FORMATION OF CITRATE IN RAT LIVER MITOCHONDRIA IN RESPONSE TO CALCIUM IONS

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1. Introduction

The energy dependent uptake of Ca^{2+} by mitochondria has attracted much attention (see reviews by Chance [1] and Rossi, Carafoli, Drahota and Lehninger [2]). A possible function of the phenomenon in the muscle cell as a means of removing ionic Ca from the cytoplasm after the ion's release during contraction has been discussed [1]. However, no such function can obtain in other organs and Ca shifts have rather been related to enzyme regulation, either of cytoplasmic pyruvate kinase [3] or of mitochondrial pyruvate carboxylase [3, 4]. It has been shown that the intramitochondrial formation of citrate depends on the redox state of the pyridine nucleotides rather than on the ATP/ADP ratio [5]. Since Ca^{2+} addition causes a cycle of pyridine nucleotide oxidation [1] it should favour citrate formation and we have investigated this.

2. Methods

Rat liver mitochondria were prepared by Schneider's method [6]. Homogenisation and the first two washes were carried out in 250 mM sucrose with 1 mM ethyleneglycol bis(2-aminoethyl)tetracetate. The third wash and resuspension was carried out in 250 mM sucrose. Portions of the suspension were incubated in the media specified in the tables. Samples were withdrawn at intervals for rapid centrifugal separation as described by Harris and Van Dam [7]. Metabolite levels in the supernatants and in the acid extracts of the mitochondrial pellets were measured using specific enzymes. Ca was measured by atomic absorption spectrophotometry. In most experiments an equimolar Ca+Mn mixture was used because this is stated [8] to have a less disruptive effect than Ca alone.

3. Results

The cycle of decreased fluorescence from the reduced form of pyridine nucleotide which occurs when Ca^{2+} is added could, in rat liver, be indicative of oxidation of either or both of NADH and NADPH. Analyses made on samples withdrawn in experiments in which Ca^{2+} additions were made showed that, in fact, only the NADH is oxidised. (See table 1.)

Since therefore the transhydrogenase activity in the non-energy-requiring direction from NADPH to NAD is inadequate to link the redox states of the two nucleotides, it is likely that the energy requirement for Ca^{2+} transport is met primarily from malate oxidation. The extent to which the oxidation of isocitrate and glutamate will be promoted depends on the concentrations and the degree of specificity of the internal enzymes to NADP.

A shift from malate to oxalacetate will favour citrate formation so long as a source of acetyl-CoA is present. With pyruvate as added substrate the endogenous malate, plus oxalacetate formed by carboxylation of pyruvate, can lead to the generation of citrate in response to Ca^{2+}. This is shown in lines 2 and 3 of table 2.

Adding malate releases the newly formed citrate into the medium (table 2, line 4) while more Ca^{2+} generates more citrate and tends to move citrate

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Table 1

Changes of NADH and NADPH contents of rat liver mitochondria in response to an addition of Ca^{2+} as $CaCl_2$. Fluorescence was monitored (excitation 360 nm, measurement at 420 nm) and samples with withdrawn at intervals for alkaline extraction and analysis.

Time of sampling	% Original fluorescence	Contents: μmole/g protein NADH	NADPH
Before addition		2.7	3.8
Just after adding Ca^{2+} 62 μmole/g protein	64	0.7	4.0
After 50 sec	87	1.7	3.7
After 132 sec	117	2.5	3.7

The medium contained sucrose 250 mM, KCl 10 mM, Tris chloride pH 7.4 20 mM, Tris glutamate and malate 3 mM each.
(We are indebted to Dr. M.P.Höfer for these analyses.)

Table 2

Generation of citrate in response to $Ca^{2+} + Mn^{2+}$ additions and the shift from mitochondria to medium caused by malate.

Additions in sequence	Citrate in Medium (μM)	Mitochondria μmole/g protein
None	60	6.4
6 μmole each Ca^{2+}+Mn^{2+} per g protein	63	8.4
8 μmole each Ca^{2+}+Mn^{2+} per g protein	60	9.9
1.3 mM malate	128	6.7
17 μmole each Ca^{2+}+Mn^{2+} per g protein	80	12.5

The medium contained KCl 120 mM, Tris chloride pH 7.4 20 mM, tris pyruvate 2.5 mM, fluorocitrate 15 μM. Equimolar $CaCl_2$ + $MnCl_2$ was used for the additions. Protein 6.3 mg/ml.

