NEW ASPECTS OF THE MECHANISM OF INORGANIC PHOSPHATE AND DICARBOXYLATE TRANSPORT IN MITOCHONDRIA

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Received 20 September 1974

1. Introduction

It is generally accepted that the transport of inorganic phosphate in mitochondria is mediated by two distinct translocating systems localized in the inner membrane [1–7]. The first mediates the exchange–diffusion of P_i with OH^- (phosphate carrier) and the second that of P_i with dicarboxylates (dicarboxylate carrier). The observation that $P_i - P_i$ exchange is inhibited when both *N*-ethylmaleimide (NEM) and butylmalonate (BM) are present [4,7], together with the finding that dicarboxylate–dicarboxylate or P_i–dicarboxylate exchanges are not inhibited by NEM [8,9], are considered as main evidence that the two carriers are well distinct functional entities. We have however, found that butymalonate interfers with the mechanism of action of NEM since it removes or prevents in intact mitochondria the extrabinding of P_i induced by NEM [10]. These findings have prompted us to reinvestigate the effect of NEM and BM on the transport of P_i and dicarboxylic acids in rat-liver mitochondria. The results reported in this paper indicate that the mitochondrial membrane contains only one transporting system which, depending on the prevailing conditions, mediates either P_i–OH^- or P_i–dicarboxylate exchange.

2. Methods

Rat-liver mitochondria were loaded with P_i, malate or succinate by preincubation for 10 min at 4° C in the presence of 250 mM sucrose, 5 μg/ml oligomycin, 0.34 μg/ml rotenone, 0.17 μg/ml antimycin and one of these anionic substrates at a 2 mM concentration. After washing, mitochondria were suspended in cold sucrose containing 10 μCi carrier free $^{32}P_i$, [^{14}C] malate or [^{14}C] succinate. Loaded mitochondria were incubated in a medium containing; 200 mM sucrose; 20 mM Tris–HCl; oligomycin; rotenone and antimycin at the concentrations indicated above. The pH was adjusted to 7.2. At the time specified in the legends nigericin, butylmalonate, *N*-ethylmaleimide and the counter-anions were added. Incubation was carried out at 4°C directly in small centrifuge tubes in a total vol of 1 ml and mitochondria separated from the suspending medium by rapid centrifugation at 20 000 *g*.

Before incubation P_i was determined chemically [11] and malate enzymatically [12]. Their specific activity was calculated by measuring the corresponding total radioactivity of 12% $HClO_4$ extracts of loaded mitochondria. P_i, malate and [^{14}C] succinate content of mitochondrial pellet was corrected for that present in the sucrose space.

3. Results

The sensitivity of the P_i carrier to NEM and BM was studied by following the efflux of P_i from mitochondria induced by the addition of nigericin [6]. Fig.1 shows that NEM at 200 μM fully inhibited the P_i–OH^- exchange whilst BM, even at a very high concentration as 5 mM (not shown), had no effect at all. However when these two compounds were added together different results, according to the sequence of additions and NEM concentration, were obtained. BM, added after NEM, increased the inhibition of P_i efflux

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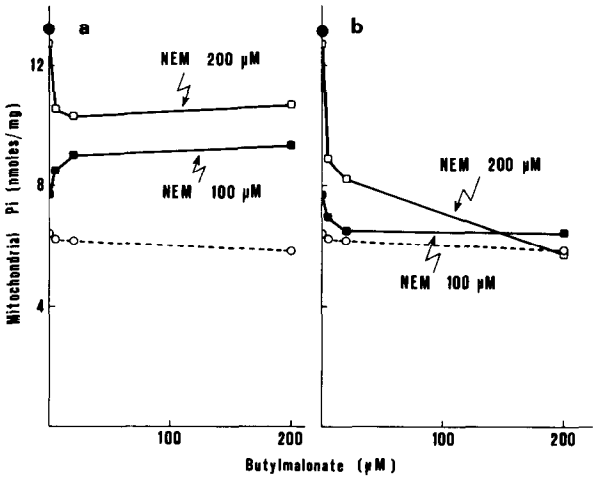

Fig. 1. Effect of BM on the inhibition of P_i transport by NEM. P_i-loaded mitochondria (3.5 mg/ml) were incubated in standard medium as described. Sequence of additions in expt. (a): (○——○) zero time, mitochondria; at 2 min, BM as indicated; at 6 min. 0.14 μM nigericin and reaction stopped at 8 min. In curves (■——■) and (□——□), at 2 min respectively 100 or 200 μM NEM; at 6 min, BM; at 10 min, nigericin and reaction stopped at 12 min. In expt. (b) the sequence of additions was as in expt. (a) with the difference that NEM was added at 6 min and Bm always at 2 min. (●), control whithout additions and stopped at 2 min.

brought about by 100 μM NEM and partially released that of 200 μM NEM (fig.1a). When added before NEM, butylmalonate completely restored the P_i efflux independently of the concentration of NEM (fig.1b). It can be noted that BM affects NEM inhibition at very low concentrations. In the experiment of fig.2 the effect of NEM an BM on the exchange between malate and succinate has been examined. In this case NEM had no effect and BM inhibited the efflux of malate promoted by the addition of succinate. NEM removed (fig.2b) or prevented (fig.2a) the inhibitory effect of BM. It appears that NEM is more effective in restoring malate efflux when added after than before BM.

