ELECTRIC CHARGE STOICHEIOMETRY OF CALCIUM TRANSLOCATION IN RAT LIVER MITOCHONDRIA

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1. Introduction

It has been shown by a variety of methods that calcium ions enter respiring mitochondria electrophoretically via a specific lanthanide-sensitive porter in the cristae membrane [1–12].

When a small amount of calcium chloride is added to a suspension of rat liver mitochondria respiring in State 4, there is a transient respiratory stimulation and a net export of H^+ ions, while virtually all the calcium ions are imported. In such experiments it is generally observed that the number of H^+ ions exported per extra oxygen atom reduced (the $\rightarrow H^+/O$ ratio) is about 2 per conventional coupling site in operation [2,7]. The number of calcium ions imported per extra oxygen atom reduced (the $\leftarrow$calcium/O ratio) is likewise about 2 per conventional coupling site in operation [2,7]. In other words, each calcium ion appears to carry only one positive charge as it is taken up electrophoretically through the porter system. The simple interpretation would be that the calcium-specific porter is not a Ca^{2+} uniporter, but is a calcium–anion symporter or a calcium/cation antiporter through which the net calcium and charge translocation stoicheiometry can be represented as $\leftarrow Ca^+$. As far as we know, this interpretation is not irreconcilable with other experimental data [1–13] concerning the calcium-specific porter. However, according to Lehninger and co-workers [14–16], calcium translocation occurs as Ca^{2+} uniport and the $\rightarrow H^+/O$ ratio must be 4 per site to account for the $\leftarrow Ca^{2+}/O$ ratio of 2 per site. They argue that $\rightarrow H^+/O$ values have been grossly underestimated because of the loss of H^+ ions back through the membrane by non-electrogenic symport with endogenous inorganic phosphate or with substrate anions included in the medium [14–16].

In view of the importance of stoicheiometry in characterising biochemical reaction systems, we have reassessed the electric charge stoicheiometry of calcium import in respiring rat liver mitochondria by measuring the electrically equivalent H^+ export, taking special precautions to prevent loss of H^+ ions by non-electrogenic H^+–anion symport.

We have found that the lanthanide-sensitive calcium porter catalyses a reaction for which the net calcium and electric charge translocation can be represented as $\leftarrow Ca^+$.

Abbreviations: NEM, *N*-ethyl maleimide; pH_o, pH of the suspension medium; $\rightarrow H^+$, quantity of protons translocated; $\rightarrow H^+$(acid), quantity of acid equivalents translocated; $\leftarrow$Ca, quantity of calcium translocated electrophoretically

2. Materials and methods

Rat liver mitochondria were isolated as described previously [17]. The methods of measuring and recording the rate of oxygen consumption and the time-course of the suspension medium pH changes (pH_o), used for calculating the quantity of acid equivalents translocated [$\rightarrow H^+$(acid)], were essentially as before [3,17]. In some experiments, the quantity of Ca^{2+} in the medium was measured at a given moment by observing the change of pH_o on injecting

excess EDTA, according to the principle originally introduced by Chappell, Cohn and Greville [1]. The calcium ion content of the mitochondria and media was routinely measured by atomic absorption spectrophotometry [3].

Two different types of experiment were used to estimate the electric charge stoicheiometry of calcium ion translocation from the number of acid equivalents that were exported through the respiratory chain system per calcium ion imported through the lanthanide-sensitive calcium porter [the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio].

2.1. *Type A experiments*

In experiments of type A, the mitochondrial suspension was initially equilibrated anaerobically at 25°C for 20 min, so that endogenous calcium and endogenous inorganic phosphate were released into the medium from the mitochondria. Respiration was initiated by injecting a small quantity of hydrogen peroxide which was decomposed by catalase routinely added to the medium [18]. The limiting quantity of acid equivalents, rapidly exported during the period of fast respiration, corresponding to the import of the calcium ions present in the suspension medium, was used to obtain the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio [3,19]. A small correction for drift in the observed limiting value of $\rightarrow H^+$(acid) was made by extrapolation back to the time of half-generation of this value of $\rightarrow H^+$(acid), as indicated by the broken lines in fig.1. In some of the type A experiments extra calcium salt was equilibrated in the suspension medium before respiration was initiated. In other experiments of this type a known quantity of calcium salt was injected with the hydrogen peroxide to ensure that this extra calcium could not have entered the mitochondria prior to the commencement of respiration.

