

LANTHANIDE-SENSITIVE CALCIUM—MONOCARBOXYLATE SYMPORT IN RAT LIVER MITOCHONDRIA

Jennifer MOYLE and Peter MITCHELL

Glynn Research Laboratories, Bodmin, Cornwall, PL30 4AU, England

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1. Introduction

Our recent observations [1,2] on lanthanide-sensitive calcium and strontium translocation in rat liver mitochondria show that the electric charge stoichiometry of calcium translocation should be represented not as $\leftarrow\text{Ca}^{2+}$, but as $\leftarrow\text{Ca}^+$, and that bivalent cation translocation may be catalysed by a $(\text{Ca}_2)^{4+}\text{--HPO}_4^{2-}$ symporter. These observations suggest that the import of calcium and monocarboxylates by respiring mitochondria [3], and the swelling of non-respiring mitochondria in isotonic calcium or strontium acetate solutions in the presence of proton-conducting uncouplers [4], may involve bivalent cation-monocarboxylate symport, either mediated by the $(\text{Ca}_2)^{4+}\text{--HPO}_4^{2-}$ symporter or by a $\text{Ca}^{2+}\text{--monocarboxylate}^-$ symporter.

In this paper we provide evidence for specific bivalent cation—monocarboxylate symport. Mitochondria respiring in State 6, in the presence of excess calcium or strontium salt, show a lanthanide-sensitive monocarboxylate-dependent respiratory stimulation and import of bivalent cation, when the recirculation

of phosphate is prevented by inhibiting the phosphoric acid uniporter with NEM. This monocarboxylate-dependent respiratory stimulation and bivalent cation import is rather specific for β -hydroxybutyrate and is not competitive with calcium—phosphate import. We propose that the mitochondrial cristae membrane contains a calcium— β -hydroxybutyrate symporter, as well as the $(\text{Ca}_2)^{4+}\text{--HPO}_4^{2-}$ symporter described previously [1,2], both symport reactions having an electric charge stoichiometry corresponding to $\leftarrow\text{Ca}^+$.

2. Materials and methods

Rat liver mitochondria were isolated as described previously [1]. The translocation of acid equivalents and of electric charge were estimated from the time-course of the pH of the suspension medium (pH_0) in respiratory experiments of the State 4-3-4, State 4-3-6 or State 6-3-6 type essentially as described before [1,2]. Experimental details are given in the legends to the figures.

Experiments on the swelling of non-respiring mitochondria suspended in isotonic salt solutions were done as before [5].

3. Results and discussion

3.1. Electric charge stoichiometry of calcium— β -hydroxybutyrate import

Under experimental conditions corresponding to those of fig.1, we found that the extra oxygen consumed during the import of pulses of given small

Abbreviations: pH_0 , pH of suspension medium; βOHbut , β -hydroxybutyrate; NEM, *N*-ethyl maleimide; nupercaine, 2-butoxy-*N*-(2-diethylaminoethyl) cinchoninamide; 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; $\rightarrow\text{H}^+(\text{charge})/\text{O}$ quotient, number of protonic charges translocated by the respiratory chain per oxygen atom reduced; $\rightarrow\text{H}^+/\sim$, number of protons translocated per bivalent reducing equivalent traversing each coupling site