Fig.3 shows that dicarboxylate–dicarboxylate exchanges are differentially affected by BM. The $malate_{in}$ → $malate_{out}$ and $succinate_{in}$ → $malate_{out}$ exchanges were not inhibited by BM (see also ref. [13]) neither by BM plus NEM. Identical results were obtained by adding phenylsuccinate instead of BM. $Malate_{in}$ → $succinate_{out}$ and $succinate_{in}$ → $succinate_{out}$ on the other hand, were inhibited by BM and the inhibition was fully or partially abolished by NEM. The efflux of P_i, induced by externally added P_i, was inhibited only when both NEM and BM were present whereas P_i efflux in exchange with malate was inhibited by BM (cf. also ref. [10]) and the further addition of NEM had practically no

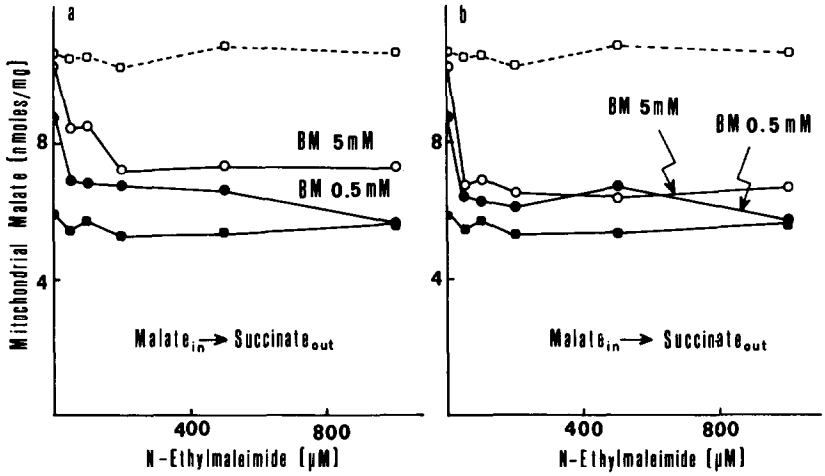

Fig.2. Effect of NEM on the inhibition of $malate_{in}$ → $succinate_{out}$ exchange by BM. Malate-loaded mitochondria (3.3 mg/ml) were incubated in standard medium as described. Sequence of additions in expt. (a): (□——□) zero time, mitochondria; at 2 min, NEM as indicated and reaction stopped at 6 min. In curve (■——■) at 6 min, 500 μM succinate and reaction stopped at 10 min. In curve (●——●) and (○——○), at 6 min respectively 0.5 and 5 mM BM; at 10 min, succinate and reaction stopped at 14 min. In expt. (b) the sequence of additions was as in expt. (a) with the difference that BM was added at 2 min and NEM at 6 min.

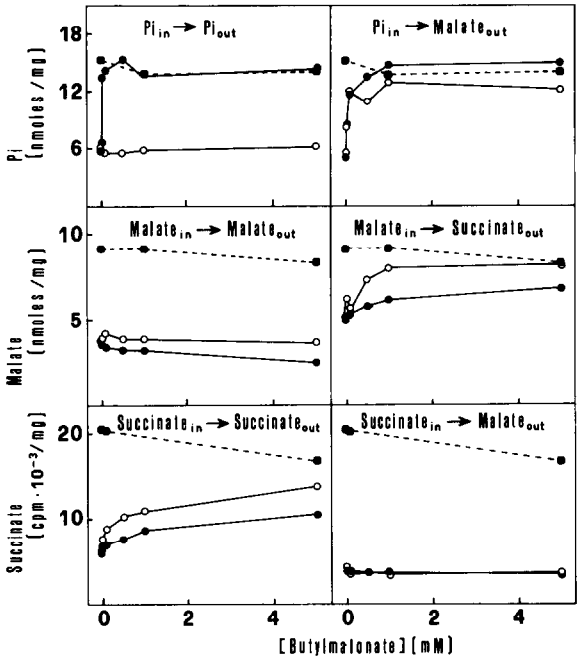

Fig.3. Effect of BM and NEM on P_i, malate and succinate efflux from mitochondria. Mitochondria (4.0 mg/ml) loaded respectively with P_i, malate or succinate were incubated in standard medium as described. Sequence of additions in control curves (■——■): zero time, mitochondria; at 2 min, BM as indicated and reaction stopped at 6 min. In curves (○——○) at 6 min, 1 mM counter-anion as specified and reaction stopped at 10 min. In curves (●——●) at 6 min, 200 μM NEM; at 10 min, counter-anion and reaction stopped at 14 min.

effect. In addition other experiments (not reported here) have shown that the transport of malonate in exchange with malate, succinate, P_i or malonate itself, was in any case inhibited by BM.

