THE LANTHANIDE-SENSITIVE CALCIUM PHOSPHATE PORTER OF RAT LIVER MITOCHONDRIA

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1. Introduction

The import of calcium (and of some other bivalent cations including strontium) into respiring mitochondria from rat liver occurs electrophoretically via a lanthanide-sensitive porter system that is insensitive to thiol reactors. We recently obtained evidence that only one electric charge is translocated through the lanthanide-sensitive porter system per Ca^{2+} imported; and we proposed the hypothesis that the so-called calcium porter may actually be a calcium phosphate porter, more precisely described as a $(Ca_2)^{4+}$–HPO_4^{2-} symporter [1].

Two other systems that catalyse phosphate translocation in rat liver mitochondria are known [2–4]. One is the NEM-sensitive and mersalyl-sensitive phosphoric acid uniporter (or phosphate/hydroxyl-ion antiporter). The other is the mersalyl-sensitive phosphate/dicarboxylate antiporter. Our hypothesis that there is a $(Ca_2)^{4+}$–HPO_4^{2-} symporter therefore predicts that it should be possible to observe a lanthanide-sensitive, but NEM- and mersalyl-insensitive, import of phosphate that is dependent on and stoicheiometric with the import of Ca^{2+} or Sr^{2+}. In the present paper we verify this prediction.

Abbreviations: NEM, *N*-ethyl maleimide; nupercaine, 2-butoxy-*N*-(2-diethylaminoethyl)cinchoninamide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; State 5, anaerobic mitochondrial state; State 3, uncontrolled state of respiration in the presence of substrate; State 4, controlled state of respiration in the presence of substrate; State 6, controlled state of respiration in the presence of substrate and excess external bivalent cation

2. Materials and methods

Rat liver mitochondria were isolated as described previously [1]. The methods of measuring and recording the rate of oxygen consumption and the time course of the changes of the pH of the suspension medium (pH_0) used for observing the translocation of acid equivalents and of electric charge in experiments of type A (respiratory State 5–3–4) and of type B (State 4–3–4 or 4–3–6) were essentially as before, using a closed glass reaction vessel [1]. Parallel experiments were done in glass centrifuge tubes covered with tinfoil caps for the purpose of measuring the import of Ca^{2+}, Sr^{2+} and P_i by direct estimations on the suspension medium after centrifugation. The mitochondrial suspensions (4.5 ml containing about 30 mg mitochondrial protein) were prepared in various media in the glass centrifuge tubes containing 1.6 g Ballotini glass-balls No. 12 (Chance Bros. Ltd, Birmingham). The Ballotini prevented dispersal of the medium immediately above the centrifuged mitochondrial pellet.

For initial anaerobic (State 5) measurements, the tubes were continuously flushed with a stream of O_2-free N_2 during preparation of the mitochondrial suspensions. Centrifuging was done at 2520 × *g* for 5 min in an angle centrifuge taking 1 min to attain full speed. The upper 3 ml of the supernatant was used for estimations of Ca^{2+}, Sr^{2+} and P_i on duplicate aliquots.

Estimations of Ca^{2+} and Sr^{2+} were done by atomic absorption spectrophotometry and by the EDTA method, as before [1]. P_i was measured by a modification of the Fiske and SubbaRow method using $SnCl_2$ as reducing agent [5].

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3. Results and discussion

Table 1 shows that, in experiments of types A and B, the ratio of Ca^{2+} or Sr^{2+} to P_i imported ($\leftarrow Ca/\leftarrow P_i$ or $\leftarrow Sr/\leftarrow P_i$) in respiring mitochondria was near 2 when the phosphoric acid uniporter was blocked by NEM. The type B experiments show that the NEM-insensitive import of Sr^{2+} and P_i is lanthanide sensitive, since 10 μM $LaCl_3$ suppressed the import of both Sr^{2+} and P_i in the initial control samples under aerobic conditions.

The relatively conventional experiments of type B have generally been done with Ca^{2+}. However, the uptake of calcium induces mitochondrial fragility [6], which is attributable to phospholipase activation. Local anaesthetics, such as nupercaine, have a stabilising effect [6]. Following observations by Carafoli [7] and Selwyn [8], we verified that similar results were obtained in type B experiments using either Ca^{2+} or Sr^{2+}. But Sr^{2+} gave more stable and reproducible respiratory transitions than Ca^{2+}, even when imported in large quantities.