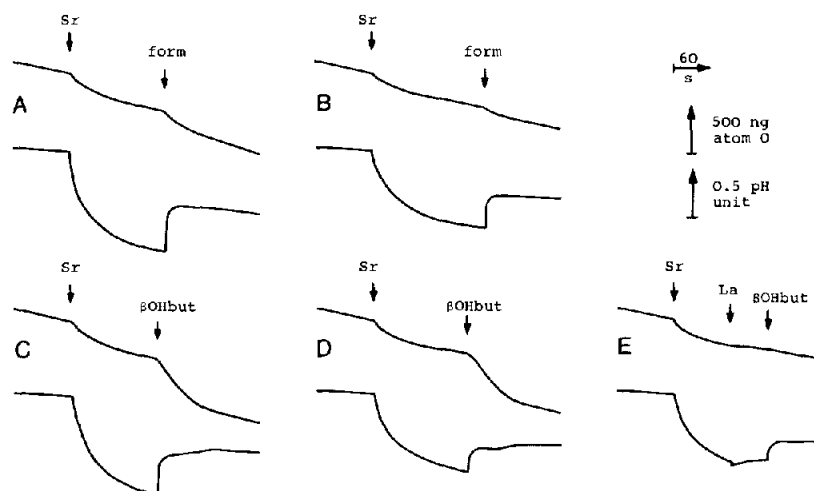


Fig.1. Time-course of oxygen concentration and of pH_O in suspensions of respiring rat liver mitochondria. Upper curves: oxygen content of 3.3 ml suspension. Lower curves: pH_O . The aerobic suspension medium (3.3 ml) at 25°C and initially at pH_O 7.2 contained 250 mM sucrose, 15 mM choline chloride, 3.3 mM glycylglycine and mitochondria (6.2 mg protein/ml). Oligomycin (1 mg/g mitochondrial protein) and carbonic anhydrase (30 $\mu\text{g}/\text{ml}$) were also present. 0.2 mM NEM was added to the suspension 4 min after the mitochondria in B, D and E. Injections were made at the arrows: Sr, 150 μmol SrCl_2/g mitochondrial protein; form, choline formate to give a concentration of 10 mM in the suspension; βOHbut , choline β -hydroxybutyrate to give a concentration of 10 mM in the suspension; La, LaCl_3 to give a concentration of 10 μM in the suspension.

quantities of calcium or strontium (up to 85 μg ion/g mitochondrial protein), in a 250 mM sucrose, 15 mM choline chloride medium containing 0.2 mM NEM, was proportional to the quantity of bivalent ion injected, and was the same whether 0.5 mM inorganic phosphate or 10 mM β -hydroxybutyrate was previously added to the medium. This was so, even when the quantity of calcium or strontium taken up from the medium containing 10 mM β -hydroxybutyrate was equivalent to three times the quantity of endogenous phosphate present, provided that 200 μM nupercaine was present to stabilise the mitochondria in the calcium-containing media [2]. The $\rightarrow\text{H}^+(\text{charge})/\text{O}$ quotient was found to be independent of the presence of β -hydroxybutyrate or phosphate in this NEM-containing medium, and the electric charge stoichiometry for calcium- or strontium-phosphate import was previously shown to correspond to $\leftarrow\text{Ca}^+$ or $\leftarrow\text{Sr}^+$ [1,2]. It follows that the electric charge stoichiometry for the import of calcium- or strontium- β -hydroxybutyrate corresponds to $\leftarrow\text{Ca}^+$ or $\leftarrow\text{Sr}^+$.

3.2. Anion specificity of bivalent cation import

Rat liver mitochondria contain about 25 μg ion of endogenous P_i per g mitochondrial protein. We attempted to deplete this endogenous P_i using various centrifuging, ion-exchange and phosphate esterification methods. But appreciable depletion of the endogenous P_i proved to be impossible without inducing mitochondrial fragility or high solute permeability. It was therefore necessary to induce the import of relatively large amounts of bivalent cation in experiments designed to observe the import of calcium- or strontium-monocarboxylates. We usually employed strontium rather than calcium salts in these experiments, because, as discussed previously [2], calcium induces mitochondrial fragility, whereas strontium does not.

Figure 1 shows experiments in which mitochondria respiring in State 4 were pulsed, first with a large quantity of SrCl_2 (150 $\mu\text{mol}/\text{g}$ mitochondrial protein) to induce import of most of the endogenous inorganic phosphate, and then with formate or β -hydroxy-

butyrate to give a concentration of 10 mM in the mitochondrial suspension. The upper traces show the respiratory transitions, and the lower traces show the transitions of pH_O . It is important to note that NEM does not inhibit respiration significantly in the choline-containing medium selected for these experiments. The addition of formate after the excess strontium (A) caused a considerable respiratory stimulation, which was largely inhibited (B) when the recirculation of phosphate was prevented by the presence of 0.2 mM NEM. The pH_O traces show that rapid alkalisation occurred when formic acid entered the mitochondria after the addition of the pulse of formate. Presumably the respiratory stimulation after addition of formate, seen in A, was due to the collapse of ΔpH by the entry of formic acid, the consequent export of phosphoric acid via the NEM-sensitive phosphoric acid porter, and the re-entry of phosphate with Sr^{2+} via the calcium-phosphate symporter. The fact that there was a small respiratory stimulation after addition of formate in B, despite the inhibition of the phosphoric acid porter by NEM, is probably explained by a small further uptake of external strontium-phosphate, as a direct result of the collapse of ΔpH by the entry of formic acid (see eq. 2 in [2]). In the experiments where 10 mM β -hydroxybutyrate was injected in place of formate (C and D), there was a much greater respiratory stimulation than with formate; and this large respiratory stimulation was not inhibited when phosphate recirculation was prevented by the presence of 0.2 mM NEM (D). However, as shown in E, the respiratory stimulation induced by β -hydroxybutyrate in the presence of 0.2 mM NEM was almost completely abolished by $10\text{ }\mu\text{M}$ La^{3+} , although the entry of β -hydroxybutyric acid, shown by the increase of pH_O after injection of the β -hydroxybutyrate, was not inhibited (compare D and E). These experiments imply that there is a lanthanide-sensitive NEM-insensitive bivalent cation-monocarboxylate symport system that is very active for strontium- β -hydroxybutyrate import but comparatively inactive for strontium-formate import.

