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Review

Regulation of mitochondrial dehydrogenases by calcium ions

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ABSTRACT

Studies in Bristol in the 1960s and 1970s, led to the recognition that four mitochondrial dehydrogenases are activated by calcium ions. These are FAD-glycerol phosphate dehydrogenase, pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase. FAD-glycerol phosphate dehydrogenase is located on the outer surface of the inner mitochondrial membrane and is influenced by changes in cytoplasmic calcium ion concentration. The other three enzymes are located within mitochondria and are regulated by changes in mitochondrial matrix calcium ion concentration. These and subsequent studies on purified enzymes, mitochondria and intact cell preparations have led to the widely accepted view that the activation of these enzymes is important in the stimulation of the respiratory chain and hence ATP supply under conditions of increased ATP demand in many stimulated mammalian cells. The effects of calcium ions on FAD-isocitrate dehydrogenase involve binding to an EF-hand binding motif within this enzyme but the binding sites involved in the effects of calcium ions on the three intramitochondrial dehydrogenases remain to be fully established. It is also emphasised in this article that these three dehydrogenases appear only to be regulated by calcium ions in vertebrates and that this raises some interesting and potentially important developmental issues.

1. Introduction

In many circumstances, increases in the concentration of calcium ions in the cytoplasm in cells of many species are important in the initiation of cellular processes such as contraction, secretion, ion- and metabolite- pumping and proliferation. These processes are energy requiring and are associated with an increased utilisation of ATP, which must be matched by an increase in ATP supply to ensure that ATP is not depleted.

One way that this increased ATP demand may be met is by an increased supply of reducing equivalents in the form of NADH or FADH to the respiratory chain. Studies in the Department of Biochemistry, University of Bristol in the 1960s and 1970s led to the identification of four important mitochondrial dehydrogenases involved in the direct supply of NADH and FADH that are activated by low concentrations of calcium ions [1–4]. There is now substantial evidence that the parallel activation of these dehydrogenases can be important in the matching of ATP supply to ATP need in at least vertebrate cells (Fig. 1) [5–10]. This is also discussed in the article by Griffiths and Rutter [11].

These four dehydrogenases are FAD-glycerol phosphate dehydrogenase, which is located on the cytoplasmic surface of the inner membrane of mitochondria, plus pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase, all found in the matrix of mitochondria. In this article, I summarise the early studies that led to the recognition of their activation by calcium ions and then review present knowledge on the interactions of calcium ions with the four dehydrogenases from studies on the enzymes and mitochondria. Studies on intact cell preparations are reviewed in [11]. There are still important gaps in our knowledge in this area and these will be highlighted in the final section.

2. Early studies

FAD-glycerol phosphate dehydrogenase together with the cytoplasmic NAD-glycerol phosphate dehydrogenase make up the glycerol phosphate shuttle that transfers reducing equivalents from NADH generated in glycolysis in the cytoplasm to the respiratory chain in the form of $FADH_2$. In 1967, Hansford and Chappell were the first to establish that FAD-glycerol phosphate dehydrogenase could be activated by micromolar concentrations of calcium ions [1]. They were studying glycerol phosphate oxidation by insect flight muscle mitochondria. It was already known that the divalent metal ion chelator EDTA inhibited this enzyme activity and that this inhibition could be overcome by magnesium ions [12]. The detailed studies of Hansford and Chappell [1] showed that EGTA which only binds magnesium ions very poorly also inhibited, and that this inhibitory effect was not reversed by magnesium ions. This suggested that calcium ions or another divalent metal ion activated this process rather than magnesium ions and these authors went on to show,

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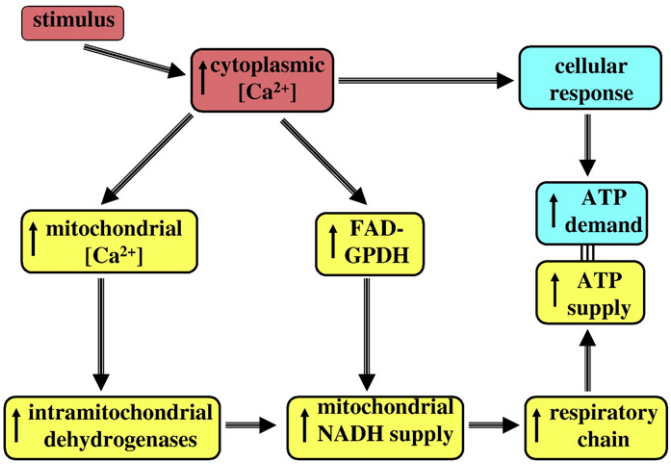

Fig. 1. Calcium ions in the co-ordinate regulation of ATP supply and demand in vertebrate tissues.

using the then newly-developed EGTA calcium buffers [13], that calcium ions activated the process by lowering the Km for glycerol phosphate and that the $k_{0.5}$ for calcium ions was about 0.1 μM. Qualitatively similar results were obtained when the dehydrogenase itself was assayed in freshly prepared mitochondrial extracts. Since the enzyme appeared to be exposed to cytoplasmic concentrations of calcium ions and had a similar sensitivity to calcium ions as the actomyosin ATPase, they concluded that activation of this enzyme was important in the increased supply of ATP needed in stimulated insect flight muscle [1].

