The stoichiometry of the exchange catalysed by the mitochondrial calcium/sodium antiporter

Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. Rat heart mitochondria respiring on succinate in the presence of Ruthenium Red (to inhibit uptake on the Ca^{2+} uniporter) released Ca^{2+} on the calcium/sodium antiporter until a steady state was reached. 2. Addition of the ionophore A23187 (which catalyses $\text{Ca}^{2+}/2\text{H}^{+}$ exchange) did not perturb this steady state. 3. Thermodynamic analysis showed that if a $\text{Ca}^{2+}/n\text{Na}^{+}$ exchange with any value of n other than 2 was at equilibrium, addition of A23187 would cause an obvious change in extramitochondrial free $[\text{Ca}^{2+}]$. 4. Therefore the endogenous calcium/sodium antiporter of mitochondria catalyses electroneutral $\text{Ca}^{2+}/2\text{Na}^{+}$ exchange.

Two systems are known to catalyse efflux of Ca^{2+} from mitochondria (see Saris & Åkerman, 1980; Nicholls & Crompton, 1980; Nicholls & Åkerman, 1982). $\text{Ca}^{2+}/2\text{H}^{+}$ antiport activity is particularly active in liver, where I have shown it to be electroneutral (Brand, 1985a). The $\text{Ca}^{2+}/n\text{Na}^{+}$ antiporter predominates in heart and brain mitochondria (Crompton *et al.*, 1976, 1977, 1978; Crompton & Heid, 1978; Nicholls, 1978a), but is also found in other tissues, including liver (Nedergaard & Cannon, 1980; Haworth *et al.*, 1980; Heffron & Harris, 1981; Harris & Heffron, 1982; Goldstone & Crompton, 1982; Goldstone *et al.*, 1983; Nedergaard, 1984). The $\text{Ca}^{2+}/2\text{H}^{+}$ antiport pathway is thought to be distinct from the $\text{Ca}^{2+}/n\text{Na}^{+}$ antiporter because of its markedly different sensitivity to lanthanides (Crompton *et al.*, 1979).

The stoichiometry of the calcium/sodium exchange has not previously been determined. Kinetic measurements suggest that there are three independent binding sites for Na^{+} , and have led to the working hypothesis that n , the number of Na^{+} ions exchanged per Ca^{2+} ion, is 3 (Crompton *et al.*, 1976, 1977; Hayat & Crompton, 1982), as it is in the analogous carrier in cardiac sarcolemma (Reeves & Hale, 1984). Affolter & Carafoli (1980) showed that $\Delta\psi$ in heart mitochondria did not alter

measurably when $\text{Ca}^{2+}/n\text{Na}^{+}$ antiport was induced, and took this result to mean that the carrier was electroneutral, i.e. with $n = 2$. However, many other fast ion translocations were occurring in their system, including respiration-driven H^{+} ejection. These would diminish the size of any change in $\Delta\psi$ that might have occurred, so it is doubtful whether $\Delta\psi$ changes would have been great enough to have been observed in their experiments. Thus their conclusion cannot be relied on.

In this paper I show by a thermodynamic method that the calcium/sodium antiporter of heart mitochondria exchanges 2Na^{+} ions for each Ca^{2+} ion exported. A preliminary report of this work has appeared (Brand, 1985b).

Experimental

Rat heart mitochondria were prepared as described by Crompton *et al.* (1976) after Polytron disintegration of hearts in medium containing 210 mM-mannitol, 70 mM-sucrose, 10 mM-Tris/HCl and 0.1 mM-EDTA (potassium salt), pH 7.4. Mitochondria were washed twice in the same medium, but without the EDTA. Mitochondrial protein was assayed by a biuret method (Gornall *et al.*, 1949).

Extramitochondrial free $[\text{Ca}^{2+}]$ was measured with a Radiometer F2112 Ca-sensitive electrode as described previously (Brand, 1985a) in a standard medium at 25°C containing 210 mM-mannitol, 70 mM-sucrose, 10 mM-Hepes, 1 mM-nitrilotriacetic acid, 1 mM-phosphoric acid, adjusted to pH 7.0 with tetramethylammonium hydroxide.

