PHOSPHATE RETENTION AND RELEASE DURING ATP HYDROLYSIS IN LIVER MITOCHONDRIA

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1. Introduction

Organic mercurials are inhibitors of the P_i carrier of mitochondria and they inhibit both P_i uptake and efflux [1–4]. Inhibition of P_i efflux by mercurials was shown in a system in which mitochondria hydrolyzed added ATP in the presence of uncouplers. ATP hydrolysis takes place at the inner surface of the inner membrane and P_i is liberated in the matrix space. If the P_i carrier is not inhibited, the P_i generated from ATP leaves the matrix immediately. If organic mercurials were added, the P_i was retained within the matrix and the increase of osmotic equivalents resulted in parallel mitochondrial swelling [4].

It was reported that mercurials, notably mersalyl, did not inhibit P_i efflux in the ATP-uncoupler system if the medium contained Mg^{2+} [5]. Furthermore, even in the absence of added Mg^{2+} a very significant part of the P_i generated from ATP was found to leave mersalyl-treated mitochondria [6]. These observations directed attention to the possibility that the reaction of organic mercurials with the P_i carrier was not complete under all conditions and subject to modulation during ATP hydrolysis. As a result of this modulation, the effect of mersalyl on P_i efflux could change from partial to total inhibition. Another possible interpretation of the P_i efflux during ATP hydrolysis, namely the operation of an alternative, mercurial-insensitive P_i transport system was however clearly recognized in [6].

The aim of this work was to find the conditions under which P_i generated from added ATP is either retained within the mitochondria or released from them. The distribution of the P_i liberated from ATP was investigated by a rapid filtration method. If ATP hydrolysis was triggered by valinomycin plus K^+, mersalyl blocked P_i efflux instantaneously and completely. If however ATP was hydrolyzed under uncoupling conditions, either in the presence of valinomycin plus K^+ and nigericin, or FCCP, then there was an initial period of P_i efflux which was followed by a total retention of the generated P_i within the matrix.

2. Materials and methods

Rat liver mitochondria were isolated in 0.25 M sucrose, 5 mM Tris–HCl at pH 7.4 by differential centrifugation.

Incubation of 5 mg mitochondrial protein in 3 ml was carried out at room temperature in the cell of the Leres spectrophotometer equipped with a Sefram recorder. The absorbance of the suspension was recorded at 546 nm. At given times a 0.4 ml aliquot of the cell content was rapidly injected into 1.1 N perchloric acid for measuring ATP hydrolysis. At the same time the rest of the cell content (2.6 ml) was rapidly and very gently filtered through Millipore 'Millex' SLHA 025-OS filters with pore size of 0.45 μm to measure extramitochondrial P_i. Filtration lasted 5 s under which 0.6–0.8 ml clear filtrate was

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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produced. More forceful filtration resulted in turbid filtrates and higher apparent extramitochondrial P_i content. The tubes in which the filtrates were collected contained 5 μl 5.5 N perchloric acid and were kept in crushed ice until the P_i assay was carried out. Total and extramitochondrial P_i content was measured according to [7]; this procedure detected 10 nmol P_i with sufficient accuracy. The difference between the total and the extramitochondrial P_i content was taken as the P_i within the matrix space.

3. Results

3.1. *The distribution of P_i during valinomycin plus K^+-induced hydrolysis of ATP*

In the presence of K^+, valinomycin induces rapid hydrolysis of ATP, and even if no inhibitor of the P_i transport is added, all the P_i liberated in the first 0.5 min is retained within the matrix with corresponding swelling of the mitochondria (fig.1). After this initial period the P_i liberated further leaves the mitochondria, the P_i within the matrix and the mitochondrial volume remaining constant. On addition of mersalyl the P_i efflux is immediately and completely blocked. The retention of the P_i liberated is also reflected in further mitochondrial swelling. After 1–2 min a small efflux of P_i is occasionally seen, < 10% of the value of ATP hydrolysis. It cannot be excluded that this small leak of P_i is due to damage of swollen mitochondria during the filtration procedure.

