Biochimica et Biophysica Acta, 1102 (1992) 62–66

PBABIO 43646

Bidirectional transport of spermine in rat liver mitochondria

Dagmar Siliprandi, Antonio Toninello and Lisa Dalla Via

Dipartimento di Chimica Biologica dell'Universita' di Padova, Centro Studio Fisiologia Mitocondriale del CNR, Padova (Italy)

(Received 11 December 1991)
(Revised manuscript received 22 April 1992)

Key words: Mitochondrion; Spermine; Bidirectional transport; Phosphate; (Rat liver)

Further study of the mitochondrial transport of spermine (Toninello et al. (1988) J. Biol. Chem. 263, 19407) shows that, after loading rat liver mitochondria with [^{14}C]spermine and [^{32}P]phosphate, these components are released together into the surrounding medium by adding mersalyl or *N*-ethylmaleimide. On later addition of dithioerythritol, both are recaptured, but if acetate or nigericin are added instead, only spermine re-enters and there is continued export of phosphate. This bidirectional transport of spermine in and out mitochondria is driven, respectively, by membrane potential and pH gradient at constant protonmotive force. Results using [^{14}C]spermine or [^{32}P]phosphate, in conjunction with the their unlabelled isomers and with or without carbonyl cyanide/*p*-trifuloromethoxyphenylhydrazone (FCCP) present suggest that there is a continuous energy-dependent efflux-influx cycling of spermine and phosphate.

Introduction

In a previous paper [1] we showed that spermine can be transported into the mitochondial matrix space at significant rates, which increase sharply and non-ohmically with increasing membrane potential.

Some of the accumulated spermine could be metabolized and some could accumulate in the inner mitochondrial compartment (either as an insoluble salt or immobilized on membranes by electrostatic attraction or other absorptive forces). A third possibility is that there could be spermine efflux into the surrounding medium. The mechanism of such efflux would differ from that of influx because of the unfavorable high negative membrane potential. In this paper we add further evidence to preliminary results [1,2] favouring the latter mechanism, by showing clearly that accumulated spermine is released from rat liver mitochondria by an energy-dependent process. This efflux occurs when, at constant protonmotive force, the membrane potential is lowered and the ΔpH concurrently increased. These necessary conditions are achieved by first loading mitochondria with spermine and P_i [1], then observing their subsequent release by blocking further P_i accumulation.

We have also shown that spermine and phosphate are not necessarily co-transported, even though the polyamine participates, like P_i, in a continuous energy-dissipating influx-efflux cycle.

Experimental procedures

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes (pH 7.4) by conventional differential centrifugation. Mitochondrial protein was assayed by a biuret method with bovine serum albumin as standard. All mitochondrial preparations used showed a respiratory control index ranging between 7–10 and a membrane potential ($\Delta\Psi$), in the presence of phosphate, of 180–190 mV.

Oxygen uptake was assayed polarographically using a Clark electrode (Yellow Spring Instruments, OH) in a water-jacket thermostated vessel, equipped with a magnetic stirrer.

Measurements of $\Delta\Psi$ and ΔpH were performed on the same sample. $\Delta\Psi$ was measured, in an open, thermostatically-controlled and stirred vessel, by monitoring the distribution of the lipophilic cation tetraphenilphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode, prepared in our laboratory according to published procedures [3,4]. Care was taken concerning concentration of TPP^+

Correspondence to: D. Siliprandi, Dipartimento di Chimica Biologica, Via Trieste, 75, 35121 Padova, Italy.

Abbreviations: DTE, dithioerythritol; $\Delta\mu_{H^+}$, protonmotive force; $\Delta\Psi$, electrical transmembrane potential difference; ΔpH, transmembrane H+ concentration gradient; DMO, 5,5-dimethyl oxazolidine-2,4-dione; NEM, *N*-ethylmaleimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

used, as previously indicated [1]. $\Delta\Psi$ measured with the TPP^+ selective electrode was corrected, as proposed by Jensen et al. [5]. ΔpH was calculated from the distribution of [^{14}C]DMO across the mitochondrial membrane [6].