$\Delta\psi$, ΔpH , ΔpNa and matrix volume were

Abbreviations used: $\Delta\psi$, electrical potential of the medium—electrical potential of the matrix; ΔpH , medium pH—matrix pH; ΔpNa , medium pNa—matrix pNa, where $\text{pNa} = -\log[\text{Na}^{+}]$; $[\text{Ca}^{2+}]_o$, extramitochondrial free Ca^{2+} concentration; TPMP, methyltriphenylphosphonium.

measured by using radioisotopes. The standard medium was supplemented with 5 μ M-rotenone, 5 mM-succinate (tetramethylammonium salt) and nigericin (1.3 μ g/mg of protein) and either 0.05 μ Ci of [3 H]TPMP bromide/ml (200 nM) for $\Delta\psi$, or 1 μ Ci of [6,6'(n)- 3 H]sucrose/ml and 0.14 μ Ci of [14 C]-methylamine hydrochloride/ml (20 μ M) for Δ pH, or 1 μ Ci of [3 H]sucrose/ml and 0.14 μ Ci of 22 NaCl/ml for Δ pNa or 1 μ Ci of 3 H $_2$ O/ml and 0.14 μ Ci of D-[14 C]mannitol/ml for matrix volume. Rat heart mitochondria (1 mg of protein/ml) were added, then incubated in parallel to the experiment shown in Fig. 3, with the same additions. At the times shown, 1 ml samples were taken in triplicate and centrifuged for 2 min in an Eppendorf bench centrifuge. Supernatant and pellet were assayed for radioactivity in an LKB Rackbeta scintillation counter with quench corrections. $\Delta\psi$, Δ pH, Δ pNa and matrix volume were calculated as described in Brown & Brand (1985), with the correction factors described below.

Correction for methylamine binding was as-

essed as described by Brand (1985a). Heart mitochondria (1 mg of protein/ml) were suspended in medium at 25°C containing different concentrations of KCl in the presence of nigericin, and the acid-inside pH gradients set up were measured by the distributions of 86 Rb and [14 C]methylamine. Fig. 1 shows that methylamine was accumulated more than Rb, so an empirical correction was applied by using the formula:

$$\text{corrected methylamine accumulation ratio} = 0.6 (\text{observed accumulation ratio}) + 1.25$$

Correction for binding of TPMP was carried out as described by Brown & Brand (1985) in standard medium at 25°C supplemented with 5 μ M-rotenone, 200 nM-TPMP bromide, 1 μ g of oligomycin/ml, 0.05 μ Ci of [3 H]TPMP/ml and 0.1 μ Ci of 86 Rb/ml. Heart mitochondria (1 mg of protein/ml) were added, followed after 1 min by 1 μ M-valinomycin and after a further 2 min by 5 mM-succinate (tetramethylammonium salt). After 4 min 1 ml

Fig. 1. Calibration of [14 C]methylamine accumulation. Heart mitochondria were suspended in a sucrose-based medium containing 0, 0.5 mM-, 3 mM- or 125 mM-KCl. pH gradients (acid inside) were established by adding nigericin and measured by using 86 Rb. For details see the Experimental section. The line drawn was calculated by linear regression and had slope of 1/0.60 and intercept on the Rb axis of 1.25.

Fig. 2. Calibration of [3 H]TPMP accumulation. Heart mitochondria were suspended in standard medium containing 0, 0.1 mM-, 0.2 mM-, 0.5 mM- or 5 mM-KCl supplemented as described in the Experimental section. Membrane potentials were established by adding valinomycin and measured by using 86 Rb. The line drawn was calculated by linear regression and had slope of 1/0.24 and intercept on the Rb axis of -13.3.

samples were removed in triplicate and assayed for radioactivity as above. Fig. 2 shows that there was considerable binding of TPMP, so again an empirical correction was applied, by using the formula:

$$\text{Corrected TPMP accumulation ratio} = 0.24 (\text{observed accumulation ratio}) - 13.3$$

Measurement of calcium by atomic absorption for Table 3 was carried out in a Perkin-Elmer 380 atomic-absorption spectrophotometer calibrated with standard CaCl_2 . Mitochondria (1 mg of protein/ml) were incubated at 25°C in standard medium containing $5\text{ }\mu\text{M}$ -rotenone, 5 mM -succinate (tetramethylammonium salt), 1.3 nmol of nigericin/ml and 200 pmol of Ruthenium Red/ml. After 5 min 4 mM -NaCl was added, followed at 7 min by $0.5\text{ }\mu\text{g}$ of A23187/ml. At 17 min 1 ml samples were removed and centrifuged for 4 min . At 20 min 100 mM -KCl was added, and at 30 min further 1 ml samples were taken and centrifuged. Supernatants were assayed for calcium.