3.2. *The distribution of P_i and the action of mersalyl on P_i efflux during hydrolysis of ATP under uncoupled conditions*

3.2.1. Valinomycin plus nigericin

Addition of nigericin after valinomycin in the state when P_i efflux from mitochondria balances P_i production results in abrupt release of P_i and simultaneous shrinkage (fig.2). On addition of mersalyl there is a tri-phasic response (table 1): in the first 30 s most of the generated P_i is retained in the matrix; over a further 30 s a large part of it is released and appears extramitochondrially; finally after 1 min most of the P_i liberated further is retained in the matrix. Mitochondrial swelling was very slow immediately after mersalyl addition and became much faster after 1 min.

3.2.2. FCCP

On addition of FCCP in the presence of mersalyl, all the P_i liberated from ATP leaves the matrix (data not shown) and no swelling is detected. After addition of mersalyl the P_i efflux continues for ~0.5 min with little increase of P_i within the matrix, but after this 0.5 min initial period P_i efflux is almost totally inhibited (fig.3). The P_i liberated and retained within

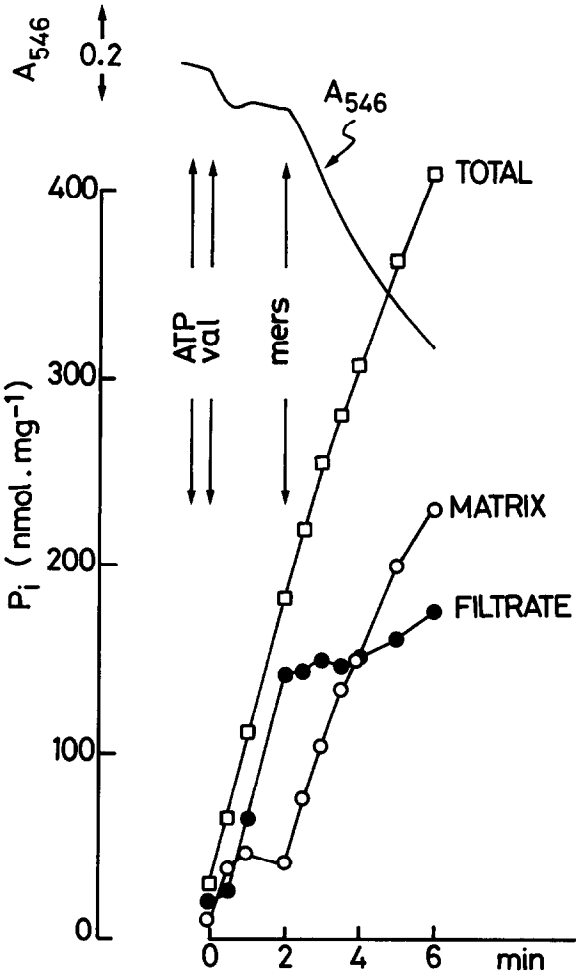

Fig.1. The distribution of P_i between the matrix and extramitochondrial space and absorbance changes during valinomycin plus K^+-induced hydrolysis of ATP. (□——□) total P_i content; (○——○) P_i within the matrix; (●——●) P_i content of the filtrate (extramitochondrial). The incubation medium contained 246 mM sucrose, 20 mM Tris–HCl, 6.6 mM KCl and 3.5 μM rotenone at pH 7.40. Further additions: 1 mM ATP Na; 21.5 ng valinomycin/mg mitochondrial protein; 20 nmol mersalyl/mg protein (33 μM).

