DIFFERENTIAL INHIBITION OF PHOSPHATE EFFLUX AND INFLUX AND A POSSIBLE DISCRIMINATION BETWEEN AN INNER AND OUTER LOCATION OF THE PHOSPHATE CARRIER IN MITOCHONDRIA

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1. Introduction

It has been shown that the transport of phosphate (P_i) in mitochondria requires the presence of SH groups in the inner mitochondrial membrane [1, 2]. The present study attempts to gain insight into the mechanism of the mitochondrial P_i transport by the use of SH reagents. If P_i is transported by a mobile carrier in the broadest sense, i.e. diffusible carrier or fixed carrier with conformational changes, at least two extreme positions can be expected where the carrier is directed towards the outer or inner side of the membrane. Furthermore, the distribution between the two locations should be variable.

In the present case the discrimination between an inner and outer location of the P_i carrier has been indicated by comparing the effect of different SH reagents on the efflux and influx of P_i through the mitochondrial membrane.

2. Results and discussion

First, the effectivity of various SH reagents on the P_i transport in mitochondria was studied by following the swelling of mitochondria due to energy-independent osmotic equilibration in isotonic ammonium phosphate buffer. Fig. 1 shows that the mercurial, mersalyl, inhibits transport at the lowest concentration, whereas the maleinimide derivative, NEM*, fully inhibits only at about 0.2 mM, and ASPM only at concentrations greater than 0.4 mM. The concentra-

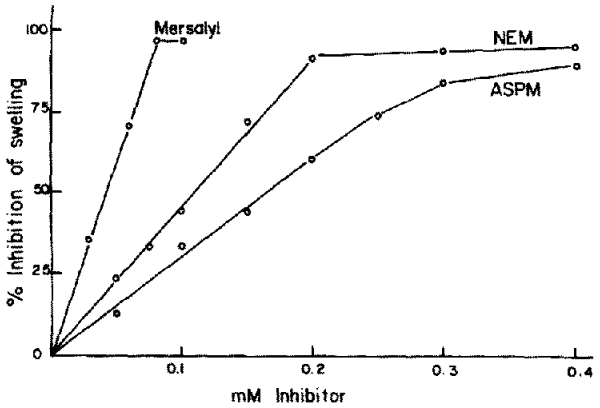


Fig. 1. Inhibition of P_i uptake by rat liver mitochondria in dependence on the concentration of various SH reagents. Indirect measurement of P_i uptake by swelling in ammonium phosphate. Rat liver mitochondria (0.62 mg protein in 0.1 ml) are preincubated for 2 min with different concentrations of mersalyl, NEM or ASPM. Then 0.1 ml mitochondria is added to 1 ml of 100 mM ammonium phosphate, pH 7.2, in a cuvette and the swelling is followed with an Eppendorf Photometer at 546 nm and at 20°.

* *Abbreviations:*
NEM: *N*-ethyl-maleinimide;
ASPM: *N*-(*N*-acetyl-4-sulfamoyl-phenyl)maleinimide [4];
DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid) [5];
EGTA: ethylene glycol bis(β-aminoethyl)-*N*,*N*-tetra-acetic acid;
FCCP: *p*-trifluoromethoxycarbonylcyanidephenylhydrazone.

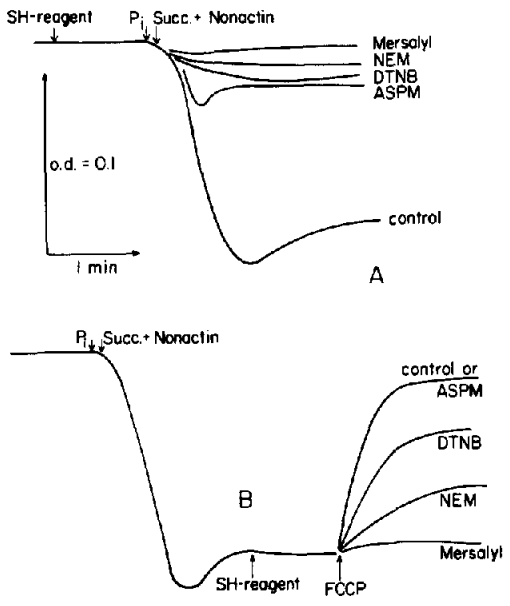


Fig. 2. Differential effects of SH reagents on the influx (A) and the efflux (B) of P_i. Mitochondria (0.5 mg) are added to 1 ml of a medium containing 0.2 M saccharose, 15 mM KCl, 1 mM EGTA, 20 mM triethanolamine-HCl buffer, pH 7.4, and 0.2 μg rotenone. Concentration of inhibitor: ASPM 0.125 mM, NEM 0.1 mM, DTNB 0.05 mM, mersalyl 0.02 mM. (A) Mitochondria first incubated for 1 min with the inhibitor, then 2 mM P_i, 5 mM succinate and 0.02 μg nonactin are added; (B) the inhibitor is added after swelling induced by the same conditions as in A. After 1 min incubation, 0.5 μM FCCP is added.

tion dependence is closely connected to the reaction rates of these reagents. ASPM reacts slowest. This can be explained in part by the relative impermeability of ASP [3] which forms the basis of the present studies.

