PHOSPHATE TRANSPORT ACROSS THE MITOCHONDRIAL MEMBRANE: THE INFLUENCE OF THIOL OXIDATION AND OF Mg^{++} ON INHIBITION BY MERCURIALS

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1. Introduction

In a recent exhaustive analysis of the reactivity of -SH reagents with the mitochondrial 'phosphate carrier', Klingenberg et al. [1] reported that P_i protects against inhibition by maleimide derivatives. The same authors also reported that endogenous P_i protects better than exogenous P_i. Unlike monothiol reagents (maleimides, mercurials etc.), diamide (diazenedicarboxylic acid bis dimethylamide), a thiol oxidizing agent, does not inhibit P_i transport across the mitochondrial membrane, but facilitates it [2]. Moreover, diamide induced swelling of rat liver mitochondria suspended in medium containing P_i is prevented by Mg^{++} [3].

In the light of these observations it appeared interesting to investigate whether diamide, on one hand, and Mg^{++}, on the other, could interfere with the action of mercurials on P_i transport.

2. Experimental

Rat liver mitochondria were isolated in 0.25 M sucrose following Schneider and Hogeboom [4]. Mitochondrial protein was determined by a biuret method. $^{32}P_i$ uptake was determined as described by Papa [5]. The P_i efflux from mitochondria was followed with the swelling method, based on the generation of P_i in the mitochondrial matrix by the FCCP stimulated ATP hydrolysis as described by Klingenberg et al. [1].

For the direct measurement of P_i efflux, ^{32}P generated in the FCCP $[\gamma^{32}P]ATP$ system was

determined both in the supernatant and in the pellet following the procedure of Ernster et al. [6]. $^{32}P_i$ was counted by Packard Tri-Carb Spectrophotometer.

3. Results

As shown in fig.1 diamide did not affect mersalyl inhibition of P_i transport. Likewise diamide did not
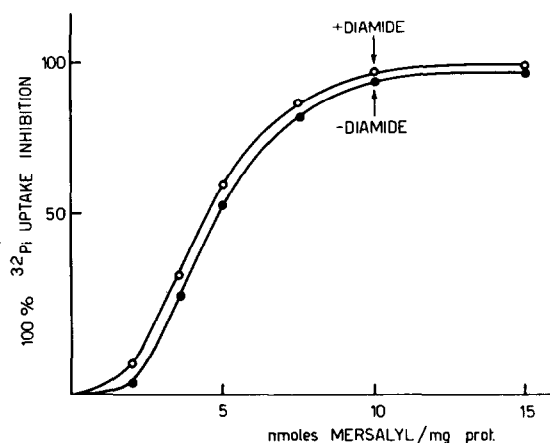

Fig.1. Dependence of $^{32}P_i$ uptake inhibition on amounts of mersalyl in the presence and absence of diamide. Rat liver mitochondria (5 mg protein/ml) were preincubated 2 min at $2°C$ in 125 mM KCl, 20 mM Tris–HCl (pH 6.55), 3 $\mu g/ml$ rotenone, 0.5 $\mu g/ml$ antimycin A and 15 $\mu g/ml$ oligomycin. When present diamide was 80 nmol/mg protein. Mersalyl was then added, followed 1 min later by 0.5 $\mu mol/ml$ $^{32}P_i$ (110 000 cpm). After 30 sec, mitochondria were rapidly centrifuged and $^{32}P_i$ was determined both in the mitochondrial extract and in the supernatant.

Table 1
Action of mersalyl and Mg^{++} on the uptake of $^{32}P_i$ by rat
liver mitochondrial

| | $^{32}P_i$ uptake | |
	CPM/mg protein	percent
Control	626	4.4
Control + Mg^{++}	625	4.4
Mersalyl	224	1.6
Mersalyl + Mg^{++}	220	1.6

Experimental conditions as in fig.1. When added, mersalyl
was 200 μM, and Mg^{++} 4 mM. 0.50 μmol/ml $^{32}P_i$ (70 000
cpm) were added.

decrease the minimum amount of mersalyl required
for complete inhibition of P_i transport.