In 1969, Reed et al. showed that the pyruvate dehydrogenase complex in mammalian tissues can be regulated by reversible phosphorylation [14]. Phosphorylation was brought about by an ATP requiring kinase that was tightly bound to the complex and resulted in essentially complete loss of pyruvate dehydrogenase activity. Reactivation could be brought about by a magnesium activated phosphatase [14]. In 1971, we reported that insulin activated pyruvate dehydrogenase activity in rat fat cells due to the dephosphorylation of the complex [15]. This seemed likely to be brought about by the activation of pyruvate dehydrogenase phosphatase and so I together with Philip Randle and Rick Martin studied the properties of this enzyme in some detail [2]. As with FAD-glycerol phosphate dehydrogenase, it was studies on the comparative effects of the bivalent metal ion chelators, EDTA and EGTA, that first suggested to us that this enzyme might also be sensitive to micromolar concentrations of calcium ions as well as, in this case requiring millimolar concentrations of magnesium ions. Use of EGTA buffers showed that the phosphatase from a number of mammalian tissues was activated by calcium ions with a $k_{0.5}$ close to 1 μM [2]. Subsequent studies showed that although insulin does act through stimulating the phosphatase, it does not act by increasing the mitochondrial concentration of calcium ions (for review see [16]). Nevertheless, the finding that pyruvate dehydrogenase phosphatase was activated by calcium ions alerted us to the possibility that calcium ions might be important in the regulation of intra-mitochondrial metabolism.

I was therefore puzzled by the reports of Newsholme and colleagues [17,18] that the intramitochondrial enzyme NAD-isocitrate dehydrogenase from invertebrate sources was inhibited by micromolar concentrations of calcium ions. It seemed strange that calcium ions should have potentially opposing effects on two key intramitochondrial dehydrogenases. Moreover, although these studies were carried out apparently with calcium-EGTA buffers, the presence of high concentrations of manganese ions meant that the buffers could not operate and the actual calcium ion concentrations in their studies were at least three orders of magnitude higher than those assumed by the authors. When I together with two undergraduate students, David Richards and Jude Chin, restudied the sensitivity of NAD-isocitrate dehydrogenase from pig heart and other mammalian sources using magnesium ions rather than manganese ions it became evident that, rather than inhibit, calcium ions in the low micromolar range could activate this enzyme [3].

Following this, Jim McCormack and I made a careful study of other intramitochondrial enzymes involved in the supply of reducing equivalents to the respiratory chain. Only one further enzyme of the many we studied proved to be calcium sensitive and that was the oxoglutarate dehydrogenase complex — again it was activated by calcium ions with a $k_{0.5}$ close to 1 μM [4].

So at the end of the 1970s, we had found three important intramitochondrial dehydrogenases that were activated by low micromolar concentrations of calcium ions. We proposed that these activations could be important in matching ATP supply to enhanced ATP demand in stimulated mammalian cells in which the cytoplasmic concentration of calcium ions had been raised [4,5] (Fig. 1). For this proposal to be correct, the intra-mitochondrial concentration of calcium ions would have to change in parallel with cytoplasmic concentrations and in the range of concentrations (10^{-7} M to about 10^{-5} M) to which the three intramitochondrial dehydrogenases were sensitive. At the time mitochondria were generally considered to be important, if not the major, reservoirs of intracellular calcium with free concentrations of calcium ions in the matrix well in excess of this value. Moreover, it was being strongly advocated that mitochondria were the source of the increased cytoplasmic calcium ions seen in many mammalian cells as the result of the action of "calcium mobilising hormones". Linked with this was the concept that mitochondria played an important role in setting or buffering the basal cytoplasmic concentration of calcium ions [19–22].

