PRELIMINARY NOTE

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Effect of butyl malonate and mersalyl on anion-exchange reactions in rat-liver mitochondria

CHAPPELL AND CROFTS[1], CHAPPELL AND HAARHOFF[2], ROBINSON AND CHAPPELL[3] and CHAPPELL *et al.*[4] have proposed that the movement of anions across the mitochondrial membranes is mediated by specific translocators, presumably located in the inner membrane. Three different techniques have been used to demonstrate the presence of these translocators, *viz.*, the swelling of mitochondria in isotonic solutions of the ammonium salts of the anions[1,2], direct measurement of the uptake (or extrusion) of the anions by the mitochondria[5–7] and the study of the intramitochondrial reactions in which the anions take part[2,5,6,8,9].

As first shown by GAMBLE[10], the accumulation of anions by mitochondria is presumably in exchange for endogenous anions. CHAPPELL AND CROFTS[1], CHAPPELL AND HAARHOFF[2] and CHAPPELL *et al.*[4] have suggested that the specific translocators mediate an exchange–diffusion process. Thus phosphate exchanges with hydroxyl *via* a phosphate translocator[1,2], dicarboxylate with phosphate *via* a dicarboxylate translocator[1,2], tricarboxylate with malate *via* a tricarboxylate translocator[2–4], α-oxoglutarate with certain dicarboxylate ions *via* an α-oxoglutarate translocator[3–6], and aspartate with glutamate *via* an aspartate translocator[11]. Direct evidence that translocators act by an exchange–diffusion mechanism has been presented by PAPA *et al.*[12]. They studied the malonate-stimulated exit of α-oxoglutarate, formed from glutamate in arsenite-poisoned rat-liver mitochondria, and found that one molecule of malonate was taken up by the mitochondria for each molecule of α-oxoglutarate extruded.

According to CHAPPELL AND HAARHOFF[2], the dicarboxylate translocator catalyses not only a dicarboxylate–phosphate exchange, but also an exchange of dicarboxylate for dicarboxylate ions. For instance, during the oxidation of succinate by isolated mitochondria, the substrate anions are continually taken up in exchange for malate ions (formed from fumarate). However, the question arises of whether this direct exchange is brought about by the dicarboxylate translocator or not. In order to investigate this problem, we have studied the effect of butyl malonate and mersalyl on various anion-exchange reactions in rat-liver mitochondria.

Butyl malonate was introduced by ROBINSON AND CHAPPELL[3] as an inhibitor of the transport of dicarboxylate ions across the mitochondrial membrane. In Table I, the effect of this inhibitor on the extrusion of malate (present in freshly prepared mitochondria) is shown. When malate extrusion was induced by adding phosphate to the medium, butyl malonate inhibited. However, no inhibition occurred when the extrusion of malate was brought about by added malonate.

FONYO[14] and TYLER[15,16] have shown that sulphydryl-blocking reagents like mersalyl inhibit intramitochondrial reactions dependent on added phosphate. This is

TABLE I

THE EFFECT OF BUTYL MALONATE ON THE EXIT OF INTRAMITOCHONDRIAL MALATE INDUCED BY ADDED MALONATE OR P_i

Rat-liver mitochondria (11 mg protein) were preincubated at 20° for 30 sec in a medium containing 15 mM KCl, 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 3 μg rotenone, 0.6% ethanol, 20 mM sucrose and (when present) 5 mM butyl malonate. After this preincubation the exchange reaction was started by adding malonate or P_i (in the concentrations indicated in the Table). After the last addition the reaction volume was 1.8 ml. 1 min later, 1 ml of the reaction mixture was centrifuged for 40 sec in an Eppendorf microfuge at full speed. The supernatant was decanted and immediately acidified with $HClO_4$. At the same time the centrifuge tube was quickly blotted dry and the mitochondrial pellet also acidified with $HClO_4$. Malate was determined with citrate synthase, malate dehydrogenase, NAD^+ and acetyl-CoA as suggested by E. J. DAVIS (personal communication); this method is based on the method of OCHOA *et al.*[13] for assaying citrate synthase. $Malate_{in}$ and $Malate_{out}$ refer to the amounts of malate found in the mitochondrial pellet and the supernatant, respectively, after centrifuging 1 ml of the reaction mixture. $Malate_{total}$ is the sum.