Table 1 shows that Mg^{++} did not affect mersalyl
inhibition of $^{32}P_i$ uptake from the medium. On the
contrary, Mg^{++} abolished mersalyl inhibition of P_i
efflux from mitochondria assayed with the 'ATP-FCCP'
system (fig.2.). This effect was also observed in the
presence of higher amounts of mersalyl (up to 2 mM).
Diamide was uneffective on the inhibition of mersalyl
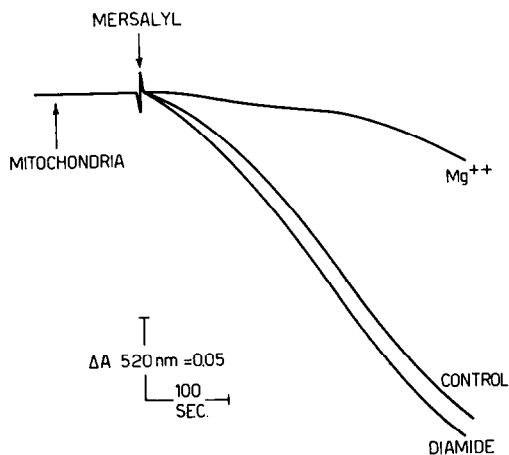of P_i efflux (fig.2).

Fig.2. Reversal of the inhibition by mersalyl of P_i efflux as
recorded by the 'FCCP-ATP system'. 2.5 mg/ml mitochon-
drial protein were added to a medium containing 250 mM
Sucrose, 20 mM Tris–HCl, pH 7.4, 1 mM ATP, 1 μM FCCP.
200 μM mersalyl was added. When present Mg^{++} was 4 mM
and diamide 80 nmol/mg protein. The changes in absorbance
are recorded in an Aminco–Chance Spectrophotometer at
520 nm with a scale extension of A = 0.5 cm^{-1}.

That the suppression of mersalyl induced swelling
by Mg^{++} was really due to the restored efflux of P_i
from mitochondria and not, for instance, to the inhibi-
tion of the uncoupler stimulated ATPase activity,
which generates P_i intramitochondrially, or to the
precipitation of phosphate salts inside the
mitochondrion, is confirmed by the results of table 2.

These results show that: 1) mersalyl, both in the
presence and in the absence of Mg^{++} did not decrease
the FCCP induced ATPase activity, which, on the
contrary, was increased (compare the amount of total
$^{32}P_i$ hydrolyzed from $[\gamma^{32}P]ATP$); 2) in spite of the
increased ATPase activity, mersalyl strongly prevented
the accumulation of $^{32}P_i$ in the supernatant; 3) when
Mg^{++} was present, much more $^{32}P_i$ was found in the
supernatant and correspondingly less in the pellet.
Consequently it appears that Mg^{++} restores mersalyl-
blocked efflux of P_i by preventing, or antagonizing,
the action of mersalyl, and not as a consequence of
the possible above-mentioned secondary effect.

4. Discussion

The action of mersalyl, as well as other thiol
reagents, on P_i transport through the inner mitochon-
drial membrane is generally interpreted as a conse-
quence of an inhibition of a more or less specific
'phosphate translocator', [7–9]. Fonyo [10] has
calculated that the amount of mersalyl that causes
total inhibition of phosphate translocation minus the
amount of mersalyl that is not inhibitory at all, equals
about 2–3 nmol/mg protein. The above reported find-
ing that diamide does not decrease the minimal amount

Table 2
Action of mersalyl and Mg^{++} on the efflux of $^{32}P_i$ formed
inside the mitochondria from ^{32}P ATP

| | $^{32}P_i$ cpm/mg protein | | |
	Supernatant	Pellet	Total
Control	2080	49	2129
+ Mersalyl	1433	1514	2947
+ Mersalyl + Mg^{++}	2785	159	2934

Rat liver mitochondria (2.5 mg/ml) were incubated 5 min at
20°C in the medium described in fig. 2 containing 2.1 μmol/
ml $[\gamma^{32}P]ATP$ (49 000 cpm). When present mersalyl was
200 μM, Mg^{++} 4 mM.

of mersalyl required for the complete inhibition of P_i transport (fig.1) indicates that the population of thiols affected by diamide is not related to the thiols involved in the P_i transport. In a parallel study (manuscript in preparation), it has been found that, at the concentrations used in the present work, diamide oxidizes 25% of the total mitochondrial thiols. These oxidized thiols, in the light of diamide properties [11], should include all the solubilized thiols and pairs of membrane bound thiols, sterically accessible to diamide interaction. This observation is in full agreement with the previous one that diamide, per se, does not inhibit, but rather facilitates, P_i transport [2].

The observation that Mg^{++} prevents the inhibition by mersalyl of P_i efflux from mitochondria is in apparent contrast with the lack of effect on the inhibition by mersalyl on P_i influx (compare table 2 and 1). The interpretation of this finding is very difficult also in consideration of the circumstance that, unlike heart mitochondria, liver mitochondria do not seem capable of accumulating Mg^{++} [12]. On the other hand, data reported in table 2 rule out the possibility of a precipitation of Mg^{++} phosphate salts inside the mitochondria. For the time being, asymmetry of the mitochondrial membrane with respect to the steric position or to the function of the thiol groups involved in the phosphate translocation appears the most plausible hypothesis.

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