Radiochemicals were from Amersham International, except for $[^3\text{H}]\text{TPMP}$, which was from New England Nuclear. Ruthenium Red was from Sigma and was purified as described by Luft (1971). Diltiazem hydrochloride was a gift from Gödecke A.G., Freiburg, Germany.

Results

The strategy that I employed was similar to that described in Brand (1985a) to demonstrate that calcium/proton exchange in liver mitochondria is electroneutral. Heart mitochondria were allowed to release Ca^{2+} by calcium/sodium exchange until the carrier was close to equilibrium. The ionophore A23187, which catalyses $\text{Ca}^{2+}/2\text{H}^+$ exchange (Reed & Lardy, 1972; Pfeiffer *et al.*, 1976), was then added to discover whether $\text{Ca}^{2+}/n\text{Na}^+$ antiport on the endogenous carrier was thermodynamically different from $\text{Ca}^{2+}/2\text{H}^+$ antiport on the ionophore. Note that Na^+/H^+ antiport is fast in these mitochondria (see below), so $\text{Ca}^{2+}/2\text{Na}^+$ antiport is thermodynamically equivalent to $\text{Ca}^{2+}/2\text{H}^+$ antiport, and the comparison between the endogenous calcium/sodium exchange and the exchange of Ca^{2+} for 2H^+ on the ionophore is valid.

Fig. 3 shows an example of this type of experiment. Heart mitochondria treated with Ruthenium Red to inhibit the endogenous Ca^{2+} uniporter had a low basal rate of Ca^{2+} efflux. This was greatly stimulated by addition of 4 mM -NaCl. The stimulation by Na^+ could be abolished by $200\text{ }\mu\text{M}$ -diltiazem, a known inhibitor of the calcium/sodium antiporter (Vaghy *et al.*, 1982),

Fig. 3. Effect of A23187 on $[\text{Ca}^{2+}]_i$ at the equilibrium of the endogenous calcium/sodium exchange. Heart mitochondria (1 mg of protein/ml) were suspended in 3 ml of standard medium at 25°C containing $5\text{ }\mu\text{M}$ -rotenone, 5 mM -succinate (tetramethylammonium salt), 2 nmol of nigericin/ml and 67 pmol of Ruthenium Red/ml. $[\text{Ca}^{2+}]_i$ was measured with a Ca^{2+} -sensitive electrode. Where indicated 4 mM -NaCl, 0.6 nmol of A23187/ml and 20 nmol of standard CaCl_2 were added. The bars marked $\text{Ca}^{2+}/3\text{Na}^+$ and $\text{Ca}^{2+}/2\text{Na}^+$ show the predicted $[\text{Ca}^{2+}]_i$ for that exchange; the difference between them shows the size of the fall in $[\text{Ca}^{2+}]_i$ expected on addition of A23187 if the endogenous antiporter catalyses $\text{Ca}^{2+}/3\text{Na}^+$ exchange.

demonstrating that the efflux that was observed was indeed on this carrier (results not shown). $[Ca^{2+}]_o$ reached a steady value after 25–30 min. Addition of A23187 caused little change in this value, whether added after 5 min or after 30 min. Thus in this system the endogenous calcium/sodium and Na^+/H^+ antiporters of heart mitochondria catalyse an exchange which is experimentally indistinguishable from the electroneutral $Ca^{2+}/2H^+$ exchange catalysed by the ionophore A23187.