Figure 2 shows a summary of data from experiments like those of fig.1, giving the quantity of strontium initially imported with endogenous P_i and subsequently imported with various monocarboxylates added to the medium at several different concentrations. As in the experiments of section 3.1 and of fig.1, the quantity

of strontium taken up was estimated from the extent of the respiratory stimulation: A, when phosphate recirculation occurred; and B, when phosphate recirculation was inhibited by the presence of 0.2 mM NEM. The broken line shows the maximum quantity of strontium that could be imported by $(\text{Sr}_2)^{4+}$ - HPO_4^{2-} symport, when recirculation of P_i was inhibited by NEM. In the absence of NEM, the ΔpH -collapsing effect and the consequent recirculation of phosphate (fig.2A), enabled all the monocarboxylates tested to increase the total quantity of strontium imported, as observed previously for calcium import by Lehninger [6]. But when the phosphate recirculation was prevented by NEM (fig.2B), the symport system for strontium-monocarboxylates was highly specific for β -hydroxybutyrate at a concentration of 2 mM. However, both acetate and butyrate showed a significant activity in this system at concentrations above 2 mM. Bicarbonate was inactive at a concentration of 1 mM (fig.2); and likewise, pyruvate, acetoacetate, succinate, L-malate and malonate (not included in fig.2) were inactive at a concentration of 2 mM.

Although the lanthanide-sensitive strontium-monocarboxylate symport system is specific for β -hydroxybutyrate at low concentration, the fact that acetate is moderately active in this system at a concentration of 20 mM in our respiratory transition experiments (fig.2) is consistent with the observation of Selwyn et al. [4] that non-respiring rat liver mitochondria swell in isotonic strontium acetate solutions (which contain 166 mM acetate) in the presence of a proton-conducting agent, and that this swelling is inhibited by lanthanides. We confirmed this observation, using similar methods [5]. We also showed that non-respiring rat liver mitochondria swell in 100 mM strontium β -hydroxybutyrate in the presence of $1\text{ }\mu\text{M}$ FCCP. This swelling was insensitive to 0.2 mM NEM or mersalyl ($10\text{ }\mu\text{mol/g}$ mitochondrial protein) or both.

It is evident that the cristae membrane of rat liver mitochondria contains a bivalent cation-monocarboxylate symporter that is specific for bivalent cation- β -hydroxybutyrate symport at concentrations around 2 mM, but is active for acetate, butyrate, and probably other monocarboxylates, at relatively high concentration. The question obviously arises whether this system is the same or different from the $(\text{Ca}_2)^{4+}$ - HPO_4^{2-} symporter described previously [1,2].

