATP did not affect the $K_{0.5}$ for D-isocitrate of this enzyme, ADP decreased the $K_{0.5}$ to approximately half the value seen in the absence of adenine nucleotides, whereas AMP also activated the enzyme by decreasing $K_{0.5}$ around 6-fold. This effect of AMP agrees with previous observations carried out on the purified enzyme (Barnes et al., 1971; Cupp and McAlister-Henn, 1993). Table 1 allows comparison of $K_{0.5}$ for D-isocitrate between the yeast and rat enzymes. The principal difference in their regulation by adenine nucleotides was that AMP had little or no effect on the rat enzyme. As noted in previous studies (Denton et al., 1978; Rutter and Denton, 1988), the presence of ADP or ATP is necessary for mammalian NAD-ICDH to exhibit Ca^{2+} -sensitivity. The present study shows that AMP does not have this property. For yeast NAD-ICDH, at 25 μM D-isocitrate, half-maximal activation was seen with about 100 μM AMP. In general, the $K_{0.5}$ values of yeast NAD-ICDH for D-isocitrate were up to an order of magnitude lower than those seen in the rat enzyme under equivalent conditions.

Effects of Ca^{2+} and adenine nucleotides on the sensitivity of OGDH to 2-oxoglutarate

As with NAD-ICDH, the maximal activity of OGDH in yeast mitochondrial extracts was essentially unchanged by addition of Ca^{2+} or adenine nucleotides. The maximal activity of this enzyme was 95 ± 20 nmol/min per mg of protein in mitochondrial extracts, compared with 133 ± 29 nmol/min per mg in extracts of

Table 1 Kinetic parameters of NAD-ICDH in extracts of yeast and rat heart mitochondria at pH 7.2

Further details of assay conditions and calculation of constants are given in the Methods section. Each parameter is calculated from a minimum of 20 assays of enzyme activity at various substrate concentrations. $K_{0.5}$ values refer to the substrate isocitrate. Maximal activities (V_{max}) of both enzymes were not significantly changed by Ca^{2+} and adenine nucleotides (see the Results section). Assay conditions for the data taken from Rutter and Denton (1988) were the same as those employed in this study.

Additions		Yeast enzyme		Rat heart enzyme			
Ca^{2+} (μM)	Adenine nucleotides	$K_{0.5}$ (μM)	h	Data from this study		Data from Rutter and Denton (1988)	
				$K_{0.5}$ (μM)	h	$K_{0.5}$ (μM)	h
< 0.001	None	177 $\pm$ 11	4.4	1200 $\pm$ 93	2.5	1080 $\pm$ 20	3.7
	AMP (1.5 mM)	25 $\pm$ 4	3.0	1310 $\pm$ 43	2.9	—	—
	ADP (1.5 mM)	74 $\pm$ 6	3.6	590 $\pm$ 83	1.8	550 $\pm$ 14	3.8
	ATP (1.5 mM)	163 $\pm$ 9	4.0	1105 $\pm$ 98	2.7	1140 $\pm$ 50	2.5
140	None	174 $\pm$ 4	4.3	1045 $\pm$ 87	3.4	990 $\pm$ 30	4.1
	AMP (1.5 mM)	33 $\pm$ 3	1.8	1260 $\pm$ 51	2.0	—	—
	ADP (1.5 mM)	80 $\pm$ 5	2.8	—	—	160 $\pm$ 4	3.0
	ATP (1.5 mM)	194 $\pm$ 7	4.7	210 $\pm$ 30	2.9	340 $\pm$ 12	3.0

Figure 1 Sensitivity of (a) yeast NAD-ICDH and (b) yeast OGDH to adenine nucleotides

Activities were measured at pH 7.2, in the presence of no adenine nucleotide (∇), 1.5 mM AMP ($\blacktriangle$), 1.5 mM ADP ($\blacklozenge$) or 1.5 mM ATP ($\blacksquare$). Activities are presented as a proportion of the maximal activity. Each activity is shown as the mean of three assays at each substrate concentration, with error bars representing S.E.M. Calculated kinetic constants are given in Tables 1 and 2.