2.2. *Type B experiments*

In experiments of type B, a small quantity of a calcium salt was injected into a mitochondrial suspension respiring in State 4 at 25°C. The quantity of acid equivalents exported during the ensuing transient period of respiratory stimulation was compared with the quantity of calcium salt injected (and virtually all imported) to obtain the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio [1,2].

3. Results and discussion

3.1. *Type A experiments*

We previously observed [3,19] that, in a 150 mM KCl medium near pH_0 7, with 2 mM succinate or

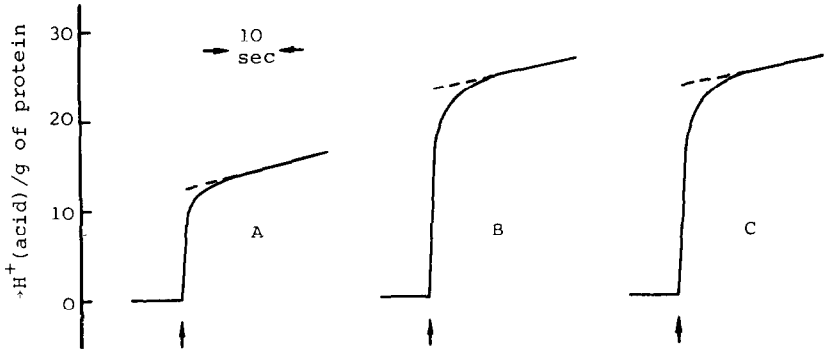

Fig.1. Time-course of $\rightarrow H^+$(acid) after initiation of respiration in anaerobic suspensions of rat liver mitochondria. The mitochondria (6.7 mg protein/ml) were equilibrated anaerobically at 25°C at pH_0 7.0–7.1 for 20 min in 3.3. ml of a medium containing 150 mM KCl, 3.3 mM glycylglycine, 2 mM potassium succinate, oligomycin (1 mg/g mitochondrial protein), carbonic anhydrase (30 μg/ml), catalase (0.05 μl of Sigma C-100/ml) 0.4 μM rotenone and 0.2 mM NEM (added 15 min after the beginning of the equilibration period). Respiration was initiated by injecting 50 μl of 10 mM H_2O_2 (at arrows). The amount of O_2 produced from the H_2O_2 was sufficient to sustain respiration for some 100 s. (A) Endogenous calcium (10.0 μg ions/g mitochondrial protein) was present. (B) 200 nmol $CaCl_2$ (corresponding to 9.1 nmol/g mitochondrial protein) was included in the suspension medium. (C) 200 nmol $CaCl_2$ was included in the H_2O_2 solution.

β-hydroxybutyrate as substrate, the limiting value of $\rightarrow H^+$(acid) in type A experiments represented the same number of H^+ ions as the number of Ca^{2+} ions in the suspension. We suggested two alternative kinds of interpretation [3]:

(1) Virtually all the Ca^{2+} ions were outside the mitochondria after anaerobic equilibration and each carried only one charge in via the porter system during respiration.

(2) Only half the Ca^{2+} ions were outside the mitochondria after anaerobic equilibration and each carried two charges in via the porter system during respiration.

To decide between these alternatives, we have now investigated the effect of injecting calcium salt with the H_2O_2 solution used to initiate respiration. As shown in fig.1, curve A, when no extra calcium salt was added and the quantity of endogenous calcium was 10.0 μg ions per gram of mitochondrial protein, the limiting value of $\rightarrow H^+$(acid) was 12.2 μg ions. However, when a further 9.1 μg ions of calcium salt was added per gram of mitochondrial protein, the same limiting value (23.4 μg ions) of $\rightarrow H^+$(acid) was obtained when the calcium salt was added before the initial anaerobic equilibration (curve B) as when it was injected with the H_2O_2 solution (curve C). In each case the $\rightarrow H^+$(acid)/Ca ratio was near 1. Control experiments, using atomic absorption spectrophotometry, and experiments in which EDTA was added at various times before and after the initiation of respiration, confirmed that virtually all the Ca^{2+} was in the suspension medium at the end of the twenty minute anaerobic equilibration. More than 95% of the Ca^{2+} was taken up during the period of rapid respiration-driven net H^+ export. Thus, the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio is equal to the $\rightarrow H^+$(acid)/Ca ratio, within experimental error, and we conclude that $\rightarrow H^+$(acid)/$\leftarrow$Ca is near 1.