Mitochondrial matrix volume was similarly calculated from the distributions of either [^{14}C]sucrose and ^3H_2O [7] or of [^{14}C]sucrose and [^{3}H]glycerol [8].

Mitochondria (1 mg protein/ml) were incubated at 20°C in a standard medium containing 200 mM sucrose, 10 mM Hepes-Cl (pH 7.4), 5 mM succinate (as the sodium salt) and 1.25 μM rotenone. Other additions or variations are indicated in the figure legends.

Uptake of [^{14}C]spermine and [^{32}P]phosphate was determined by a centrifugal filtration method, as previously described [1,9]. The [^{32}P]P_i used was first heated in a boiling water bath for 3 h in 0.1 M HCl (to remove polyphosphates).

Results

When mersalyl, a known inhibitor of the P_i-H^+ transporter [10], is added to a mitochondrial suspension, previously loaded with [^{14}C]spermine and [^{32}P]P_i, there is a concurrent efflux of both ions. However, the two rates are not the same, P_i efflux occurring at a faster rate than that of spermine (Fig. 1). Similar results are obtained on replacing mersalyl with NEM (not shown). If the mersalyl is now removed by adding DTE [11], there is a rapid and synchronous reuptake of both spermine and P_i. Both rates are higher than the original uptake rates (Fig. 1).

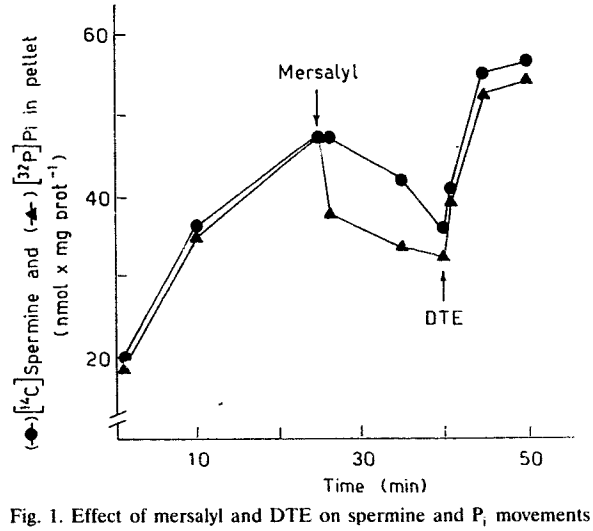

Fig. 1. Effect of mersalyl and DTE on spermine and P_i movements across the mitochondrial membrane. Rat liver mitochondria were incubated in the standard medium containing 1 mM [^{14}C]spermine (50 μCi/mmol) and 1 mM [^{32}P]P_i-sodium P_i (50 μCi/mmol). Additions, 10 μM mersalyl at 25 min; 2 mM DTE at 40 min.