Table 2 Kinetic parameters of OGDH in extracts of yeast and rat heart mitochondria

Maximal activities (V_{max}) of both enzymes were not significantly changed by Ca^{2+} and adenine nucleotides (see Results section). $K_{0.5}$ values refer to the substrate 2-oxoglutarate. Assay conditions for the data taken from Rutter and Denton (1988) were the same as those employed in this study.

Additions		Rat heart enzyme		
Ca^{2+} (μM)	Adenine nucleotides	Yeast enzyme $K_{0.5}$ (μM)	Data from Rutter and Denton (1988)	
			Data from this study $K_{0.5}$ (μM)	$K_{0.5}$ (μM)
< 0.001	None	1260 $\pm$ 90	1710 $\pm$ 250	1655 $\pm$ 120
	AMP (1.5 mM)	750 $\pm$ 100	1805 $\pm$ 220	—
	ADP (1.5 mM)	510 $\pm$ 43	330 $\pm$ 20	220 $\pm$ 21
	ATP (1.5 mM)	869 $\pm$ 97	1850 $\pm$ 250	1280 $\pm$ 170
140	None	1050 $\pm$ 91	259 $\pm$ 52	360 $\pm$ 22
	AMP (1.5 mM)	780 $\pm$ 50	260 $\pm$ 82	—
	ADP (1.5 mM)	402 $\pm$ 70	—	71 $\pm$ 6
	ATP (1.5 mM)	960 $\pm$ 132	420 $\pm$ 93	—

rat heart mitochondria. Kinetic parameters for OGDH from both yeast and rat mitochondria in the presence and absence of Ca^{2+} and adenine nucleotides are given in Table 2. As with NAD-ICDH, the yeast OGDH was not activated by Ca^{2+} .

Figure 1(b) shows the effects of AMP, ADP and ATP on the activity of yeast OGDH across a range of substrate concentrations. Table 2 allows a comparison of the effects of adenine nucleotides on OGDH from yeast and rat heart. The responses of both enzymes to adenine nucleotides were broadly similar. They were activated by ADP, whereas AMP did not have a large effect in either case, though it may activate the yeast OGDH to some extent. The action of ATP on rat OGDH is not completely defined. Rutter and Denton (1988) did not see any inhibition by ATP (regardless of the amount of Ca^{2+} present) in either mitochondrial extracts or toluene-permeabilized mitochondria.

Table 3 Variation of kinetic parameters for NAD-ICDH and OGDH with pH, in yeast and rat heart mitochondrial extracts

All assays were carried out in the presence of 1.5 mM ADP, and with $\text{Ca}^{2+} < 0.001 \mu\text{M}$. $K_{0.5}$ values refer to the substrates isocitrate or 2-oxoglutarate. Maximal activities (V_{max}) are given relative to the maximal activity at pH 7.2. Each set of parameters is based on a minimum of 12 assays of enzyme activity at various substrate concentrations.

Enzyme		Kinetic properties at different pH values		
		pH 6.8	pH 7.2	pH 7.6
Rat NAD-ICDH	$K_{0.5}$	221 ± 14	590 ± 83	990 ± 90
	h	1.9	1.8	2.3
	V_{max}	0.54	1	1.05
Yeast NAD-ICDH	$K_{0.5}$	22 ± 3	53 ± 3	60 ± 9
	h	2	3.4	3.5
	V_{max}	0.67	1	1.08
Rat OGDH	$K_{0.5}$	79 ± 12	330 ± 20	2400 ± 310
	V_{max}	0.77	1	1.13
Yeast OGDH	$K_{0.5}$	350 ± 40	450 ± 55	1620 ± 332
	V_{max}	0.95	1	0.93

The results presented here show a marginal increase in $K_{0.5}$ for 2-oxoglutarate by ATP in the presence of 0.14 mM Ca^{2+} . In contrast, early studies using purified pig heart OGDH showed a clear inhibition by ATP at sub-saturating 2-oxoglutarate concentrations (McCormack and Denton, 1979), and we have confirmed this (B. J. Nichols, M. Rigoulet, R. M. Denton and E. Sotharan, unpublished work).