3. Present knowledge of the interactions of calcium ions with the mitochondrial calcium-sensitive dehydrogenases

3.1. FAD-glycerol phosphate dehydrogenase

It is now well established that this enzyme from both vertebrate and invertebrate sources is activated by calcium ions with a typical $k_{0.5}$ in the region of 0.1 μM. The effect of calcium ions is to lower the Km for glycerol phosphate very substantially [8,23–26]. Studies with mitochondrial preparations indicated that this enzyme is associated with the inner membrane of mitochondria with binding sites for both glycerol phosphate and calcium ions on the outside of this membrane and essentially directly accessible to cytoplasmic concentrations of both of these small molecules [27,28]. The amino acid sequences of the enzyme from a number of sources is in agreement with this arrangement [26,29]. Hydropathy plots indicate three transmembrane segments towards the amino terminus. With this terminus placed on the matrix side of the inner mitochondrial membrane, about 70% of the enzyme lies on the cytoplasmic side within the intermembrane space. The predicted FAD binding site is within the first of the transmembrane segments whereas that for glycerol phosphate lies within the part of the molecule predicted to be on the outside of the inner membrane together with two EF-hand calcium ion binding motifs. The more C-terminal of the two domains contains all the features to form a functional EF-hand; however, the other potential domain has a lysine residue in a position likely to be critical for calcium ion binding suggesting that it is not involved in high affinity calcium binding (Fig. 2). There are many other examples of calcium regulated proteins containing pairs of EF-hands with one probably unable to bind calcium [30–32]. Direct binding of calcium ions to glycerol phosphate dehydrogenase has not been intensively studied but binding to the enzyme from rat testis has been demonstrated [26]. The enzyme from yeast and plants has a strong similarity to the enzyme from mammalian sources with over 50% of aligned amino acid

calcium binding loop position	1	2	3	4	5	6	7	8	9	10	11	12
consensus sequence	asp	X	O	X	O	gly	X	J	O	X	X	glu
FAD-GPDH EF hand 1	asp	glu	asp	glu	lys	gly	phe	iso	thr	iso	val	asp
FAD-GPDH EF hand 2	asp	leu	asn	lys	asn	gly	gln	val	glu	leu	his	glu
PDH-Pase1 "EF" sequence	asp	asn	asp	ile	ser	leu	glu	ala	gln	val	gly	asp

Fig. 2. Sequences of possible calcium binding loops within potential EF-hands. An EF-hand Ca^{2+} binding site is typically 29 amino acids and consists of a calcium binding loop flanked by helical regions [31,32]. The calcium binding loop of 12 residues has the consensus sequence shown with the residues that co-ordinate the calcium ion being residues 1 and 12 (the acidic groups of asp and glutamate respectively) plus residues 3, 5 and 9 (asn, asp, glu, gln, ser or thr — shown as "O") and the carbonyl group of residue 8. Residue 6 is invariably gly allowing a 90 degree turn essential for the co-ordinations with the calcium ion to take place while residue 8 is either ile, leu or val involved with hydrophobic links with the corresponding residue in the paired loop (shown as "J").

residues being conserved [33,34]. However, the yeast and plant enzymes are smaller than their mammalian counterparts and lack the two EF hands. It is reasonable to assume that the yeast and plant enzymes are insensitive to calcium ions but this does not seem to have been shown directly.

3.2. Pyruvate dehydrogenase phosphatase

In mammalian tissues, the pyruvate dehydrogenase complex has a molecular weight of about 8 MDa. The complex is based on a hollow dodecahedron core. Most of the 60 subunits that make up this core are the dihydrolipoate acetyltransferase (E2) subunits but probably 12 are the related E3 binding (E3BP) subunits [35]. To this core are attached the pyruvate decarboxylase (E1) subunits and the dihydrolipoate dehydrogenase (E3) subunits. The reaction catalysed by the E1 subunits is the irreversible step and the enzyme is composed of two subunits and exists as a tetramer, $\alpha_2\beta_2$ [36]. The pyruvate dehydrogenase complex has a central role in mammalian metabolism, in particular it represents "the point of no return" in carbohydrate metabolism. The complex is therefore subject to stringent regulation [37,38]. The activity of the complex can be directly influenced by end-product inhibition by increasing concentrations of acetylCoA/CoA and $NADH/NAD^+$ ratios as well as more importantly by reversible phosphorylation of three sites on the E1 subunits by highly specific kinases and phosphatases found in mitochondria.

In mammalian mitochondria, there appears to be two isoforms of pyruvate dehydrogenase phosphatase, PDP1 and PDP2, each with a Mg^{2+}-dependent catalytic subunit of about 55 kDa, designated PDP1c and PDP2c. They are related to the protein phosphatase 2C/PPM family of protein phosphatases. In the case of PDP1, its catalytic subunit is bound to a regulatory subunit of around 95 kDa that contains FAD. The role of the regulatory subunit is poorly understood but it may influence the sensitivity of the catalytic subunit to Mg^{2+} [39–43].

Important to the topic of this article, only PDP1c is activated by calcium ions [42]. There is mounting evidence that expression of the two isoforms may differ in different tissues and may also be affected by nutrition status [40,41]; thus the possibility arises that the calcium sensitivity of the pyruvate dehydrogenase system in different vertebrate tissues and circumstances may vary. However, it is important to note that the actual relative catalytic activities within mitochondria have not been measured directly and we will return to this point later.