Table 1
Stoicheiometry of Ca^{2+} and Sr^{2+} import with P_i into rat liver mitochondria respiring in the presence of NEM

A	Ca^{2+}	P_i	Ca^{2+}	P_i
Anaerobic outer medium	25.0	20.7	27.4	24.5
Aerobic outer medium	7.9	11.3	8.3	14.3
Imported	17.1	9.4	19.1	10.2
$\leftarrow Ca/\leftarrow P_i$	1.80		1.88	
B	Sr^{2+}	P_i	Sr^{2+}	P_i
Aerobic outer medium La before Sr	42.0	13.4	41.0	10.7
Aerobic outer medium La after Sr import	32.7	8.8	32.8	6.8
Imported	9.3	4.6	8.2	3.9
$\leftarrow Sr/\leftarrow P_i$	2.04		2.10	

Results of two separate experiments of type A and of type B are shown. Quantities of Ca^{2+}, Sr^{2+} and P_i are expressed as μg ion/g mitochondrial protein. Each value is the mean of duplicate (A) or triplicate (B) estimations, and the standard deviation was not greater than ±2%.

In A, the mitochondrial suspensions were prepared in glass centrifuge tubes in anaerobic medium containing 250 mM sucrose, 10 mM KNO_3, 3.3 mM glycylglycine, 200 μM nupercaine, catalase (0.05 μl Sigma C-100/ml), oligomycin (1 mg/g protein), 0.4 μM rotenone, 2 mM ascorbate, 40 μM TMPD and $Ca(NO_3)_2$ equivalent to about 20 μmol/g protein, at pH_0 7.1 and at 20°C. After 15 min equilibration, 0.2 mM NEM was added. After a further 5 min, 0.1 ml 30 mM H_2O_2 was added to half the tubes to initiate respiration. Three minutes later, all tubes were centrifuged.

In B, the mitochondrial suspensions were prepared in glass centrifuge tubes in aerobic medium containing 250 mM sucrose, 15 mM choline chloride, 3.3 mM glycylglycine and oligomycin (1 mg/g protein) at pH_0 7.1 and at 25°C. After 4 min, 0.2 mM NEM was added. Two min later, 10 μM $LaCl_3$ was added to half the tubes and after 1 min, 0.3 mM $SrCl_2$ (about 45 μmol/g protein) was injected into all the tubes. After a further 4 min, 10 μM $LaCl_3$ was added to the tubes that did not already contain $LaCl_3$, and all tubes were then centrifuged.

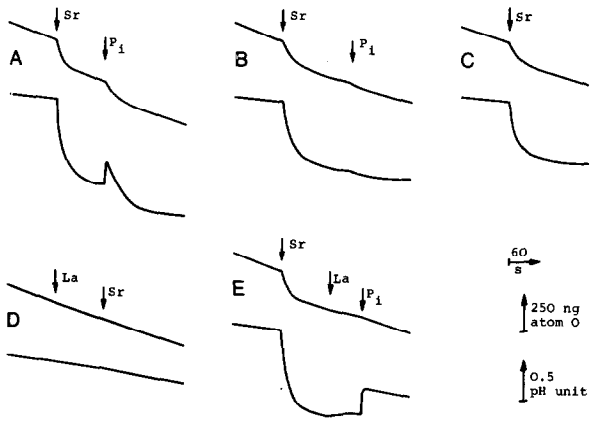

Fig.1. Time-course of oxygen concentration and of pH_0 in suspensions of respiring rat liver mitochondria. Upper curves: oxygen content of 3.3 ml suspension. Lower curves: pH_0. The aerobic suspension medium (3.3 ml) at 25°C and initially at pH_0 7.2 contained 250 mM sucrose, 15 mM choline chloride, 3.3 mM glycylglycine and mitochondria (7.0 mg protein/ml). Oligomycin (1 mg/g mitochondrial protein) and carbonic anhydrase (30 μg/ml) were also present. Other additions to the suspension medium (added 4 min after the mitochondria) were: (B) 0.2 mM NEM; (C) 0.06 mM mersalyl. Injections were made at the arrows: Sr, final concentration 0.6 mM $SrCl_2$; P_i, final concentration 0.2 mM choline phosphate; La, final concentration 10 μM $LaCl_3$.