The $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio can be taken to represent the number of positive charges carried per Ca translocated through the Ca-specific porter system only if $\rightarrow H^+$(acid) represents the actual number of H^+ ions translocated outwards electrogenically by the respiratory chain system. In the experiments of fig.1, the substrate was 2 mM succinate. 0.2 mM NEM was present to prevent the decay of $\rightarrow H^+$(acid) due to non-electrogenic proton–anion symport reactions [20].

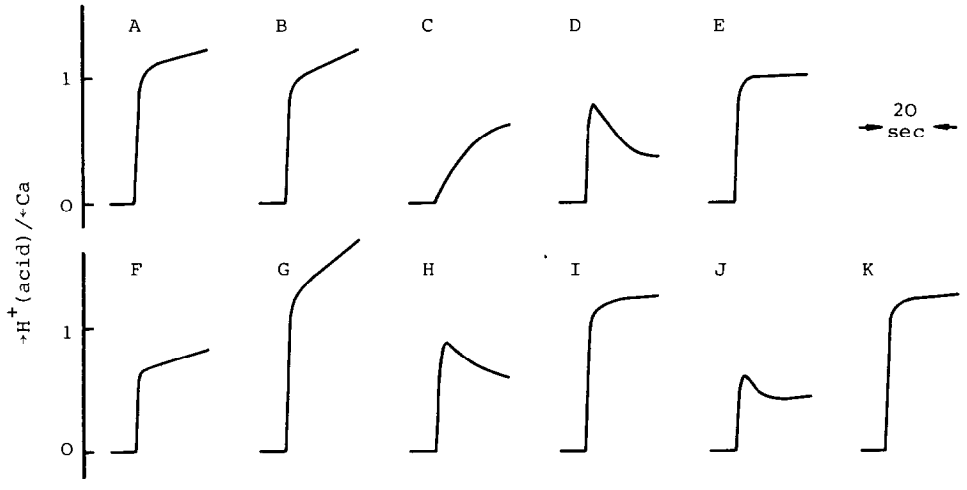

Fig.2 Time-course of $\rightarrow H^+$(acid) after initiation of respiration in anaerobic rat liver mitochondria suspended in a variety of media. The procedure was as fig.1. All anaerobic suspension media contained quantities of glycylglycine, carbonic anhydrase, catalase, and oligomycin as for fig.1 and known quantities of mitochondria (about 6 mg protein/ml) of measured Ca^{2+} content. In addition, the media contained: (A) 250 mM sucrose, 10 mM choline chloride, 2 mM potassium β-hydroxybutyrate. (B) 250 mM sucrose, 10 mM KCl. (C) As (B) with 10 μM $LaCl_3$. (D) 250 mM sucrose, 10 mM K_2SO_4. (E) as (D) with 0.2 mM NEM. (F) 150 mM KCl, 2 mM potassium succinate and 0.4 μM rotenone. (G) as (F) with 0.2 mM NEM. (H) 250 mM sucrose, 10 mM potassium succinate and 0.4 μM rotenone. (I) as (H) with 0.2 mM NEM. (J) as (H) with 1 mM potassium orthophosphate. (K) as (J) with 0.2 mM NEM.

Figure 2 shows that, in the absence of added porter-specific anions, the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio was near 1. But, as expected [3], there was a decay of $\rightarrow H^+$(acid) to values considerably less than 1 when porter-specific anions, such as succinate, sulphate or phosphate, were present. The decay of $\rightarrow H^+$(acid) was abolished, and the limiting $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio was restored to near 1, when the phosphoric acid porter was inhibited [20] by the presence of 0.2 mM NEM. Curve C confirms the lanthanide sensitivity of the calcium import. The rate of calcium import indicated by $\rightarrow H^+$(acid) was inhibited by 92% by the 10 μM $LaCl_3$ present in this experiment.

These results indicate that the net electric charge stoicheiometry of calcium translocation through the lanthanide-sensitive calcium porter should be represented as $\leftarrow Ca^+$.