Progress has been made into understanding the mechanism whereby calcium ions regulate PDP1c. Binding studies using purified PDP1c and components of the pyruvate dehydrogenase complex show that PDH-phosphatase binds to E2 provided that calcium ions are present and that this association greatly increases the activity of the phosphatase towards the phosphorylated sites on the E1 subunits [43,44]. The $k_{0.5}$ for calcium ions for this is close to 1 μM in agreement with the effects of calcium ions on activity. Binding studies have also been carried out showing that neither PDP1c nor E2 bind calcium ions alone, but in combination form a single calcium binding site with a $k_{0.5}$ of about 10 μM [44]. Each of the E2 subunits contains two covalently attached lipoyl groups within independently folded domains — an NH2-terminal one (L1) and an interior one (L2). It is the L2 domain that is involved in binding PDP1c [45–47]. Recently the crystal structure of PDP1c has become available [48] and as expected the structure confirmed that PDP1c belongs to the 2C/PPM family of protein serine/threonine phosphatases. On the basis of this crystal structure a model has been proposed for the role of calcium ions in the binding of PDP1c to the lipoic acid within the L2 domain of E2 but confirmation of this model must await the determination of the structure of the PDP1c/L2/calcium ion complex[48].

The deduced amino acid sequence of PDP1c contains a putative EF-hand calcium binding motif (Fig. 2) and early studies suggested that this may also be involved in calcium binding [45]. However, this sequence does not contain the essential glycine at position 6 and does not take up the characteristic EF-hand conformation in the PDPc1 crystal structure [44,48]. A similar sequence is also present in PDPc2 which is calcium insensitive [40] and as mentioned above recent careful binding studies were unable to detect any specific calcium binding to the PDPc1 in the absence of the L2 domain of E2 [44]. Thus it must be concluded that this putative EF-hand domain has no role in calcium binding.

Extensive studies have been carried out to explore the regulation by calcium ions of pyruvate dehydrogenase phosphatase(s) within mitochondria from a number of different mammalian tissues including heart [49,50], liver [51], fat cells [52] brain [53] and kidney [54]. These have shown using permeabilised, uncoupled or coupled mitochondria that the pyruvate dehydrogenase systems within mitochondria from all these sources are indeed sensitive to calcium ions with a $k_{0.5}$ in all cases close to 1 μM. The major differences in calcium sensitivity that would be predicted from the apparently large variations in relative amounts of the calcium-sensitive PDP1 and the calcium-insensitive PDP2 are not found. An explanation of this may be that the actual specific activity of PDP2 within mitochondria may be much less than that of PDP1 perhaps because it has a much higher $k_{0.5}$ for Mg^{2+}. The $k_{0.5}$ values for PDP2 have been reported to be about 15 mM (although this can be reduced by the polyamine, spermine) and hence are at least an order of magnitude greater than that of PDP1

[55] or the likely concentration of magnesium ions within mitochondria [56]. Methods are needed to be able to measure accurately the contribution of each of the two isoforms to overall pyruvate dehydrogenase phosphatase activity within intact mitochondria. Studies with toluene-permeabilised mitochondria may be a useful approach here since these mitochondria are permeable to all small molecules and thus the effects of calcium and magnesium ions and other potential regulators/inhibitors can be studied in detail.

Fig. 3 summarises results obtained in 1980 using coupled rat heart mitochondria [49]. These studies showed for the first time that increasing extramitochondrial calcium ion concentrations in the physiological range altered the activity of both pyruvate dehydrogenase and oxoglutarate dehydrogenase within mitochondria (Fig. 3) and hence furnished excellent early evidence for the basic hypothesis set out in Fig. 1 [5]. It should be noted that if such studies are carried out in the absence of extramitochondrial sodium and magnesium ions then half-maximal effects of extra-mitochondrial calcium ions are seen at about 0.04 μM — well below the physiological range. However, from the properties of the uptake and efflux components of the calcium-transporting systems in rat heart mitochondria it would be expected that addition of both sodium ions (which are necessary for the efflux pathway) and magnesium ions (which inhibit uptake) should diminish the gradient of calcium ions across the mitochondrial inner membrane and thus a relatively higher extramitochondrial calcium ion concentration would be needed to cause half-maximal activation of pyruvate dehydrogenase and oxoglutarate dehydrogenase. This prediction is completely fulfilled in rat heart mitochondria (Fig. 3) and