4. Discussion

The findings that P_i–P_i exchange was fully inhibited only when both BM and NEM were present, has been taken till now as an evidence in favour of the possibility that P_i penetrates the mitochondrial membrane by utilizing alternatively its own carrier or the dicarboxylate carrier. In agreement with this would also be the finding that the efflux of P_i in exchange with OH^- is inhibited by NEM but not by BM whilst the efflux of malate in exchange with succinate is inhibited by

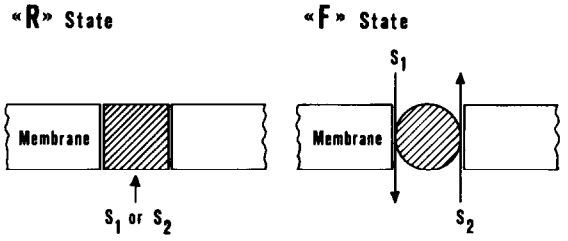

Fig. 4. Transition of the P_i carrier from resting (R) to functional (F) state. The carrier shifts into (F) state when a specific couple of substrates is present and the two substrates (S_1 and S_2) are localized one inside and the other outside mitochondrial membrane.

BM and insensitive to NEM. However the existence of two distinct functional carriers is inconsistent with the finding that the inhibitory effect of NEM on P_i efflux and that of BM on malate efflux, were both abolished or prevented by BM and NEM, respectively. Similar results were obtained studying the binding of P_i in intact mitochondria [10]. It has also been reported that dicarboxylate analogues protect the uptake of malonate and P_i against mersalyl inhibition [14]. These data would, on the other hand, suggest that there exists in the mitochondrial membrane only one system which mediates both P_i–OH^- and P_i–dicarboxylate exchanges.

Further support to this hypothesis is given by the expts. reported in fig.3 wich clearly show that the inhibitory effect of BM on P_i and dicarboxylate transport varied widely, depending both on the nature of the substrates involved and on their localization on the two sides of mitochondrial membrane. Indeed the concentration of BM to obtain half maximal inhibition ranged 50 μM for $P_{i\ in} \rightarrow malate_{out}$ exchange or 10 μM for $P_{i\ in} \rightarrow P_{i\ out}$ in the presence of NEM, to 500 μM for $succinate_{in} \rightarrow succinate_{out}$ exchange. The inhibitory effect of BM on the efflux of intramitochondrial succinate and malate disappeared on changing the external counter-anion. In this regard very interesting was the finding that malate–succinate exchange was or was not sensitive to BM, just by changing the relative position of the two substrates in the extra- and in the intra-mitochondrial phase. Thus it is unlikely that the transport of malate and succinate can be mediated by different translocating systems wich are differentially sensitive to BM [2,8]. This seems also to be excluded

by the finding that the inhibition by BM of malate and succinate efflux was removed or prevented by NEM and that dicarboxylate analogues at concentrations even lower than those used in expts. of fig.3, inhibited also tricarboxylate and oxoglutarate carriers [15]. It is well established that P_i moves across the mitochondrial membrane only when the side of the membrane opposite to that occupied by P_i, is exposed to specific substrates or the OH^- concentration is raised. This indicates that the translocating system can be considered to be in 'resting state' (R) when it is bound to its subtrates on only one side of the membrane and is in the 'functional state' (F) when it is bound on both sides to a specific couple of substrates, namely P_i–OH^-, P_i–P_i, P_i– dicarboxylate or dicarboxylate–dicarboxylate. The different activity elicited by the carrier in translocating P_i, malate, succinate and malonate [13,16,17] may be ascribed to different conformational changes induced by these substrates. In swelling experiments it has been found that malonate increased whereas succinate protected against the inhibition of P_i transport by a NEM analogue [18]. On the other hand recently it has been shown that phenyl phosphate and phenylphosphonate inhibit competitively succinate oxidation but they do not influence P_i-stimulated swelling of mitochondria in NH_4Cl and inhibit in a non competitive manner P_i-stimulated swelling in ammonium malate [19]. The finding reported by Robinson and Williams [13] that the malate–malonate exchange has different K_m's and different initial rates, according to the relative position of the two substrates on the two sides of the mitochondrial membrane, could be interpreted on the basis of the mechanism proposed. A conformational change of the carrier is also suggested by the finding that the inhibition of P_i efflux by NEM which is firmly bound to the membrane, is removed by the further addition of BM and that NEM restoring malate efflux is more effective when added after than before BM. The effect of SH reagents and dicarboxylate analogues may consist in counteracting directly or indirectly the transition of the carrier and since this is substrate-dependent a differential effect of these inhibitors, according to the substrates used, can be found.

Acknowledgements

The technical assistance of L. Gargano is gratefully acknowledged.

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