CONFORMATIVE RESPONSE OF THE MITOCHONDRIAL P_i -DICARBOXYLATE TRANSPORT SYSTEM TO INHIBITORS AND SUBSTRATES

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1. Introduction

The distribution of inorganic phosphate between intra- and extra-mitochondrial space has been established to be governed by the ΔpH [1–3] and the Δ concentration of anionic substrates [1–5] imposed across the inner membrane by respiration and metabolic activity. The hypothesis that the transport of Pi is mediated by two well distinct translocating systems, is mainly based on the differential effect of two classes of inhibitors: SH reagents and dicarboxylate analogues [6–8]. Recently we have proposed and presented evidences [9–11] that the inner mitochondrial membrane contains only one carrier which, irrespective of the driving force, mediates either the P_i-OH^- or the P_i-dicarboxylate exchange–diffusion reactions. Kinetic studies carried out in presence and absence of *N*-ethylmaleimide (NEM), butylmalonate (BM) and bathophenanthroline (BP) further substantiate this hypothesis. The results reported in this paper indicate that the SH groups of the membrane are not directly involved in the transport process and that the functional state of the P_i-dicarboxylate translocator is promoted by the binding of specific couples of complementary substrates. Conformational change of the transport system appears to be a suitable mechanism to account for the stimulatory and inhibitory effect of NEM and for the finding that the effectiveness of non-competitive inhibition of BM is related to the nature of intra- and extra-mitochondrial substrates.

2. Materials and methods

The kinetic of P_i and dicarboxylates transport was determined by using the multi-layer centrifugation technique. Rat liver mitochondria loaded as previously described [10], with [^{14}C]malate, [^{14}C]succinate and $^{32}P_i$ were preincubated in a medium containing 120 mM sucrose, 65 mM choline-Cl, 25 mM Tris-HCl, oligomycin 5 μg/mg protein, rotenone 1 μg/ml antimycin 0.5 μg/ml and inhibitors as specified in legends of figs. and table. Aliquots of 0.3 ml mitochondrial suspension were then pipetted into plastic tubes with 4 mm in diameter, containing from bottom to top the following layers: (a) 50 μl of 'stop medium' containing 250 mM sucrose, 25 mM Tris-HCl and dextran 22.5 mg/ml; (b) 100 μl of 'incubation medium' containing 200 mM sucrose, 25 mM choline-Cl, 25 mM Tris-HCl, dextran 18 mg/ml, rotenone, oligomycin, antimycin, counter-anion or X-464 and inhibitors; (c) 80 μl of 'washing medium' containing 170 mM sucrose, 40 mM choline-Cl, 25 mM Tris-HCl, dextran 4.5 mg/ml, rotenone, oligomycin and antimycin. The pH of these mixtures was carefully checked and adjusted to 7.5. Mitochondria, through washing and incubation layers, were precipitated into stop medium centrifuging the tubes at 20 000 *g* for 4 min. The pellet was immediately rinsed and acidified with 12% $HClO_4$. $^{32}P_i$, [^{14}C]malate and [^{14}C]succinate content of mitochondrial pellet was determined as previously described [10]. In all the steps of this procedure the temperature

was kept constant at 4°C. Incubation time, given by the time-period in which the mitochondria were in contact with the incubation layer, was 7 sec. This was determined by measuring in exactly the same conditions above described, the reduction of cytochrome *c* added to the incubation layer. The reduction was promoted by the rotenone-insensitive NADH-cytochrome *c* reductase localized on the outer mitochondrial membrane [12].