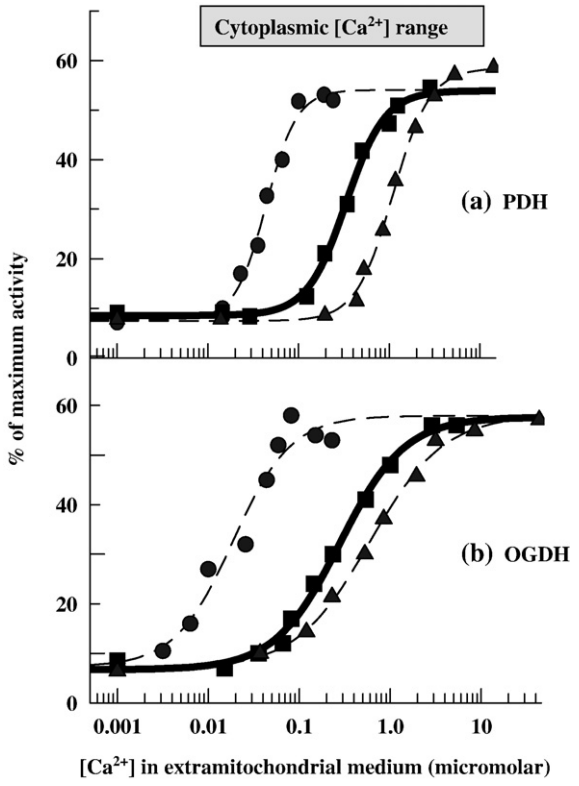

Fig. 3. Sensitivity of the pyruvate dehydrogenase system and oxoglutarate dehydrogenase in coupled rat heart mitochondria to changes in extramitochondrial calcium ion concentration. (a) Pyruvate dehydrogenase (PDH) activity: mitochondria were incubated in medium containing phosphate, oxoglutarate and malate plus CaEGTA buffers to give required extramitochondrial calcium ion concentration and in the presence of (●), no additions; (■), 15 mM NaCl and 0.5 mM Mg_2Cl; (▲) uncoupler FCCP plus ATP. (b) Oxoglutarate dehydrogenase (OGDH) activity: mitochondria were incubated in the same conditions as (a) but 2 mM ADP was added and the initial concentration of oxoglutarate was 0.5 mM; no ATP was added with uncoupler ATP. Results from [49] where full details can be obtained.

subsequently comparable results have been obtained in mitochondria from other tissues [7,51,52,57]. Fig. 3 also shows the sensitivity of the pyruvate dehydrogenase system in uncoupled mitochondria when little or no gradient of calcium ions would be expected across the inner mitochondrial membrane is close to 1 μM. Similar values are found with toluene permeabilised mitochondria and purified enzymes as summarised above. The greater sensitivity to extra-mitochondrial calcium ions in coupled mitochondria in the presence of physiological concentrations of sodium and magnesium ions would predict that in intact cells the concentration of calcium ions in mitochondria tissues would not be normally more than 2 to 3 times that in the cytoplasm. Subsequent studies in which calcium concentrations have been measured directly at the same time in both cytoplasmic and mitochondrial compartments are in general agreement with this (see [11] for review).

There is mounting evidence that the pyruvate dehydrogenase complex is regulated by phosphorylation in yeast, plants and invertebrates [39,58–61] but the properties of the pyruvate dehydrogenase phosphatases involved have not been as extensively studied as those from mammalian tissues and in particular their sensitivity to calcium ions appears unknown.

3.3. NAD-isocitrate dehydrogenase

NAD-isocitrate dehydrogenase from mammalian sources appears to exist primarily as an octamer of composition $2(\alpha_2\beta\gamma)$. All three different subunits are structurally related and have molecular masses close to 40 kDa [62–66]. Recent site-directed mutagenesis studies have suggested complementary roles of the different subunits with substrate binding and active sites being shared between the subunits [67,68]. No crystal structure of this enzyme is currently available. However, structures of the yeast enzyme have been published [69]. The yeast enzyme is again an octamer but composed of equal numbers of just two different subunits. The crystal structures again suggest that substrate and regulatory sites are positioned at the interfaces between the two different subunits[69]. Both the mammalian and yeast enzymes have complex regulatory properties consistent with a controlling role in the formation of NADH for the respiratory chain. In particular, the enzymes are inhibited by increasing ATP/ADP and $NADH/NAD^+$ ratios, properties that are shared with the pyruvate dehydrogenase system and oxoglutarate dehydrogenase.

Calcium ions activate mammalian NAD-isocitrate dehydrogenase by causing a marked decrease in the Km for the substrate threo-DS-isocitrate [3,70,71]. The presence of either ADP or ATP is an absolute requirement for calcium sensitivity. It should be noted that no such equivalent requirement is observed for the actions of calcium ions on pyruvate dehydrogenase phosphatase and oxoglutarate dehydrogenase. The calcium ion sensitivity of NAD-isocitrate dehydrogenase is influenced by the ATP/ADP ratio — becoming more sensitive at lower ratios. The range of $k_{0.5}$ values for calcium ion activation that are observed range from 5–50 μM depending on the ATP/ADP ratio [70,71]. This range is observed both with isolated enzymes and with the enzyme within mitochondria and is about an order of magnitude higher than that observed for pyruvate dehydrogenase phosphatase and oxoglutarate dehydrogenase. This important difference is discussed further in Section 4.1.