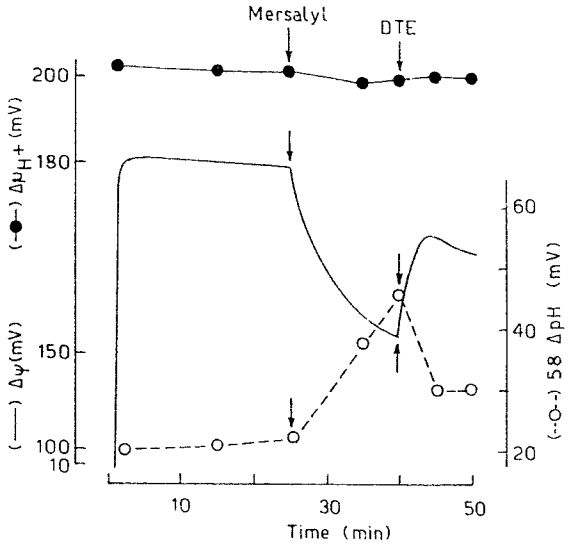

Fig. 2. Effect of mersalyl and DTE on $\Delta\Psi$ and ΔpH of mitochondrial membrane. Rat liver mitochondria were incubated in the standard medium in the presence of 1 mM spermine, 1 mM P_i, 2 μM TPP^+, 400 μM [^{14}C]DMO (1 μCi/mmol), 5 mM [^{3}H]glycerol (100 μCi/mmol). Additions, 10 μM mersalyl at 25 min; 2 mM DTE at 40 min.

Considerable changes in ΔpH and $\Delta\Psi$ accompany, and may explain, these ion movements. Thus, after adding mersalyl, $\Delta\Psi$ falls sharply but ΔpH rises, whereas later addition of DTE reverses these changes (Fig. 2). The result is that the protonmotive force ($\Delta\mu_{H^+}$) is unchanged, establishing that mersalyl has not induced non-specific uncoupling. This is also confirmed by the fact that when spermine uptake is induced by acetate [1], mersalyl added afterwards does not then induce efflux (not shown). Hence, at the concentrations used mersalyl is not an uncoupler and its effect is solely due to the inhibition of the P_i-H^+ transporter.

The mitochondrial level of Ca^{2+} is unaffected during the movements of spermine and phosphate and these movements are also unresponsive to the addition of ruthenium red to inhibit Ca^{2+} uptake (results not shown).

DTE alone has no effect on spermine accumulation (Fig. 3), suggesting that the observed increase in the rate of spermine uptake, upon addition of mersalyl, and then its removal by DTE, may be due to a mersalyl-induced rearrangement of membrane SH groups.

Spermine released from mitochondria by the action of mersalyl is also recaptured, though less efficiently, on adding acetate (Fig. 4A), but the induced reuptake is now no longer linked to that of P_i, which continues to be released throughout. This difference is readily

explicable because, though acetate (like P_i, but less potently) can increase $\Delta\Psi$, its own transport is insensitive to mersalyl. Similar results are obtained by substituting nigericin for acetate (Fig. 4B) but, in this case, the spermine reuptake is small and transient, corresponding to a similar transient increase in $\Delta\Psi$ (Fig. 4B inset).

We have followed the flux of spermine in mitochondria after achieving a steady state by the sequential use of unlabelled and labelled spermine. By direct incubation with [^{14}C]spermine in a P_i-containing medium, a steady state is achieved after 40 min (Fig. 5Aa). If unlabelled spermine (at the same concentration) is used instead and then [^{14}C]spermine is added after this period, an exchange between the pools is demonstrable, as shown by the appearance of considerable [^{14}C]spermine in the mitochondrial pellet (Fig. 5Ab). This spermine/[^{14}C]spermine exchange is energy-dependent because it is abolished by adding FCCP (Fig. 5Ac). This technique has also been used to study phosphate flux in the presence of unlabelled spermine by using P_i and [^{32}P]P_i. The results (Fig. 5B) also indicate a continuous energy-dependent uptake and release of P_i.

Discussion

Spermine enters the mitochondrial matrix driven by the high $\Delta\Psi$ achieved by P_i uptake [1]. Conversely, spermine increases the extent of P_i accumulation [9] remarkably. This reciprocity is attributable to their opposite effects on $\Delta\Psi$, which is, respectively, increased by P_i and decreased by spermine. Accumulated spermine is retained within the matrix space, as long as $\Delta\Psi$ remains above a certain critical value. A decrease of $\Delta\Psi$, as that caused by uncouplers, produces an immediate outflow of the spermine taken up [9]. However, as shown in the present paper, spermine can also be released from energized mitochondria by inhibiting the influx of P_i by mersalyl, thus decreasing $\Delta\Psi$ and, concomitantly, increasing ΔpH, i.e., mantaining $\Delta\mu_{H^+}$ unmodified (Fig. 2). Under this condition ΔpH, no longer utilizable for P_i influx, becomes available for driving spermine efflux.