If the carrier were not electroneutral, then addition of A23187 would have caused a change in $[Ca^{2+}]_o$. It was important to show that any such change would have been large enough to have been measured. I did this by carrying out the experiments in low- K^+ medium in the presence of nigericin, conditions which make ΔpH positive, i.e. acid inside. This maximizes the size of the expected changes for values of n greater than 2. I then measured the relevant potential gradients to allow calculation of the extent of the expected change in $[Ca^{2+}]_o$ for different values of n in Ca^{2+}/nNa^+ exchange. These procedures are described in more detail below. They showed that $Ca^{2+}/2Na^+$ antiport was experimentally distinguishable from exchanges with any other value of n .

For a Ca^{2+}/nNa^+ antiport at equilibrium the following relationship holds (Brand, 1985a):

$$[Ca^{2+}]_o = Ca^{2+}_{i,o} / (B_o \cdot V + B_i \cdot v \cdot 10 \exp[(2-n)\Delta\psi/59 + n\Delta pNa]) \quad (1)$$

Superscripts f and t denote free and total, subscripts i and o denote intramitochondrial and extramitochondrial (thus $Ca^{2+}_{i,o}$ is the total Ca^{2+} in the system), B is $[Ca^{2+}]_i/[Ca^{2+}]_o$, V is extramitochondrial volume and v is intramitochondrial volume. Since each of the other terms on the right-hand side can be determined (see Brand, 1985a), the value of $[Ca^{2+}]_o$ can be predicted for any value of n . There is some circularity in the calculation, as B_i is calculated from $[Ca^{2+}]_o$ in the presence of A23187. However, the absolute values of $[Ca^{2+}]_o$ do not affect the conclusions, since it is the differences in predicted $[Ca^{2+}]_o$ for different n values that are important here.

Table 1 shows the values of the relevant parameters determined for the experiment of Fig. 3. ΔpH was reversed, as expected, giving a matrix pH about 1 unit more acid than the medium. ΔpH and ΔpNa were very similar, showing that Na^+/H^+ antiport was sufficiently rapid to equilibrate Na^+ and H^+ gradients in these experiments. The protonmotive force was low, presumably because of substrate limitation: succinate will be largely excluded from the matrix under these conditions.

The mean value of B_i from three determinations

Table 1. *Parameter values for the experiment in Fig. 3*
For details see Fig. 3 and the Experimental section.
The value of v was $0.27 \mu\text{mol/mg}$ of protein (mean value from all samples).

	Sample		
	1	2	3
Measured			
$\Delta\psi$ (mV)	161	156	153
ΔpH (pH units)	+0.99	+0.97	+0.96
ΔpNa (units)	0.86	0.92	0.91
$[Ca^{2+}]_o$ (μM)	6.37	6.47	7.60
V (μl)	3123	3124	3126
Calculated			
B_o	5.19	5.19	5.19
B_i (from samples 2 and 3)	28	28	28
$[Ca^{2+}]_i$ (μM)	—	495	566
$Ca^{2+}_{i,o}$ (nmol/mg of protein)	38.6	38.6	45.3

Table 2. *Predicted values of $[Ca^{2+}]_o$ in the experiment of Fig. 3 for different modes of Ca^{2+}/nNa^+ antiport*
Values were calculated from the data in Table 1, sample 1, by using eqn. 1.

n	Predicted $[Ca^{2+}]_o$ (M)
0	1.836×10^{-8}
1	9.951×10^{-7}
2	6.525×10^{-6}
3	7.159×10^{-6}
4	7.170×10^{-6}
5	7.170×10^{-6}

was 17 ± 11 (S.D.). This is similar to that found in liver by Nicholls (1978b) and myself (Brand, 1985a), and in heart by Crompton & Heid (1978). It is much lower than the values reported by Coll *et al.* (1982) and Denton & McCormack (1980) under rather different conditions. In the present experiments the matrix was very acid, about pH 6, which may lead to considerably less binding of Ca^{2+} than the more physiological conditions used by others. My estimate of B_i is not particularly accurate. However, if it is too low then the conclusion of the present paper is strengthened, since the predicted differences in $[Ca^{2+}]_o$ become greater as B_i is increased.