3.2. *Type B experiments*

This type of experiment confirmed the observations of Lehninger and others [2,7,14,21,22] that the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio was near 1 in a variety of media and with several different substrates. In particular, we observed (table 1) that the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio was not changed significantly when the phosphoric acid porter was inhibited by 0.2 mM NEM, nor was the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio increased by substituting the relatively permeant nitrate ion for chloride, as would have been the case if the calcium porter catalysed Ca^{2+}–NO_3^- symport.

In the experiments of table 1, the mean $\leftarrow Ca^+/O$ ratio estimated from the extra oxygen consumption during calcium import corresponded to a value of 1.8 per site, consistent with our measurements of $\rightarrow H^+/O$ ratios [3,23] corresponding to near 2 per site.

Table 1
Values for the $\rightarrow H^+$(acid)/$\leftarrow$Ca ratio, obtained by injecting small quantities of calcium salts into suspensions of rat liver mitochondria respiring in State 4

Medium	Additions	$\rightarrow H^+$(acid)/$\leftarrow$Ca
150 mM KCl,	–	0.92, 0.93, 1.16, 0.93
2 mM β-hydroxy-butyrate	Oligomycin	0.95
	Oligomycin + NEM	1.00
150 mM KNO_3,	–	0.98, 0.94, 1.07, 0.91
2 mM β-hydroxy-butyrate	Oligomycin	0.95
	Oligomycin + NEM	0.93
150 mM choline chloride	Oligomycin	1.08, 1.04
150 mM choline nitrate	Oligomycin	0.93, 0.92
250 mM sucrose, 10 mM choline chloride, 5 mM β-hydroxy-butyrate	Oligomycin	0.95, 0.90

Conditions were as for fig.1, except that the mitochondria were initially respiring in State 4. All aerobic suspension media contained quantities of glycylglycine, carbonic anhydrase, and other additions indicated in the table, at the same concentrations as for fig.1 and known quantities of mitochondria (about 6 mg protein/ml) of measured Ca^{2+} content. The quantity of calcium salts injected (200 nmol) corresponded to about 10 μmol/g mitochondrial protein.

4. Conclusions and research prospect

The lanthanide-sensitive calcium porter of rat liver mitochondria catalyses a reaction for which the net calcium and electric charge translocation corresponds to $\leftarrow Ca^{+}$.

Azzone and co-workers [13] observed that the steady-state concentration ratio of Ca^{2+} ions across the cristae membrane of respiring rat liver mitochondria, under conditions of limited calcium uptake, was about the same as the concentration ratio of K^{+} in the presence of valinomycin. However, it should have been about the square of this concentration ratio if calcium were taken up by Ca^{2+} uniport through the lanthanide-sensitive porter. They regarded their observations as evidence against a chemiosmotic type of mechanism for energy coupling in ion transport. However, the univalent charge stoicheiometry of the calcium porter reaction, described in the present paper, leads to the conclusion that the observations of Azzone and co-workers [13] actually support a chemiosmotic mechanism of calcium translocation. Moreover, since the charge stoicheiometry of calcium translocation corresponds to $\leftarrow Ca^{+}$ and not to $\leftarrow Ca^{2+}$, the observed $\leftarrow Ca^{+}/O$ stoicheiometry of near 2 per site agrees with the $\rightarrow H^{+}/O$ ratios of 2 per site observed in our laboratory [3,23] contrary to the contentions of Lehninger and co-workers [14–16].

Our observations raise the obvious question: what is the overall translocation reaction catalysed by the lanthanide-sensitive calcium porter of rat liver mitochondria? Presumably the net charge stoicheiometry of calcium translocation, corresponding to $\leftarrow Ca^{+}$, could result either from calcium–anion symport or from calcium/cation antiport. Such reactions have been suggested for mitochondria [24–26]. The results described in the present paper, together with our earlier measurements of $\rightarrow H^{+}/O$ ratios [3,19,23], support the conclusion of Lehninger and Brand [14] that the calcium porter is not a calcium/proton antiporter.

Observations on the stimulatory effect of inorganic phosphate on calcium import by respiring mitochondria [10,27,28], and some evidence for the bivalency of the calcium porter [7,8,10,12] have prompted us to consider the hypothesis that the lanthanide-sensitive so-called, calcium porter might actually be a calcium–phosphate porter catalysing $(Ca_2)^{4+}$–HPO_4^{2-} symport. This hypothesis is now being tested in our laboratory.

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