The process is reversible, since the released P_i and spermine are quickly, and synchronously, recaptured on removing mersalyl with DTE. This reversibility also shows that the membrane permeability properties required for the preservation of $\Delta\mu_{H^+}$ have not been substantially altered by the successive addition and removal of mersalyl.

The transports of spermine and P_i are not obligatorily linked because, when acetate or nigericin are sub-

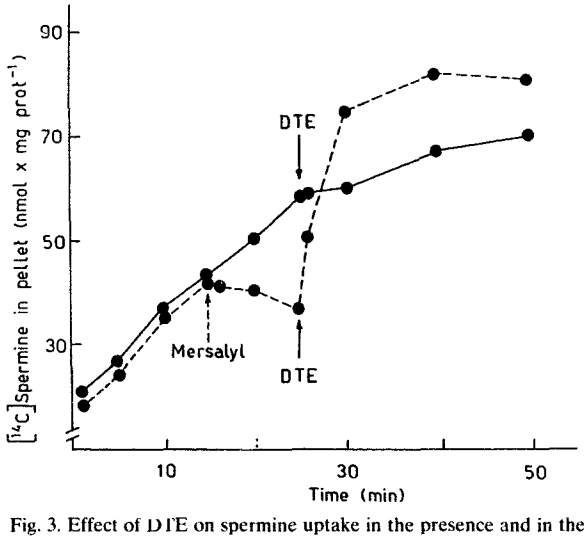

Fig. 3. Effect of DTE on spermine uptake in the presence and in the absence of mersalyl. Rat liver mitochondria were incubated in the standard medium containing 1 mM [^{14}C]spermine (50 μCi/mmol) and 1 mM P_i. 2 mM DTE is added at 25 min. When added 10 μM mersalyl at 15 min. ●———●, in the absence of mersalyl; ●------●, in the presence of mersalyl.

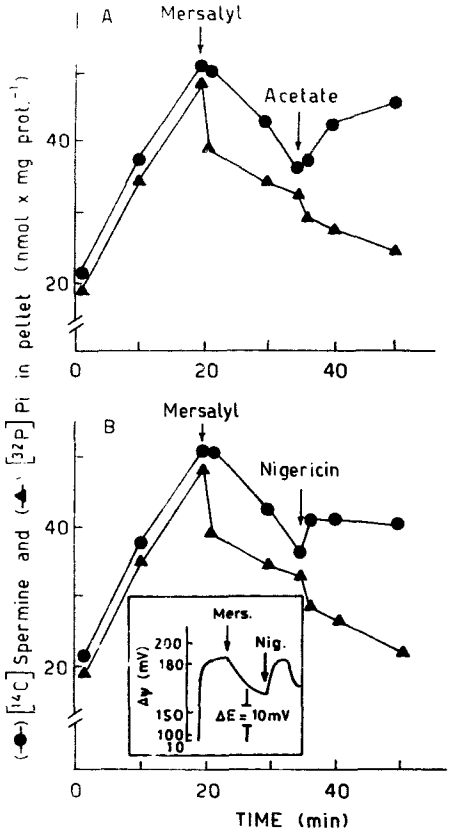

Fig. 4. Spermine and P_i movements in liver mitochondria driven by $\Delta\Psi$ variations. Spermine reuptake induced by acetate (A) and by nigericin (B). Experimental conditions, as in Fig. 1 Additions, 10 μM mersalyl at 20 min; 30 mM acetate at 35 min; 0.03 μg/mg nigericin at 35 min.