Direct measurements of calcium ion binding also fit these kinetic properties [71]. Binding requires the presence of ADP or ATP as well as isocitrate and magnesium ions and the dissociation constant for calcium binding is about 5 μM in the presence of ADP but much greater, about 40 μM, in the presence of ATP, consistent with calcium ion effects on activity. The stoichiometry of calcium binding appears to be about 2 calcium ions per octamer. This would be consistent with binding to either β or γ subunits but given the mounting evidence that the binding of isocitrate and regulators may be at interfaces between subunits it seems quite possible that this may be also the case for

calcium ions. No putative EF-hands or other calcium binding sites have been identified by comparing the sequences of the three subunits with other known calcium binding proteins [64,65].

Calcium sensitivity appears only to be observed with NAD-isocitrate dehydrogenase from vertebrate sources. Certainly the enzyme from yeast, blowfly and locust flight muscle, potato and the spadix of the arum lily did not show any stimulation by calcium ions whereas the enzyme from the hearts of frog, trout and pigeon were sensitive [72,73].

It should be noted that mitochondria from vertebrate sources contain activities of NADP-isocitrate dehydrogenase that are often greatly in excess of those of NAD-isocitrate dehydrogenase. This enzyme shows no allosteric regulation including regulation by calcium ions [3]. However, the reducing equivalents on the $NADPH^+$ produced can be transferred to NAD^+ to give NADH by nicotinamide transhydrogenase which is present in the inner membrane of mitochondria [74]. Thus it is possible that this might be an alternative route whereby isocitrate is converted to oxoglutarate in mitochondria rather than involving NAD-isocitrate dehydrogenase. Indeed, this has recently been proposed by Hartong et al. [75] who have found in two families with retinitis pigmentosa that affected family members were homozygous for an essentially complete loss of function mutation in the β-subunit of NAD-isocitrate dehydrogenase associated with a massive increase in the Km for NAD^+. The affected individuals had no health problems except the retinitis pigmentosa suggesting that NAD-isocitrate dehydrogenase may not be an essential component of the citrate cycle in tissues other than the retina, which may have particularly low levels of the NADP-linked enzyme. Further studies are needed in a range of coupled mitochondria and intact cell preparations that express different activities of the two dehydrogenases to explore this possibility. However, it should be noted that in intact coupled rat fat, liver and kidney mitochondria increases in calcium ion concentrations clearly stimulate oxidative metabolism with isocitrate as the main substrate [51,54]strongly enhancing the widespread view that it is the NAD^+ linked enzyme that normally participates in the citrate cycle [76–78].

3.4. Oxoglutarate dehydrogenase

Oxoglutarate dehydrogenase is a multienzyme complex with some similarities with pyruvate dehydrogenase. It has a hollow core made up of predominantly dihydrolipoamide succinyl-transferase (E2) subunits to which are attached 2-oxoglutarate decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3) subunits [79] although the latter attachment may be via binding to E1 subunits [80]. As with pyruvate dehydrogenase, the enzyme is end-product inhibited by increases in the succinyl CoA/CoA and $NADH/NAD^+$ ratios. However, unlike pyruvate dehydrogenase the enzyme is not regulated by reversible phosphorylation. The action of calcium ions is directly on the enzyme and is associated with a marked decrease in the Km for oxoglutarate [4]. The Km for oxoglutarate is also decreased by a decrease in the ATP/ADP ratio and also, as with NAD-isocitrate dehydrogenase, decreases in this ratio also markedly increase the sensitivity of the enzyme to calcium ions [4,70,81]. The Km in the presence of ADP alone is about 0.2 μM, whereas that with ATP alone is close to 2 μM [70].

Studies into the binding of calcium ions to purified pig heart oxoglutarate dehydrogenase indicate that between 2.5 and 5 calcium ions bind to each complex and that the dissociation constant for this binding is in the range 1–7 μM [71] close to the range of $k_{0.5}$ values for calcium activation. As with pyruvate dehydrogenase it is likely that the step catalysed by E1 normally regulates overall activity and it is this step that appears to be sensitive to calcium ions [81]. Each oxoglutarate complex probably contains 12 E1 subunits arranged as 6 dimers so it is possible that each E1 dimer binds a calcium ion. However, this has not been shown directly.

Extensive studies have been carried out on the properties of oxoglutarate dehydrogenase within permeabilised, uncoupled and coupled mitochondria from a variety of mammalian tissues [49–52,54]. Taken together these show that the regulatory properties of the enzyme within mitochondria are essentially the same as the isolated enzyme. In intact mitochondria, flux through oxoglutarate dehydrogenase can be rather straightforwardly studied by following oxygen uptake with added oxoglutarate as the oxidisable substrate. With coupled mitochondria incubated with media containing concentrations of sodium and magnesium ions likely to be present in the cytoplasm of cells, increases in extramitochondrial calcium ions in the physiological range result in increases in oxoglutarate oxidation. There is a decrease in $k_{0.5}$ for oxoglutarate oxidation as would be predicted from the properties of oxoglutarate dehydrogenase. An example of such studies using coupled rat heart mitochondria are shown in Fig. 3 [49]. This figure also illustrates that in the conditions used in these studies the pyruvate dehydrogenase and oxoglutarate dehydrogenase systems have very similar sensitivities to increases in extramitochondrial calcium. Broadly similar results have been obtained with mitochondria from rat adipose tissue, liver and kidney [51,52,54]. It is important to note as mentioned above that the sensitivity of oxoglutarate dehydrogenase to calcium ions is quite markedly increased by decreases in the ATP/ADP ratio whereas the sensitivity of pyruvate dehydrogenase phosphatase may be unaffected. Hence under some conditions activation of oxoglutarate dehydrogenase may be observed at lower calcium ion concentrations than that of the pyruvate dehydrogenase system [82].