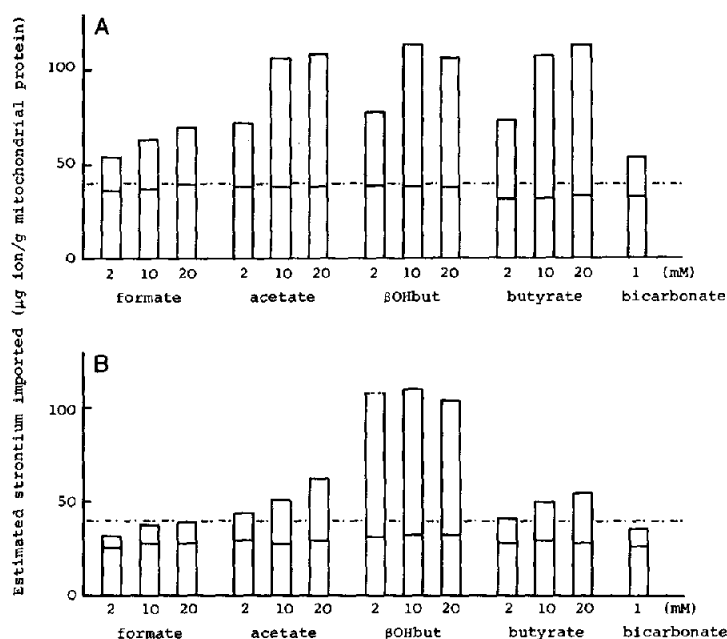


Fig.2. Import of strontium with endogenous P_i and added monocarboxylates. General conditions were as for the experiments of fig.1. A, in the absence of NEM; B, in the presence of 0.2 mM NEM. Values for the quantity of strontium imported were estimated from the extent of the stimulated (State 3) respiration during transitions from State 4 or State 6, and from appropriate \leftarrow Sr⁺/O calibrations (see section 3.1). Each histogram represents the mean value obtained from experiments done with two preparations of mitochondria. Lower section of each histogram represents Sr⁺ imported during the respiratory State 4-3-6 transition induced by injection of excess SrCl₂ (150 µmol/g mitochondrial protein). Upper section of each histogram represents Sr⁺ imported during the subsequent State 6-3-6 transition induced by injection of amounts of choline salts to give the stated concentrations in the suspensions. The broken lines indicate the maximum quantity of Sr²⁺ that could be imported with the endogenous P_i, as (Sr₂)⁴⁺-HPO₄²⁻, when recirculation of P_i is prevented. The value shown for 2 mM β-hydroxybutyrate (βOHbut) in the presence of NEM is a minimum value because the stimulated respiration did not subsequently return completely to the State 6 rate under these conditions.

3.3. Kinetic independence of calcium-phosphate and calcium-β-hydroxybutyrate import

Table 1 shows the rates of calcium import via the calcium-phosphate porter and via the porter system used for calcium-β-hydroxybutyrate import estimated in respiratory experiments of the State 4-3-4 or State 6-3-6 type by measuring the initial State 3 respiratory rate achieved either on adding CaCl₂, in media containing endogenous P_i (about 0.1 mM) or endogenous P_i + 10 mM β-hydroxybutyrate, or on adding 10 mM β-hydroxybutyrate after uptake of most of the endogenous phosphate had been induced by the addition of excess CaCl₂. Previous experiments [1] showed that the endogenous P_i was sufficiently concentrated

to give the maximal respiratory stimulation and the maximal activity of the calcium-phosphate symporter. In the present work, it was shown that the porter system for calcium-β-hydroxybutyrate import was fully active in media containing 10 mM β-hydroxybutyrate. The table shows that the estimated rate of Ca⁺ import in the presence of both P_i and β-hydroxybutyrate (line 2) was the sum of the estimated rates of Ca⁺ import in the presence of P_i (line 1) and in the presence of β-hydroxybutyrate (line 3). It seems likely, therefore, that calcium-β-hydroxybutyrate symport occurs via a separate lanthanide-sensitive porter that is independent of the lanthanide-sensitive (Ca₂)⁴⁺-HPO₄²⁻ symporter.

Table 1
Rate of calcium import with phosphate and/or
 β -hydroxybutyrate

| Anion | Estimated Ca^{2+} import rate ($\mu\text{g ion s}^{-1} (\text{g mitochondrial protein})^{-1}$) |
|----------------------------------|--|
| P_i | 0.99, 0.82 |
| $\text{P}_i + \beta\text{OHbut}$ | 2.10, 2.16 |
| βOHbut | 1.23, 1.36 |

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, 200 μM nupercaine, and mitochondria (6.1 mg protein/ml). Oligomycin (1 mg/g mitochondrial protein) and carbonic anhydrase (30 $\mu\text{g/ml}$) were also present. 0.2 mM NEM was added 4 min after the mitochondria. Calcium import was induced: by injecting 1 μmol CaCl_2 when the medium contained endogenous P_i (c. 0.1 mM) (line 1); by injecting 1 μmol CaCl_2 when the medium contained endogenous P_i + 10 mM β -hydroxybutyrate (line 2); and by injecting 10 mM β -hydroxybutyrate (in the presence of external Ca^{2+}) after first injecting 1 μmol CaCl_2 to induce import of the endogenous P_i (line 3). The Ca^{2+} import rate was estimated, in two separate sets of experiments, from the initial State 3 rate of respiration induced under the appropriate conditions and from appropriate Ca^{2+}/O calibrations

4. Conclusion and research prospect

The observations of this and our previous two papers [1,2], together with work by Azzzone et al. [7], indicate that calcium and some other bivalent cations including strontium may permeate the cristae membrane of rat liver mitochondria with an electric charge stoichiometry corresponding to Ca^{2+} via at least two systems that are sensitive to lanthanides but insensitive to NEM. One is the $(\text{Ca}_2)^{4+}\text{-HPO}_4^{2-}$ symporter described previously [1,2] and the other is a $\text{Ca}^{2+}\text{-}\beta\text{-hydroxybutyrate}^-$ symporter of unknown but equal valency for Ca^{2+} and $\beta\text{-hydroxybutyrate}^-$ described in the present paper. The latter system accounts for the NEM-insensitive swelling of non-respiring mitochondria in isotonic strontium acetate or strontium β -hydroxybutyrate solutions, and for the massive uptake of calcium acetate or calcium β -hydroxybutyrate in respiring mitochondrial suspensions with a stoichiometry corresponding to 4 acetate $^-$ or 4 β -hydroxybutyrate $^-$ anions per reducing equivalent traversing each coupling site or per \sim [3]. This conclusion is illustrated by fig.3. A shows the system assumed by Lehninger et al. [3], involving a Ca^{2+} uniporter for which there is no