In fig. 2 the effects of various SH reagents on the uptake and release of P_i are compared by following the energy-dependent swelling and contraction of mitochondria at low concentrations of P_i. Fig. 2A shows that the swelling, i.e. uptake of P_i initiated by addition of nonactin is fully inhibited by various SH reagents including ASPM. In these experiments the proportion of ASPM to protein is higher than in those of fig. 1. The release of P_i as followed by the contraction of the mitochondria in fig. 2B is completely inhibited by mersalyl, partially by NEM and to a lesser degree by DTNB, whereas ASPM does not inhibit. Thus, under the same conditions and the same concentrations of ASPM, where the influx of P_i is fully inhibited, the efflux remains fully active. At a higher concentration of ASPM, the efflux can be partially inhibited.

Another method for following the inhibition of P_i uptake is to generate P_i inside by an FCCP-induced ATP-hydrolysis. The inhibition by mersalyl causes an accumulation of P_i and therefore a swelling, as shown by Tyler [2]. This is confirmed in fig. 3, where NEM as well as mersalyl is able to produce swelling. In the presence of ASPM and also DTNB at the same concentrations as in fig. 2, no swelling is observed. This indicates that ASPM and DTNB do not inhibit the efflux of P_i, as shown in the previous experiments. The possible explanation that ASPM inhibits the ATPase can be excluded by the subsequent addition of mersalyl which then still induces swelling due to the P_i accumulation from ATP hydrolysis.

These results may be interpreted to show that the carrier for P_i has at least two locations in the membrane: position 1 is accessible to all four SH reagents, position 2 is inaccessible to ASPM and DTNB, but not to NEM and mersalyl. Since it is feasible that ASPM and DTNB do not easily penetrate the membrane, one can further specify that in position 1 the SH groups are directed to the outside and in position 2 to the inside.

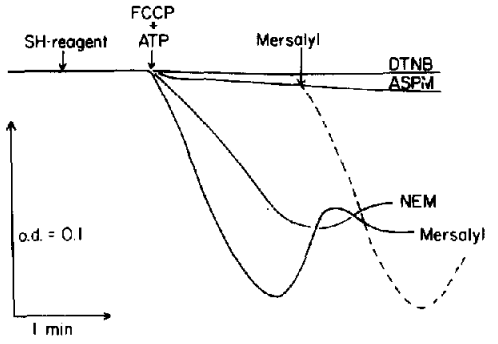
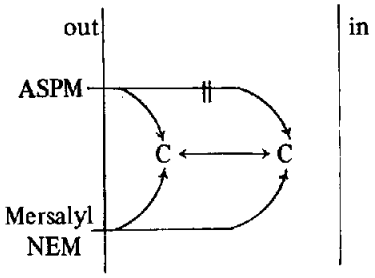


Fig. 3. Comparative action of the SH reagents on the efflux of P_i produced by a FCCP-induced hydrolysis. Mitochondria (0.5 mg) are suspended in 1 ml of medium as in fig. 2. SH reagent is added at the same concentrations as in fig. 2. Then 0.5 μM FCCP and 1 mM ATP are added.



In principle this could be rationalized by a mobile or reoriented carrier in which the binding sites and SH groups can be distributed to the inner and outer locations to a varying degree. In the experiment of P_i uptake (fig. 2A) the SH groups are located mainly to the outside and accessible to ASPM. In the experiment of P_i release (fig. 2B) the SH groups are located to the inside and inaccessible to ASPM.

The inhibition by ASPM only of the uptake and not of the release of P_i might be considered to contradict the principles of thermodynamics unless P_i transport in the two directions is catalyzed by different transport mechanisms. However, it should be considered that for the two directions the conditions are different. As a result an unequal in and out distribution of the same carrier can be expected which is different for both directions.