At 0.5 mM 2-oxoglutarate, half-maximal activation of yeast OGDH occurred at approx. 500 μM ADP. Whereas for rat OGDH ADP caused a 7-fold decrease in $K_{0.5}$ for 2-oxoglutarate (compared with the $K_{0.5}$ in the absence of any adenine nucleotides), in yeast OGDH it caused only a 2-fold decrease. $K_{0.5}$ values under various assay conditions ranged from 71 μM to 1850 μM for rat heart OGDH, whereas for the yeast enzyme they ranged from 402 μM to 1260 μM .

Effect of variations in pH on NAD-ICDH and OGDH activity

Table 3 shows kinetic parameters for NAD-ICDH from rat and yeast mitochondria at three different pH values, in the presence of 1.5 mM ADP. Both enzymes showed a decrease in $K_{0.5}$ for D-isocitrate and a decrease in maximal activity with increasing $[\text{H}^+]$. There is no great difference in the ratios between these parameters at the different pH values between the mammalian and yeast enzymes, except for a slightly greater increase in $K_{0.5}$ at pH 7.6 for rat NAD-ICDH. As described above, $K_{0.5}$ values are consistently about one order of magnitude lower for the yeast enzyme.

Table 3 also shows kinetic parameters for OGDH at different pH values. As with NAD-ICDH, the $K_{0.5}$ decreases with an increase in $[\text{H}^+]$. This was seen in both the rat and the yeast enzyme. However, changes in maximal activity are much less marked, so that the effect of decreasing pH is to effectively activate the enzyme at most substrate concentrations. The effects of pH on the rat heart dehydrogenase are in agreement with previous studies (McCormack and Denton, 1979).

DISCUSSION

General catalytic properties of NAD-ICDH and OGDH

OGDH from both rat heart and yeast mitochondrial extracts

show similar pH-dependencies, hinting that the pK values of catalytically important amino acid residues are approximately equivalent in both enzymes. NAD-ICDH enzymes also show similar patterns of pH-dependence in both rat heart and yeast mitochondrial extracts. However, the yeast NAD-ICDH has a much lower $K_{0.5}$ for D-isocitrate than does its mammalian counterpart. In mammalian mitochondria, NAD-ICDH activity is around half that of OGDH (Rutter and Denton, 1988), whereas in yeast mitochondria this ratio is reversed. The combination of a decrease in $K_{0.5}$ and an increase in V_{max} for yeast NAD-ICDH implies that the concentration of D-isocitrate present in yeast mitochondria is probably considerably less than in mammalian mitochondria.

Regulation of NAD-ICDH and OGDH by adenine nucleotides

NAD-ICDH from yeast mitochondria is activated by AMP and ADP, but is not sensitive to ATP. In contrast, the mammalian NAD-ICDH is activated by ADP, but not by AMP. ATP has complex effects on the kinetic properties of the mammalian enzyme, since, like ADP, its presence allows activation by Ca^{2+} , while at the same time ATP is able to counteract the effects of ADP (Rutter and Denton, 1988, 1989). Overall, decreases in mitochondrial ATP are likely to result in the activation of NAD-ICDH in both yeast and mammalian mitochondria, but the molecular interactions causing this activation are different. The cDNA sequences of the α and β subunits of mammalian NAD-ICDHs are not known, but examination of the deduced amino acid sequence of the γ -subunit (Nichols et al., 1993) shows a P-loop-type adenine-nucleotide-binding motif (Walker et al., 1982), shown in Figure 2. This motif is not present in either subunit of the yeast NAD-ICDH (Cupp and McAlister-Henn, 1991, 1992), despite the fact that the mammalian γ -subunits show 40–45% sequence identity with both yeast subunits. As shown in Figure 2, the ATP-binding motif is found immediately between areas of high similarity between the yeast and mammalian enzymes.

Human OGDH E1 (Koike et al., 1992) shows 40% amino acid sequence identity with yeast OGDH E1 (Repetto and Tzagoloff, 1989). This similarity occurs throughout the sequence of the enzymes. The results presented here do not demonstrate any major qualitative differences in the regulation by adenine nucleotides of the yeast and mammalian enzymes by adenine nucleotides. Inspection of the deduced amino acid sequences of human OGDH E1 and of yeast OGDH E1 does not reveal any recognizable adenine-nucleotide-binding motif.