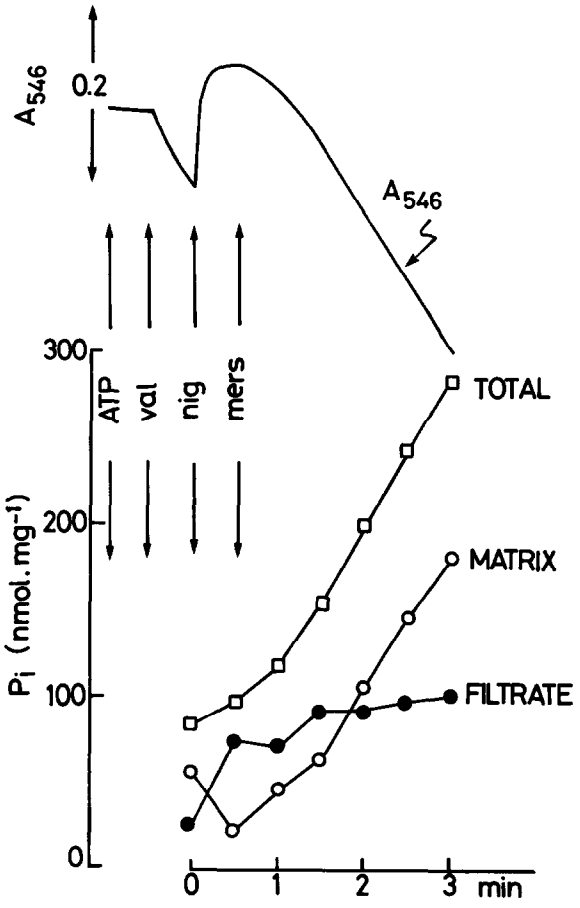

Fig.2. The distribution of P_i between the matrix and extramitochondrial space and absorbance changes during valinomycin plus K^+ plus nigericin-induced hydrolysis of ATP. (□——□) total P_i content; (○——○) P_i within the matrix; (●——●) P_i content of the filtrate (extramitochondrial). The incubation medium contained 246 mM sucrose, 20 mM Tris–HCl, 6.6 mM KCl, 3.5 μM rotenone at pH 7.40. Further additions: 1.6 mM ATP Na; 10 ng valinomycin/mg mitochondrial protein; 50 ng nigericin/mg protein; 20 nmol mersalyl/mg protein (33 μM).

Table 1
Changes in extramitochondrial P_i content following the addition of mersalyl during valinomycin plus nigericin-induced ATP hydrolysis

Time after mersalyl addition (s)	Δ extramitochondrial P_i (nmol/mg protein)		
	Exp. 1	Exp. 2	Exp. 3
0–30	5.6	−4.2	−3.4
30–60	25.3	33.0	22.0
60–90	2.9	−1.2	−0.3
90–120	1.5	4.7	4.9

Experimental conditions were as described in fig.2

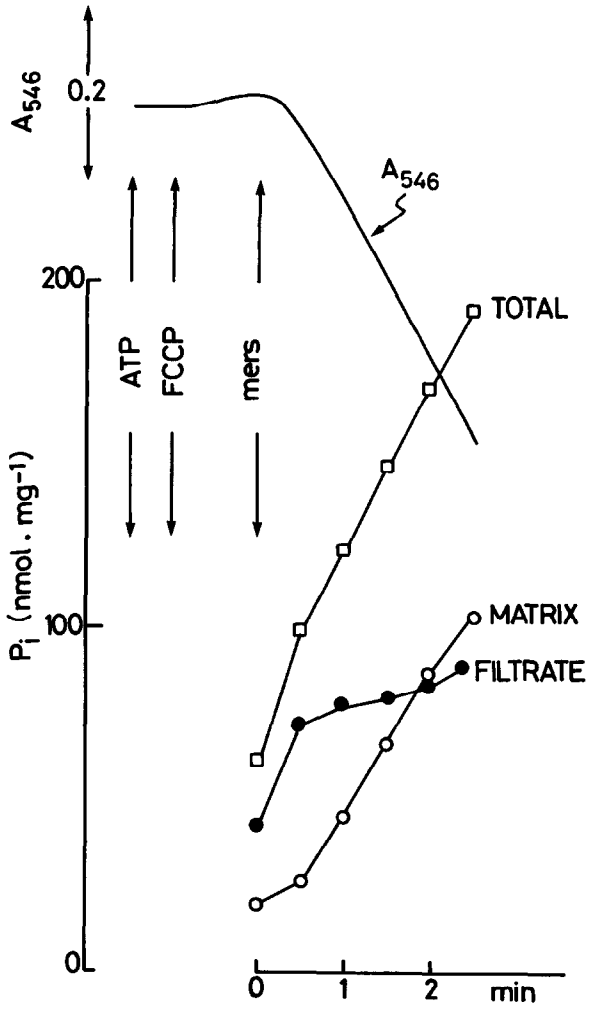

Fig.3. The distribution of P_i between the matrix and extramitochondrial space and absorbance changes during FCCP-induced hydrolysis of ATP. (□——□) total P_i content; (○——○) P_i within the matrix; (●——●) P_i content of the filtrate ATP Na; 0.67 μM FCCP; 20 nmol mersalyl/mg mitochondrial 246 mM sucrose, 20 mM Tris–HCl, 0.83 mM Na-EDTA and 3.5 μM rotenone at pH 7.40. Further additions: 1.6 mM ATP Na; 0.67 μM FCCP; 20 nmol mersalyl/mg mitochondrial protein (33 μM).

the matrix causes swelling of the mitochondria: there was little swelling immediately after addition of the mersalyl.