In the case when the same carrier catalyzes transport in both directions, if one direction of transport is inhibited, the other direction should also be abolished. This can in fact be demonstrated in the experiments shown in fig. 4, where first the uptake of P_i (according to conditions of fig. 2) is inhibited by ASPM. If now the efflux is assayed with ATP + FCCP, swelling is observed as a result of the inhibition of the P_i release. This rate is nearly the same as with mersalyl which completely inhibits the P_i efflux (cf. fig. 3). Once ASPM has inhibited uptake, it also inhibits efflux. It can be concluded that the same carrier catalyzes uptake and release of P_i and that an asymmetric distribution of the SH groups may cause a different degree of inhibition in both directions.

The reactivity of the carrier with ASPM can be expected to depend on its steady state distribution at the outside. This is influenced by the presence of exogenous P_i, as demonstrated by the experiment in fig. 5. First ASPM is allowed to react with the carrier, with or without the presence of P_i, and then the unreacted ASPM is removed by cysteine in order to prevent further reaction of ASPM during P_i uptake. Subsequently, the remaining activity of the carrier is

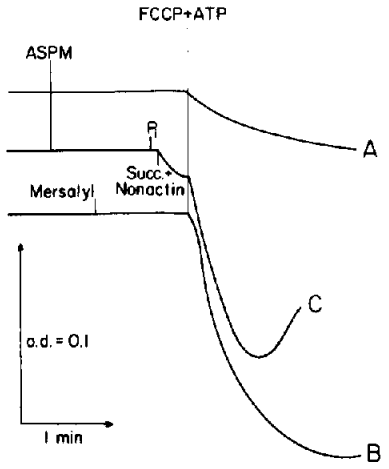


Fig. 4. Demonstration that inhibition by ASPM of P_i influx also gives inhibition of P_i efflux. (A and B) under similar conditions as in fig. 3. ASPM cannot inhibit the release of P_i; (C) before FCCP and ATP, 2 mM P_i, 5 mM succinate and 0.02 μg nonactin are added.

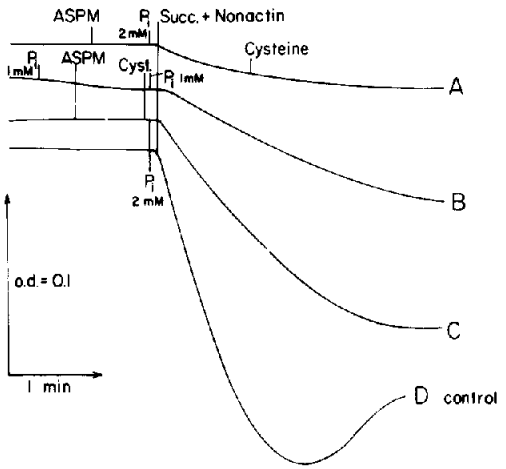


Fig. 5. Dependence on external P_i of the inhibition by ASPM. The conditions are the same as in fig. 2A, but cysteine is added to eliminate free ASPM which has not reacted. 0.7 mg protein, 0.2 mM ASPM, 1 min incubation. 2 mM P_i, 5 mM succinate 0.02 μg nonactin, 1 mM cysteine.

assayed by the P_i influx. As shown in fig. 5A, the inhibition by ASPM of the P_i influx is not abolished by addition of cysteine. When free ASPM is removed by cysteine before the addition of P_i (fig. 5C), the P_i uptake is still active to about 60% as compared to the uptake without ASPM (fig. 5D). In the opposite case, when ASPM can react in the presence of P_i (fig. 5B) and the cysteine is added, only 30% of the activity remains. In order to obtain nearly complete inhibition, ASPM must be present during the P_i influx, as shown in figs. 5A and 4C.

These results show that the position of the SH group of the P_i carrier, as assayed by the accessibility to ASPM, varies depending on several parameters. In the absence of added P_i, the carrier SH groups are directed mainly to the inside. Therefore an impermeable SH reagent such as ASPM cannot react with the SH group protected by the membrane barrier. On addition of P_i, the carriers are distributed at least partially towards the outside, so that ASPM can now react with a larger ratio of the SH groups. During the active P_i influx, apparently all the SH groups of the P_i carrier become available at the external position for reaction with ASPM. This situation may be summarized briefly as follows:

$[P_i]_e = 0 \quad C_e/C_i = 0, \quad$ ASPM noninhibiting

$[P_i]_e > 0 \quad C_e/C_i > 0, \quad$ ASPM inhibiting

C = carrier, e = external, i = internal

This interpretation of the results indicates that the P_i carrier can be regarded as a mobile carrier which can move its reactive site during the transport to both sides of the inner mitochondrial membrane. In the steady state, the distribution of the carrier at the inner or outer localization appears to vary according to the direction of the P_i flux.

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