Figure 1 shows typical recordings of oxygen concentration and of pH_0 in type B, State 4–3–6, experiments. The respiration of the rat liver mitochondria was supported by endogenous substrates and by 15 mM choline chloride present in the 250 mM sucrose medium. The quantity of Sr^{2+} injected in these experiments (final concentration 0.6 mM) corresponded to about 110 μg ion Sr^{2+}/g mitochondrial protein, whereas the quantity of endogenous phosphate present in the suspension medium in State 4 corresponded to about 0.1 mM or about 15 μg ion P_i/g mitochondrial protein. Thus, if Sr^{2+} entered the mitochondria as $(Sr_2)^{4+}$–HPO_4^{2-}, not more than 30% of the Sr^{2+} in these large pulses could be imported.

Figure 1A shows that respiration returned to a rate very close to the State 4 rate after the respiratory stimulation and acidification of the medium accompanying Sr^{2+} uptake. The fact that respiratory stimulation was subsequently induced by the injection of P_i (final concentration 0.2 mM) confirms earlier work on respiratory State 6 [9–11]. The P_i injected was previously adjusted to the same pH as pH_0 in this experiment, and the rapid alkalinisation was therefore attributable to the import of phosphate into the alkaline mitochondrial matrix via the phosphoric acid uniporter. This was confirmed in fig.1B, where the phosphoric acid uniporter was inhibited by the presence of 0.2 mM NEM, and no alkalinisation occurred when the P_i was added. There was a small, but significant, respiratory stimulation following the P_i injection, and a correspondingly small fall in pH_0, attributable to the entry of $(Sr_2)^{4+}$–HPO_4^{2-} via the lanthanide-sensitive porter.

Accordingly, the presence of 10 μM $LaCl_3$ (fig.1E) completely suppressed respiratory stimulation by P_i, but did not suppress the alkalinisation attributable to entry of P_i through the phosphoric acid uniporter. In fig.1C, 0.06 mM mersalyl was used to inhibit both the phosphoric acid uniporter and the phosphate/dicarboxylate antiporter. The result indicated that respiratory stimulation by $(Sr_2)^{4+}$–HPO_4^{2-} import was mersalyl insensitive. However, fig.1D showed that respiratory stimulation by Sr^{2+} was sensitive to 10 μM $LaCl_3$.

The univalent electric charge stoicheiometry of calcium import [1], the measured import of $2Ca^{2+}$ or $2Sr^{2+}$ per P_i in a lanthanide-sensitive but NEM-insensitive reaction (table 1), and the dependence of the release of respiratory control in State 6 on the import of P_i with Sr^{2+} (fig.1) are consistent with our hypothesis that the so-called calcium porter is actually a calcium phosphate porter catalysing $(Ca_2)^{4+}$–HPO_4^{2-} symport.

At pH_0 values on the acid side of pH_0 7.5 a significant proportion of the P_i in the suspension medium is present as $H_2PO_4^-$. Therefore, the entry of P_i as a porter-specific $(Ca_2)^{4+}$–HPO_4^{2-} complex should result in deprotonation of $H_2PO_4^-$ and acidification of the suspension medium. To test this prediction we measured the net appearance of H^+ in the medium [ΔH^+ (acid)] during Ca^{2+} import in type A experiments at three pH_0 values, and expressed the results as a ΔH^+(acid)/←Ca^{2+} ratio as shown in table 2. For comparison, we calculated the expected ratios for three conceivable calcium phosphate porter complexes. The experimental observations agree closely with the predicted result for the putative $(Ca_2)^{4+}$–HPO_4^{2-} porter complex, but not for the alternatives shown in the table.

Table 2
Dependence of the ΔH^+(acid)/$\leftarrow Ca^{2+}$ ratio in respiring rat liver mitochondria on pH_o

pH_o	ΔH^+(acid)/$\leftarrow Ca^{2+}$ ratio			
	Observed	Calculated for:		
		Ca^{2+}–$H_2PO_4^-$	$(Ca_2)^{4+}$–HPO_4^{2-}	$(Ca_3)^{6+}$–PO_4^{3-}
6.6	1.34 ± 0.05 (5 values)	0.66	1.33	1.55
7.1	1.13 ± 0.04 (6 values)	0.33	1.16	1.44
7.6	1.01 ± 0.04 (5 values)	0.14	1.07	1.38

Rat liver mitochondria (about 6 mg protein/ml) were equilibrated anaerobically in the glass reaction vessel for 20 min at 25°C at the given pH_o values in medium (3.3 ml) containing 250 mM sucrose, 10 mM KCl, 3.3 mM glycylglycine, 2 mM ascorbate, 40 μM TMPD, 0.4 μM rotenone, oligomycin (1 mg/g protein), carbonic anhydrase (30 μg/ml) and catalase (0.05 μl Sigma C-100/ml). NEM (0.2 mM) was added 15 min after the beginning of equilibration. Respiration, which was sustained for about 100 s, was initiated by injecting 50 μl 10 mM H_2O_2.