Further studies will be required to understand the nature of adenine-nucleotide-binding sites in both dehydrogenases.

Yeast NAD-ICDH1	GRDYAVFEPGSRHVGIDIKGQNVANPTAMI
Rat NAD-ICDH γ	GHVYAVFETATRNITGKSIVNKNIANPTATL
Yeast NAD-ICDH2	GHKISIFEAV.HGSAPDIAGQDKANPTALL

Figure 2 P-loop-type ATP-binding motif in the γ -subunit of rat NAD-ICDH

A selected area of the amino acid sequences of both subunits of yeast NAD-ICDH and of the rat NAD-ICDH γ -subunit is shown. Identity between the rat and yeast sequences is shown as a vertical bar (|). The ATP-binding motif, which has the consensus sequence A/G-X-X-X-X-G-K-S/T (Walker et al., 1982), is underlined.

Regulation of NAD-ICDH and OGDH by Ca^{2+}

This study has demonstrated for the first time that NAD-ICDH and OGDH from yeast mitochondria are not activated by Ca^{2+} . This is in marked contrast with the dehydrogenases from rat heart and other mammalian sources. The regulatory role of Ca^{2+} within yeast cells is not fully defined. External Ca^{2+} is essential for yeast cell survival during cell division, and increases in cytoplasmic Ca^{2+} occur within the sub-micromolar range when yeast cells mate (Iida et al., 1990; Anraku et al., 1991; Nakajima-Shimada et al., 1991). Other studies using purified yeast mitochondria loaded with the Ca^{2+} indicator Fluo-3 have reported variations in matrix Ca^{2+} concentration between 140 and 400 nM, depending on the amount of extramitochondrial Ca^{2+} present (Uribe et al., 1992). However, the range of extramitochondrial concentrations used was at least two orders of magnitude greater than the concentrations found in intact yeast cells.

The molecular basis for Ca^{2+} activation of mammalian OGDH and NAD-ICDH is not understood. Ca^{2+} binds to both enzymes after purification to homogeneity (Rutter and Denton, 1989). Ca^{2+} binds to pig NAD-ICDH with a stoichiometry of 1 mol of Ca^{2+} :1 mol of $\alpha_2\beta\gamma$ tetramer (Rutter and Denton, 1989), suggesting that either Ca^{2+} binds between subunits or Ca^{2+} binds to the β or γ subunit. Ca^{2+} binds to pig OGDH with a stoichiometry of 3–4 mol of Ca^{2+} :1 mol of OGDH complex, which is consistent with the Ca^{2+} -binding site being composed of homodimers of E1.

X-ray-crystallographic studies have provided much information about how Ca^{2+} binds to the calmodulin (Strynadka and James, 1989) and annexin (Huber et al., 1990a,b) families of proteins. Pyruvate dehydrogenase phosphatase, a mitochondrial enzyme activated by Ca^{2+} (Denton et al., 1972), has recently been cloned and contains a region with some weak similarity to the calmodulin-like Ca^{2+} -binding motif (Lawson et al., 1993). None of the short stretches of the mammalian γ -subunit of NAD-ICDH and the E1 subunit of OGDH which differ from the corresponding yeast proteins contain any putative annexin- or calmodulin-type Ca^{2+} -binding sites. In the case of NAD-ICDH it is possible that cloning of cDNAs for the α - and β -subunits may reveal the Ca^{2+} -binding domain. However, Ca^{2+} -binding sites on both enzymes appear to be quite distinct from those reported previously, and may involve, for example, more than one component of these multi-subunit enzymes. Furthermore, it is difficult to eliminate completely the possibility that separate low-molecular-mass Ca^{2+} -binding subunits are present in both dehydrogenases. If such subunits exist, they would have to be poorly stained by Coomassie Blue or AgNO_3 , insensitive to

calmodulin inhibitors (Rutter, 1990; Aogaichi et al., 1980) and very tightly bound, as no loss of Ca^{2+} binding or sensitivity is apparent on purification of both enzymes to homogeneity (Rutter and Denton, 1989).

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