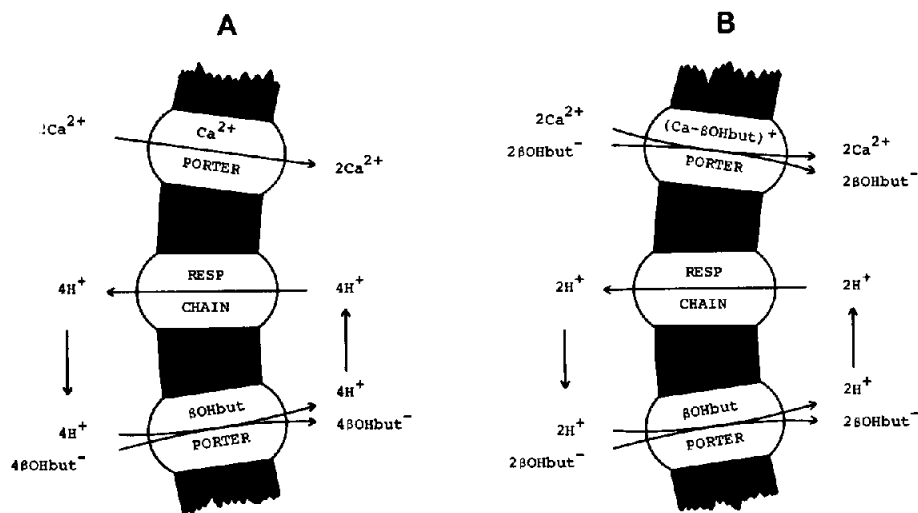


Fig.3. Alternative mechanisms proposed for import of 2Ca^{2+} with $4 \beta\text{-hydroxybutyrate}^-$ per \sim , requiring: A, $\text{H}^+/\sim = 4$; and B, $\text{H}^+/\sim = 2$. Mechanism A is as proposed by Lehninger et al. [3], and requires a Ca^{2+} uniporter for which there is no experimental evidence. Mechanism B is our proposal, and involves the $\text{Ca}^{2+}\text{-}\beta\text{-hydroxybutyrate}^-$ symporter described in this paper.

experimental support [1,2], and requiring a hypothetical respiratory $\rightarrow H^+/\sim$ stoichiometry of 4. B shows the system involving the Ca^{2+} - β -hydroxybutyrate $^-$ symporter described in the present work, the experimentally observed import of β -hydroxybutyric acid (probably catalysed by a uniporter [8]), and respiratory proton translocation with an observed $\rightarrow H^+/\sim$ stoichiometry of 2 [9].

Spencer and Bygrave [10] suggested that calcium translocation may be coupled to that of ATP in the atractyloside-sensitive ATP/ADP antiport reaction. Our observations on the import of calcium with phosphate and with β -hydroxybutyrate imply that it is now especially important to investigate whether calcium export may be coupled to that of ATP because such coupling would influence the overall stoichiometry of ADP phosphorylation and would affect the mechanism of respiratory stimulation by ADP in mitochondria.

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References

- [1] Moyle, J. and Mitchell, P. (1977) FEBS Lett. 73, 131–136.
- [2] Moyle, J. and Mitchell, P. (1977) FEBS Lett. 77, 136–140.
- [3] Brand, M. D., Chen, C.-H. and Lehninger, A. L. (1976) J. Biol. Chem. 251, 968–974.
- [4] Selwyn, M. J., Dawson, A. P. and Dunnett, S. J. (1970) FEBS Lett. 10, 1–5.
- [5] Moyle, J. and Mitchell, P. (1973) Biochem. J. 132, 571–585.
- [6] Lehninger, A. L. (1974) Proc. Natl. Acad. Sci. USA 71, 1520–1524.
- [7] Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M. and Dell'Antone, P. (1977) FEBS Lett. 78, 21–24.
- [8] Paradies, G. and Papa, S. (1975) FEBS Lett. 52, 149–152.
- [9] Mitchell, P. (1977) Ann. Rev. Biochem. 46, 996–1005.
- [10] Spencer, T. and Bygrave, F. L. (1972) Biochem. J. 129, 355–365.