Additions	$Malate_{in}$ (*nmoles*)	$Malate_{out}$ (*nmoles*)	$Malate_{total}$ (*nmoles*)
None	23.7	47.0	70.7
Malonate (1 mM)	12.1	66.7	78.8
Malonate (3 mM)	6.1	66.7	72.8
Malonate (10 mM)	4.1	67.6	71.7
Butyl malonate	27.8	44.7	72.5
Butyl malonate + malonate (1 mM)	13.7	59.8	73.5
Butyl malonate + malonate (3 mM)	6.1	63.5	69.6
Butyl malonate + malonate (10 mM)	4.1	65.3	69.4
P_i (10 mM)	4.1	66.3	70.4
Butyl malonate + P_i	22.8	49.7	72.5

TABLE II

THE EFFECT OF MERSALYL AND BUTYL MALONATE ON THE EXIT OF INTRAMITOCHONDRIAL P_i AND MALATE INDUCED BY ADDED MALONATE

Rat-liver mitochondria (10.6 mg protein) were preincubated at 20° for 30 sec in a medium containing 15 mM KCl, 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 3 μg rotenone, 0.8% ethanol, 20 mM sucrose and (when present) 16.5 nmoles mersalyl per mg protein and 5 mM butyl malonate. After this preincubation the exchange reaction was started by the addition of 10 mM malonate (or water in the control incubations). After the last addition the reaction volume was 1.3 ml. 1 min later 1 ml of the reaction mixture was centrifuged and treated as described in Table I. P_i was determined according to the method of WAHLER AND WOLLENBERGER[18].

Additions	$(P_i)_{in}$ (*nmoles*)	$(P_i)_{out}$ (*nmoles*)	$(P_i)_{total}$ (*nmoles*)	$Malate_{in}$ (*nmoles*)	$Malate_{out}$ (*nmoles*)	$Malate_{total}$ (*nmoles*)
None	112	157	269	14.5	48.5	63.0
Mersalyl	139	124	263	33.8	27.6	61.4
Malonate	83	200	283	3.0	65.1	68.1
Mersalyl + malonate	134	143	277	3.0	62.7	65.7
Butyl malonate	111	176	287	17.9	44.8	62.7
Butyl malonate + malonate	102	171	273	5.8	55.8	61.6

due to inhibition by these reagents of the uptake of phosphate[16,17]. Table II shows the effect of mersalyl on the extrusion of phosphate from rat-liver mitochondria. Mersalyl inhibited the phosphate extrusion brought about by added malonate. Butyl malonate also inhibited. In the absence of malonate, the extrusion of P_i was inhibited

in part by mersalyl (lines 1 and 2) but not by butyl malonate (lines 1 and 5). This sensitivity of the P_i–hydroxyl exchange to mersalyl and lack of sensitivity to butyl malonate was confirmed in other experiments (not shown). In the experiment of Table II, the exit of intramitochondrial malate was also measured. The malonate-induced extrusion of malate was insensitive to both butyl malonate (see Table I) and mersalyl. It should be noted that a considerable extrusion of malate occurred in the absence of added malonate, probably in exchange for the extramitochondrial phosphate always found in mitochondrial preparations (Table II, line 1). This malate extrusion was inhibited by mersalyl (Table II, line 2).

The P_i-dependent uptake of dicarboxylate ions[1,2,19] was also studied. In mitochondria preincubated with ADP and P_i in order to deplete them of malate (and other respiratory-substrate anions), the uptake of 2 mM L-[^{14}C]malate was about 80 % inhibited by mersalyl at a concentration of 32 nmoles/mg protein. This was to be expected, since under these conditions the added malate could enter the mitochondria only in exchange for intramitochondrial P_i.

These results show that at 20° dicarboxylate ions move across the mitochondrial membrane by at least two different mechanisms. In the one, dicarboxylate ion exchanges with phosphate; this process is inhibited by butyl malonate or mersalyl. In the second, dicarboxylate ion exchanges with dicarboxylate ion in a butyl malonate- or mersalyl-insensitive process. Furthermore the dicarboxylate–phosphate exchange may be distinguished from the phosphate–hydroxyl exchange in that the former is sensitive to butyl malonate and the latter not. An anion–proton symport as postulated by MITCHELL[20] does not readily explain the differential effects of the inhibitors on the anion-exchange reactions.

We have also studied the effect of butyl malonate and mersalyl on exchange reactions involving tricarboxylate and α-oxoglutarate ions. Neither butyl malonate nor mersalyl has any inhibitory effect on the α-oxoglutarate–dicarboxylate exchange[12] or on the tricarboxylate–malate exchange. It may be concluded that butyl malonate is not a specific inhibitor of the movement of dicarboxylate ions across the mitochondrial membrane; it inhibits only the exchange of dicarboxylate ions with phosphate ions.

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