The transports of spermine and P_i are not obligatorily linked because, when acetate or nigericin are sub-

stituted for DTE, the reuptake of spermine is observed although P_i continues to escape (Fig. 4). This indicates that spermine is not transported in symport with P_i on the same carrier and further confirms that P_i promotes spermine uptake by increasing mitochondrial $\Delta\Psi$ as acetate and nigericin do, though less efficiently (see also Ref. 1).

Our postulated mechanism for P_i efflux is its extrusion from the matrix space as an anion down a $\Delta\Psi$-positive outside (see also Ref. 12). On the other hand spermine efflux, like that of other cations [13], could occur electroneutrally, by exchange with incoming protons. It is conceivable that, under physiological conditions, the effluxes of P_i and spermine utilize $\Delta\Psi$ and

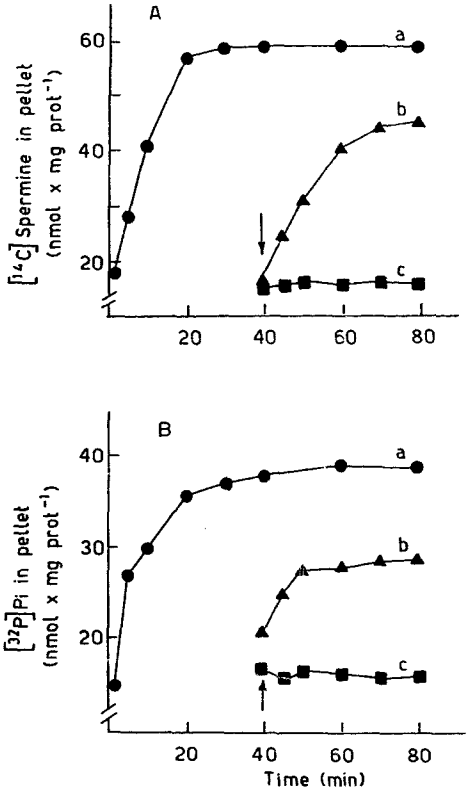

Fig. 5. Spermine and P_i cycling across the mitochondrial membrane. Rat liver mitochondria were incubated in the standard medium. (A) ●, 1 mM [^{14}C]spermine (50 μCi/mmol) and 1 mM P_i (curve a); ▲, 1 mM spermine and 1 mM P_i. At 40 min. [^{14}C]spermine carrier-free (50 nCi/ml) was added (curve b); ■, 1 mM spermine and 1 mM P_i. At 40 min 0.1 μg/mg protein FCCP and [^{14}C]spermine carrier-free (50 nCi/ml) were added (curve c). (B) ●, 1 mM [^{32}P]P_i (50 μCi/mmol) and 1 mM spermine (curve a); ▲, 1 mM spermine and 1 mM P_i. At 40 min [^{32}P]P_i carrier-free (50 nCi/ml) was added (curve b). ■, 1 mM spermine and 1 mM P_i. At 40 min 0.1 μg/mg protein FCCP and [^{32}P]P_i carrier-free (50 nCi/ml) were added (curve c).

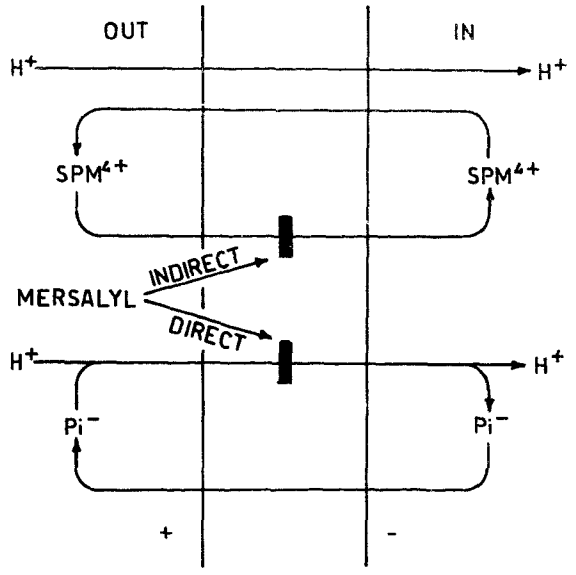

Fig. 6. Suggested mechanism for spermine and P_i flux in energized rat liver mitochondria. The uptake of spermine and P_i into the mitochondrial matrix is driven by $\Delta\Psi$ and ΔpH, respectively, whereas their efflux into the cytosol is driven by ΔpH and $\Delta\Psi$, respectively. Mersalyl, by blocking the P_i^-/H^+ symporter, inhibits, indirectly, spermine uptake and promotes spermine and P_i efflux.