Again as with NAD-isocitrate dehydrogenase, only oxoglutarate dehydrogenases from vertebrate sources have been shown to be activated by calcium ions. The enzyme from yeast, plant and insect sources that have been studied show no sensitivity to calcium ions [72,73].

4. General conclusions and areas for future research

4.1. Regulatory role of calcium ions within mitochondria of vertebrates

The studies on isolated enzymes and with mitochondrial preparations I have summarised above all fit with the general concept illustrated in Fig. 1. Namely, that the increased ATP synthesis needed in many cells in which stimuli have increased cytoplasmic concentrations of calcium ions may be brought about, at least in part, by parallel increases in mitochondrial concentrations of calcium ions activating the intramitochondrial calcium-sensitive dehydrogenases and hence through increases in NADH supply enhance respiration.

Studies on intact mammalian cell preparations have supplied further strong support for this basic hypothesis. The most extensively studied systems have been the effects of positive inotropic stimulation of the heart and the effects of “calcium-mobilising” hormones in the liver. Early approaches before 1990 are reviewed in [6]. Two in particular were useful. One was the rapid preparation of mitochondria from control and stimulated intact cell preparations under conditions in which the transfer of calcium in and out of mitochondria was blocked [83]. With this technique it was possible to show that the activations of pyruvate dehydrogenase and oxoglutarate dehydrogenase were associated with increases in intra-mitochondrial calcium ion concentration [83,84]. The other was the use of the Ruthenium Red which inhibits calcium uptake into mitochondria in the perfused rat heart. In the presence of the Ruthenium Red, the activations of pyruvate dehydrogenase that are seen with inotropic stimulation is greatly diminished in agreement with the view that the increase requires transfer of calcium ions into the mitochondria [85,86]. Since 1990, the development of methods

of measuring the intramitochondrial concentration of calcium ions within intact mammalian cell preparations have shown clearly that normally the intramitochondrial concentration of calcium is within the range necessary to regulate three calcium sensitive intramitochondrial dehydrogenases (0.1–10 μM) and moreover have shown directly that increases in cytoplasmic calcium result in increases in mitochondrial calcium [11].

The activation of FAD-glycerol phosphate dehydrogenase by cytoplasmic calcium is another way that the supply of reducing equivalents to mitochondria may be enhanced. It has been shown recently that aspartate/glutamate transporters in the inner membrane of mammalian mitochondria are also activated by cytoplasmic calcium through EF-hand domains and because of the role of such transporters in the aspartate/malate NADH shuttle this is potentially a further mechanism whereby increases in calcium ions may stimulate the supply of reducing equivalents to the respiratory chain [87].

It is important to view the model set out in Fig. 1 as an oversimplification.

Firstly, the regulation of ATP synthesis in mitochondria is complex and that in many circumstances changes in ATP synthesis will be brought about by a combination of mechanisms including increases in ADP and substrate supply as well as increases in calcium ion concentrations in both the cytoplasm and mitochondria. It should be noted that the activities of all three of the calcium-sensitive dehydrogenase are also potentially enhanced by increases in ADP, NAD^+ and/or increases in the concentration of their substrates. A continuing challenge is to develop means of measuring alterations simultaneously in the intramitochondrial concentrations of calcium ions, ATP, ADP, NAD^+, NADH and key substrates in intact cell preparations under conditions where measurable changes in the flux through the dehydrogenases are occurring. A task made more difficult by the likelihood that there may well be marked differences in the roles and activities of mitochondria in the different regions of many cells.

Secondly, there are also a number of additional potential targets of calcium action within mitochondria that may also directly or indirectly influence respiration and hence ATP synthesis. It has been proposed that the mitochondrial F_1F_0ATPase itself may be activated by micromolar concentrations of calcium ions by a mechanism involving the release of a small inhibitory protein [88–91] There is also evidence, at least in liver mitochondria, that pyrophosphatase activity is inhibited by low micromolar concentrations of calcium ions and that this may be linked to increases in mitochondrial volume and hence in respiratory chain activity [6,92,93]. At rather higher concentrations, calcium may participate in the mechanisms leading to pore opening as described in other articles in this volume [94,95]. Finally, it has recently been proposed that the mitochondria of mammalian tissues may contain an isoform of a calcium sensitive NO synthase (n-NOS) [96,97]. The apparent sensitivity to calcium ions would appear to be via calmodulin and to be less than that of oxoglutarate dehydrogenase in the same mitochondria but still in the low micromolar region. Increases in mitochondrial NO concentration that might occur by this mechanism could influence respiration by inhibiting cytochrome oxidase and if so would oppose the effects of calcium ions on the dehydrogenases.