Table 3

Changes in mitochondrial citrate, Ca^{2+} and respiratory rate after $Ca^{2+} + Mn^{2+}$ additions followed by citrate lyase.

Addition	In medium (μM) Ca	Citrate	In mitochondria (μmole/g) Ca	Citrate	Resp. Rate (μg atom oxygen/min/g protein)
None	20	280	6	7	5
6 μmole Ca^{2+}+Mn^{2+} each per g protein	48	180	13	n.d.	falls to 7 after transient
8 μmole Ca^{2+} + Mn^{2+} each per g protein	66	72	16.5	9.9	falls to 7 after transient
1.6 mM malate	90	90	13	10.4	no change
Citrate lyase	160	n.d.	8	5.7	12

Medium as in expt. of table 2, but pyruvate at 1 mM.
n.d. = not determined.

Table 4

Accumulation of citrate and pyruvate in mitochondria caused by $Ca^{2+} + Mn^{2+}$ additions in presence of malate. Palmityl carnitine added alternately with $Ca^{2+} + Mn^{2+}$.

Addition	Citrate in Medium (μM)	Mitochondria (μmole/g protein)	Pyruvate in Medium (μM)	Mitochondria (μmole/g protein)
None	56	0.7	56	1.0
l-(−)palmityl carnitine 26 μM	76	3.3	127	2.2
6 μmole Ca^{2+}+Mn^{2+} each per g protein	86	4.2	92	2.6
l-(−)palmityl carnitine 38 μM	99	7.9	94	2.7
18 μmole Ca^{2+}+Mn^{2+} each per g protein	94	10.1	94	5.6

The medium contained KCl 120 mM, Tris Cloride pH 7.4 20 mM, Tris malate 2.5 mM. Protein 9 mg/ml.

from medium to mitochondria (table 2, line 5).

In a similar experiment (table 3) the final addition was of citrate lyase. This enzyme splits the extramitochondrial citrate and so promotes exodus of the internal citrate, which is accompanied by Ca^{2+}. In a parallel experiment in an oxygen polarograph it was noted that the respiratory rate increased after the addition of the lyase (last column, table 3).

This observation raises the suggestion that one factor determining the resting respiration of mitochondria is the availability of citrate to accumulate with the Ca^{2+} carried by the preparation from the homogenisation step. We have confirmed that Ca^{2+} applied to the mitochondria as equimolar Ca-citrate causes a cycle of pyridine nucleotide oxidation and a respiratory burst. The $Ca^{2+}$is taken up along with citrate and the internal:external citrate concentration ratio can be as high as 100:1 when external citrate is 250 μM.

The oxidation of malate to energise Ca^{2+} transport can also be important in the context of fatty acid oxidation. This process requires a sustained supply of CoA which can be provided if oxalacetate condenses with the acetyl-CoA being formed from the fatty acid. In a system having malate and palmityl carnitine as substrates the accumulation of both citrate and pyruvate is promoted by Ca^{2+} (table 4). The pyruvate presumably arises from oxalacetate decarboxylation.

The energy requirement imposed by Ca^{2+}, in common with that set up by uncouplers [9] or fatty acid [10] can also lead to appearance of phosphoenolpyruvate, particularly when oxoglutarate is the added substrate and inorganic phosphate is present. For example, in the presence of 1 mM oxoglutarate and phosphate the mitochondrial phosphoenolpyruvate rose from the endogenous level (1 μmole/g protein) to 2.7 μmole/g, while citrate increased from 3.0 to 8.9 μmole/g in response to addition of 30 μmole Ca^{2+}/g protein.

4. Discussion

The generation of internal citrate in response to Ca^{2+}, coupled with the ability of the citrate to emerge [11, 12] could explain the observation that citrate in body fluids tends to adjust to the level of Ca [13]. Whether citrate production in muscle mitochondria in response to Ca freed by muscular contraction plays a part in feeding the cation as its citrate complex to the reticular sacs is an interesting question.

The manganese accumulation was not measured in these experiments because of the lesser sensitivity of the atomic absorption method to this element. However, the fact of there being an uptake of Mn^{2+}, especially when Ca^{2+} is also added, has been shown by other authors [14].

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