ΔpH, respectively, in an integrated process occurring at constant $\Delta\mu_{H^+}$.

The exchange between internal and external P_i and spermine (Fig. 5) implies a constant expenditure of energy. The function of this energy-consuming 'influx-efflux' cycle (see scheme of Fig. 6) could be that of buffering the spermine concentration between mitochondrial matrix and cytosol in a way which is controlled by the equilibrium between $\Delta\Psi$ and ΔpH.

Calcium ions do not significantly interfere with this cycle, since their mitochondrial concentration is unaffected and the movements of either spermine or phosphate are insensitive to ruthenium red. Spermine transport occurs at a much lower rate than that of Ca^{2+} but resembles it in that both ions are taken up electrophoretically and both are released electroneutrally.

Since the synthesis of polyamines is known to be an extra-mitochondrial process, the reversible transport of spermine may be of physiological importance. An intramitochondrial function is suggested by effects of spermine on such mitochondrial enzymes as protein phosphatase 2A from bovine kidney mitochondria [14] and citrate synthase from porcine heart [15]. Polyamines might also affect mitochondrial genoma, since selective depletion of mitochondrial DNA has been observed to follow spermine depletion [16].

Acknowledgments

This work was supported, in part, by a grant from Sigma-Tau (Pomezia-Roma). We are grateful to Mr. Mario Mancon for skillful technical assistance.

References

1 Toninello, A., Miotto, G., Siliprandi, D., Siliprandi, N. and Garlid, K.D. (1988) J. Biol. Chem. 263, 19407–19411.
2 Toninello, A., Siliprandi, D., Siliprandi, N. and Garlid, K.D. (1988) EBEC Rep., Vol. 5, 208.
3 Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.
4 Affolter, H. and Siegel, E. (1979) Anal. Biochem. 97, 315–319.
5 Jensen, B.D., Gunter, K.K. and Gunter, T.E. (1986) Arch. Biochem. Biophys. 248, 305–323.
6 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
7 Palmieri, F. and Klingenberg, M. (1979) Methods Enzymol. 56, 279–301.
8 Azzone, G.F., Pietrobon, P. and Zoratti, M. (1984) Curr. Top. Bioenerg. 13, 1–77.
9 Toninello, A., Di Lisa, F., Siliprandi, D. and Siliprandi, N. (1984) Biochim. Biophys. Acta 815, 399–404.
10 Fonyo, A. (1978) J. Bioenerg. Biomembr. 10, 171–194.
11 Cleland, W.W. (1964) Biochemistry 3, 480–482.
12 Azzone, G.F., Massari, S. and Pozzan, T. (1976) Biochim. Biophys Acta 426, 15–26.
13 Garlid, K.D. (1988) in Integration of Mitochondrial Function (Lemasters, J.J., Hackenbrock, C.R., Thurman, R.G. and Westerhoff, H.V., eds.), pp. 257–276, Plenum, New York.
14 Damuni, Z., Reed, J.L. (1987) J. Biol. Chem. 262, 5133–5138.
15 Yoshino, M., Yamada, Y., Murakami, K. (1991) Biochim. Biophys. Acta 1073, 200–203.
16 Vertino, P.M., Beerman, T.A., Kelly, E.J., Bergeron, R.J. and Porter, C.W. (1991) Mol. Pharmacol. 39, 487–494.