Table 2 shows the predicted values of $[Ca^{2+}]_o$ in the steady state before addition of ionophore. They were calculated from the data in Table 1 by using eqn. (1) for different values of n . Clearly, changing equilibrium from that of $Ca^{2+}/1Na^+$ antiport to that of $Ca^{2+}/2H^+$ antiport would have very large effects on $[Ca^{2+}]_o$. This was not seen, so $Ca^{2+}/1Na^+$ antiport is excluded. The change expected in $[Ca^{2+}]_o$ on moving from equilibrium

Table 3. Release of residual calcium from mitochondria by abolition of ΔpH

Total extramitochondrial calcium was measured by atomic absorption in experiments similar to that in Fig. 3 as described in the Experimental section. Values are quoted as means $\pm$ S.E.M. for 11 determinations.

	[Ca] (μM)
$[Ca]_o$, steady state	45.1 ± 7.6
$[Ca]_o$, after 100 mM-KCl	48.9 ± 8.2
$\Delta[Ca]_o$	3.8 ± 1.4

of $Ca^{2+}/3Na^{+}$ antiport to that of $Ca^{2+}/2H^{+}$ antiport would be less, but it would still have been easy to observe in the system I used. The size of the predicted change is indicated on Fig. 3 to show this. Thus $Ca^{2+}/3Na^{+}$ antiport is also excluded, as are other values of n greater than 3 or less than 1.

A second way to distinguish between the equilibria catalysed by $Ca^{2+}/3Na^{+}$ and $Ca^{2+}/2Na^{+}$ exchanges was to add valinomycin instead of A23187 (both in the presence of nigericin). This brought $\Delta\psi$ to the same clamped value as ΔpH . It would not affect $[Ca^{2+}]_o$ at the equilibrium of $Ca^{2+}/2Na^{+}$ antiport, but would alter it from the equilibrium of $Ca^{2+}/3Na^{+}$ exchange. The change in $[Ca^{2+}]_o$ would be similar to that predicted for A23187. Addition of 200 nM-valinomycin did not affect $[Ca^{2+}]_o$ in the steady state (results not shown), supporting the conclusion that the endogenous carrier is electroneutral.

The calculations predict that at the equilibrium of the $Ca^{2+}/2Na^{+}$ antiporter or of $Ca^{2+}/2H^{+}$ exchange on A23187 a significant proportion of the total Ca^{2+} in the system should be in the matrix, held in by the reversed ΔpH . The amount of Ca^{2+} retained was calculated to be about 7% of the total Ca^{2+} involved in these equilibria. Table 3 reports experiments in which this calcium was released when ΔpH was abolished by adding extramitochondrial K^{+} to clamp ΔpH close to zero. The increase in extramitochondrial calcium was about 8% of the calcium in the system, consistent with release of the internal pool as predicted above.

Discussion

These experiments show that the endogenous calcium/sodium exchange of heart mitochondria catalyses a reaction thermodynamically indistinguishable under these conditions from that catalysed by A23187, i.e. it is electroneutral. Since the endogenous Na^{+}/H^{+} antiport is very fast, the Na^{+} and H^{+} gradients are the same. The observed

efflux was dependent on Na^{+} and inhibited by diltiazem, characteristics diagnostic for the calcium/sodium exchanger, so I conclude that the carrier catalyses $Ca^{2+}/2Na^{+}$ antiport.

This conclusion was also reached by Affolter & Carafoli (1980). As discussed above, it is doubtful whether their experiments could have excluded electrophoretic calcium/sodium exchanges, and so their work cannot be taken as definitive.

Several studies have provided kinetic evidence for three independent binding sites for Na^{+} on the calcium/sodium antiporter (Crompton *et al.*, 1976, 1977; Hayat & Crompton, 1982). A simple hypothesis is that the carrier catalyses $Ca^{2+}/3Na^{+}$ antiport, but other explanations may be invoked. For example, these sites might be involved in control of the carrier's activity. Thus these kinetic results are not inconsistent with the present conclusion that the carrier catalyses exchange of Ca^{2+} for $2Na^{+}$.

I conclude that both the $Ca^{2+}/2Na^{+}$ antiporter typical of heart mitochondria (this paper) and the $Ca^{2+}/2H^{+}$ exchange that predominates in liver mitochondria (Brand, 1985a,b) are electroneutral.

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