4. Conclusion and research prospect

The experiments described in this and the preceding paper [1] are consistent with our hypothesis that calcium translocation in rat liver mitochondria is catalysed by a lanthanide-sensitive $(Ca_2)^{4+}$–HPO_4^{2-} symporter that is insensitive to NEM and mersalyl.

Our observations suggest that the state of respiratory control in the presence of excess bivalent cation, known as State 6, is due to the dependence of bivalent cation import on the distribution of P_i across the $(Ca_2)^{4+}$–HPO_4^{2-} symporter in the cristae membrane. Thus, the equilibrium poise of Ca^{2+} distribution across the membrane should depend on the distribution of HPO_4^{2-} according to the relationship:

$$\Delta pCa = \frac{\Delta\psi}{Z} - \frac{\Delta p(HPO_4^{2-})}{2} \quad (1)$$

where the operator p means $-\log_{10}$ (ion activity), $\Delta\psi$ represents the electric membrane potential and Z is the conventional factor 2.303 RT/F (see [12]). When the phosphate is equilibrated via the phosphoric acid uniporter, eq. (1) gives the relationship

$$\Delta pCa = \frac{\Delta\psi}{Z} + \Delta pH \quad (2)$$

where ΔpH represents the pH difference across the membrane. Thus, the force inducing Ca^{2+} import would be less than that corresponding to the uniport of an electrically univalent Ca species because ΔpH represents an outwardly directed force. This is nicely illustrated by the prediction of eq. (2) that if the $\Delta\psi$ and $-Z\Delta pH$ components of the total protonmotive potential were equal, ΔpCa would be zero. The distribution of Ca^{2+} according to eq. (2) has interesting implications for the migration and control function of calcium between the mitochondrial calcium phosphate porter system and the Ca^{2+}-motive ATPase of the endoplasmic reticulum or sarcoplasmic reticulum membrane.

The translocation of phosphate electrophoretically via the calcium phosphate porter as well as electrically neutrally by the phosphoric acid porter may require some revision of ideas concerning the function of the mitochondrial porter systems in the overall conversion of cytosolic ADP and P_i to cytosolic ATP.

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References

[1] Moyle, J. and Mitchell, P. (1977) FEBS Lett. 73, 131–136.
[2] McGivan, J. D. and Klingenberg, M. (1971) Eur. J. Biochem. 20, 392–399.
[3] Johnson, R. N. and Chappell, J. B. (1974) Biochem. J. 138, 171–175.
[4] Palmieri, F., Passarella, S., Stipani, I. and Quagliariello, E. (1974) Biochim. Biophys. Acta 333, 195–208.
[5] Mitchell, P. (1954) J. Gen. Microbiol. 11, 73–82.
[6] Aleksandrowicz, Z., Swierczynski, J. and Wrzolkowa, T. (1973) Biochim. Biophys. Acta 305, 59–66.
[7] Carafoli, E. (1965) Biochim. Biophys. Acta 97, 107–117.
[8] Selwyn, M. J., Dawson, A. P. and Dunnett, S. J. (1970) FEBS Lett. 10, 1–5.
[9] Ernster, L., Hollander, P., Nakazawa, T. and Nordenbrand, K. (1969) in: The Energy Level and Metabolic Control in Mitochondria (Papa, S. et al. eds) pp. 97–113, Adriatica Editrice, Bari.
[10] Quagliariello, E., Papa, S. and Chance, B. (1970) in: Electron Transport and Energy Conservation (Tager, J. M. et al. eds) pp. 61–73, Adriatica Editrice, Bari.
[11] Lehninger, A. L. (1974) Proc. Natl. Acad. Sci. USA 71, 1520–1524.
[12] Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research, Bodmin, England.