It has to be stressed that under our experimental conditions the efflux of P_i was almost totally inhibited if the mitochondria were preincubated first for 1 min with mersalyl and ATP and FCCP were added after the preincubation. Under these conditions > 90% of the P_i derived from ATP was retained in the matrix (data not shown).

The ATPase activity measured in the presence of FCCP was always lower than that measured in the presence of valinomycin plus K^+ (cf. fig.1,3). Furthermore the ATPase activity under most experimental conditions was not linear. The mechanism of the non-linearity was not investigated, but it may have its origin in the changing intramitochondrial P_i and pH values.

4. Discussion

4.1. *The relationship between P_i retention and release during ATP hydrolysis*

It was found here that all the P_i derived from ATP hydrolysis was retained within the mitochondria:

1. In the absence of mersalyl during the initial phase of the valinomycin plus K^+-induced hydrolysis of ATP;
2. Immediately after the addition of mersalyl if ATP hydrolysis was stimulated by valinomycin plus K^+;
3. 0.5–1 min after the addition of mersalyl if ATP hydrolysis was stimulated either by FCCP or by valinomycin plus nigericin plus K^+.

These findings rule out an obligatory coupling between the inward movement of ATP with the exit of P_i as suggested in [6].

4.2. *The onset of mersalyl action: the delay period of P_i transport inhibition under uncoupled conditions*

The onset of inhibition by mersalyl on P_i efflux was within the resolution time of our method if ATP hydrolysis was induced by valinomycin plus K^+. This corresponds to earlier findings in which mersalyl inhibited in < 1 s the efflux of endogenous P_i from mitochondria [8] or the exchange of $^{32}P_i$ with $^{31}P_i$ [9].

The immediate inhibitory effect of mersalyl on P_i efflux under the above conditions is in marked contrast with the delayed effect seen in the presence of FCCP and with the tri-phasic effect observed when uncoupling was achieved with the combination of valinomycin plus nigericin plus K^+. There are two possibilities to explain the delay of inhibition of P_i transport:

(1) Under uncoupled conditions a transient mersalyl-insensitive P_i efflux pathway is opened for a short time;
(2) In uncoupled mitochondria the reaction between the mercurial and the SH-groups of the P_i carrier is retarded and even already formed mercaptide bonds may temporarily dissociate.

The mechanism by which strategic SH-groups are either readily or sluggishly available to mersalyl depending on environmental conditions is still speculative. In this respect a possible effect of the protonmotive force on the orientation or the conformation of the P_i carrier in the mitochondrial membrane may be envisaged. It is noteworthy that the energy state of mitochondria appears to play a role in the inhibitory effect of another SH-group reagent, *N*-ethyl maleimide, on the ADP/ATP transport, the inhibition by this latter reagent being markedly decreased in uncoupled mitochondria [10].

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References

[1] Fonyó, A. and Bessman, S. P. (1968) Biochem. Med. 2, 145–163.
[2] Fonyó, A. (1968) Biochem. Biophys. Res. Commun. 32, 624–628.
[3] Tyler, D. D. (1968) Biochem. J. 107, 121–123.
[4] Tyler, D. D. (1969) Biochem. J. 111, 665–678.
[5] Siliprandi, D., Toninello, A., Zoccarato, F. and Bindoli, A. (1975) FEBS Lett. 51, 15–17.

[6] Reynafarje, B. and Lehninger, A. L. (1978) Proc. Natl. Acad. Sci. USA 75, 4788–4792.
[7] Wahler, B. E. and Wollenberger, A. (1958) Biochem. Z. 329, 508–520.
[8] Hoek, J. B., Lofrumento, N. E., Meijer, A. J. and Tager, J. M. (1971) Biochim. Biophys. Acta 226, 297–308.
[9] Coty, W. A. and Pedersen, P. L. (1974) J. Biol. Chem. 249, 2593–2598.
[10] Vignais, P. V., Lauquin, G. J. M. and Vignais, P. M. (1976) in: Mitochondria, Bioenergetics, Biogenesis and Membrane Structure (Packer, L. and Gomez-Puyou, A. eds) pp. 109–125, Academic Press, New York.