Thirdly, returning to the three calcium sensitive dehydrogenases, it seems clear that there are differences in the $k_{0.5}$ values for calcium at least under certain conditions. The most sensitive is oxoglutarate dehydrogenase in the presence of low ATP/ADP ratios followed closely by pyruvate dehydrogenase and finally NAD-isocitrate dehydrogenase which is distinctly less sensitive especially at high ATP/ADP ratios. What is the physiological importance of these differences? Is there a hierarchy of activation as calcium ions are progressively increased in mitochondria of stimulated tissues. If so how do the other potential effects of calcium ions both in mitochondria and on processes such as FAD-glycerol phosphate dehydrogenase and aspartate/glutamate transporters in the inner-membrane of mitochondria fit into this hierarchy?

4.2. Molecular basis of the calcium activation of the mitochondrial dehydrogenases

The binding site for calcium ions on FAD-glycerol phosphate dehydrogenase is clearly an EF-hand. In the case of pyruvate dehydrogenase, it seems likely that crystallographic studies on an appropriate ternary complex will confirm the already mounting evidence that calcium binds in this system at an interface made from the L2 domains of E2 together with the phosphatase PDP1c. The putative EF hand in PDP1c does not seem to be involved in calcium binding as it cannot take up an appropriate conformation. In contrast, we have no clear picture as to how calcium ions may bind to NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase beyond a measure of the number of binding sites. None of the amino acid sequences of any of the subunits of the two enzymes appear to contain any clearcut calcium ion binding sites and there appears to be a dearth of useful crystal studies that might help with the issue.

One obvious possibility that we have raised in the past is that calcium ions may interact with these dehydrogenases via a distinct calcium binding subunit that has gone undetected to date [9]. However, recent studies using mass spectrophotometry on highly purified preparations of both NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase that show full sensitivity to calcium ions have not shown evidence of any such extra subunits ([98]; unpublished observations Heesom, KJ and Denton RM). Moreover, there are important differences in calcium binding that also might argue against these dehydrogenases sharing a calcium binding protein. In particular, calcium binding to NAD-isocitrate dehydrogenase requires the presence of isocitrate, adenine nucleotide and magnesium ions whereas that to oxoglutarate dehydrogenase has no equivalent requirements. In summary, it seems highly likely that the molecular basis of calcium binding to all three of the intramitochondrial calcium sensitive enzymes will be distinct. Perhaps the only common feature may be that in every case the calcium binding sites may occur at the interfaces between subunits!

4.3. Are calcium-sensitive dehydrogenases only found within the mitochondria of higher animals?

This is an important question which has probably not had the attention which it deserves. The limited studies carried out to-date have only shown calcium-sensitive dehydrogenases in the mitochondria of vertebrates. No sensitivity has been reported for any of the enzymes studied in yeast, plants and insects. It is clear that further studies are required to refine the knowledge of the appearance of intramitochondrial calcium-sensitive dehydrogenases during evolution. Are there species in which only one or two of the intramitochondrial dehydrogenases are calcium sensitive?

The pattern of distribution of calcium-sensitive dehydrogenases that has emerged so far supports the idea that this calcium sensitivity emerged as a means of complementing more basic mechanisms of regulating respiration and ATP synthesis such as changes in ADP concentration. The likely advantage of involving calcium ions in the regulation of NADH production within mitochondria is that it allows increases in ATP demand to be met in stimulated cells without the need for the all-important ATP/ADP ratio in cells to fall. The situation in insect flight muscle is of considerable interest in this regard. Huge increases in ATP utilisation occur in this tissue during flight initiated by increases in cytoplasmic calcium ions and these increases also stimulate the supply of reducing equivalents to respiration by stimulating FAD-glycerol phosphate dehydrogenase on the outside of the inner membrane of mitochondria. However, intramitochondrial dehydrogenases in this tissue appear to be insensitive to calcium ions

and so other mechanisms including a decrease in ADP are probably involved in increasing their activities as well as respiration [99].

Detailed study of the amino acid sequences of a wide range of subunits of calcium-sensitive and calcium-insensitive dehydrogenases may well be helpful in supplying clues as to the amino acids involved in calcium binding. Moreover, comparative studies on the calcium transport systems in the mitochondria of a range of species may well help with understanding the developmental relationships between these systems and that of the intramitochondrial calcium sensitive dehydrogenases.

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