3. Results

Exchange–diffusion reactions, occurring at the inner mitochondrial membrane, were studied by following the efflux of one substrate when loaded mitochondria were centrifuged through the incubation layer containing various concentrations of well known counter-anion. Together with the very short incubation time, two basic requirements were fulfilled with this procedure: reaction specificity and intactness of transport process. A typical experiment illustrating the malate $_{in} \rightarrow$ succinate$_{out}$ exchange, is reported in fig.1. It is shown that, the inhibition brought about by BM is of a purely non-competitive type, and that NEM stimulates the reaction giving a two time increase of the affinity of the counter-anion to the translocating system without affecting the V_{max}. BM inhibition is partially released when NEM is also added to the preincubation medium. This result, which can also be interpreted in the sense that BM prevents the stimulatory effect of NEM, is identical to the one obtained in equilibrium conditions [10]. As shown in fig.2 also BP inhibits the malate efflux

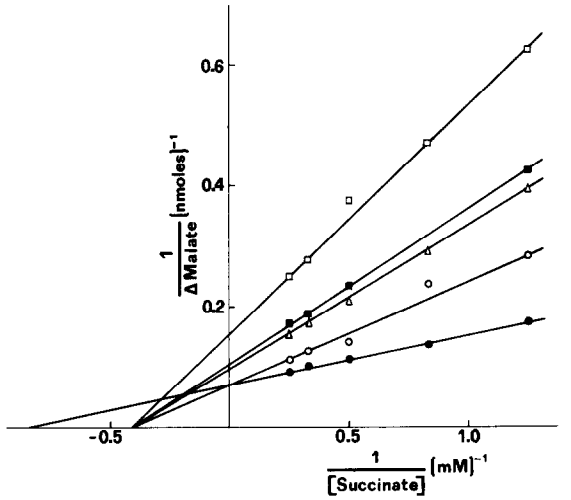

Fig.1. Effect of NEM and BM on the kinetic of malate efflux in exchange with external succinate. The efflux of malate from preloaded mitochondria was followed with the multi-layer centrifugation technique as described under Materials and methods. Succinate at the concentrations indicated was added to the incubation layer. Preincubation time and additions: (○——○) zero time mitochondria and centrifugation at 5 min; (●——●) at 5 min 0.5 mM NEM and centrifugation at 10 min; (△——△) and (□——□) at 5 min respectively 0.8 and 1.6 mM BM, centrifugation at 10 min; (▲——▲) at 5 min 0.8 mM BM, at 10 min NEM and centrifugation at 15 min. In the absence of counter-anion the malate content of matrix space relative to 3.3 mg protein, was 17 nmoles. Δ Malate was determined by subtracting the amount found in the presence of counter-anion from that in its absence.

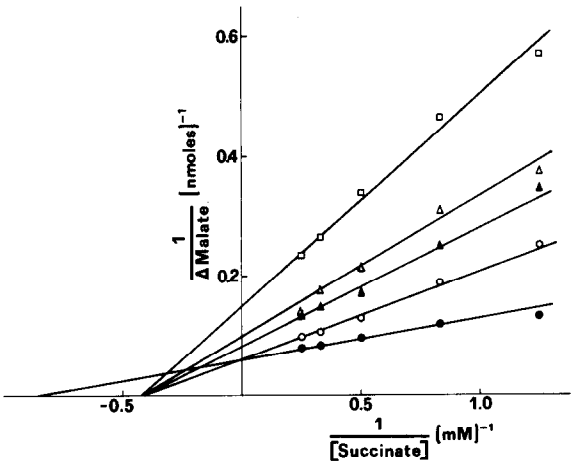

Fig.2. Effect of NEM and BP on the kinetic of malate efflux in exchange with external succinate. The efflux of malate was followed as described under Materials and methods and fig.1. Preincubation time and additions: (○——○) zero time mitochondria and centrifugation at 5 min: (●——●) at 5 min 0.5 mM NEM and centrifugation at 10 min; (△——△) and (□——□) at 5 min respectively 0.1 and 0.2 mM BP, centrifugation at 10 min; (■——■) at 5 min 0.2 mM BP, at 10 min NEM and centrifugation at 15 min. In the absence of counter-anion the malate content of matrix space relative to 3.0 mg protein, was 18 nmol.

Table 1
Effect of NEM and BM on the kinetic of exchange–diffusion reactions involving P_i and dicarboxylate anions

Exchange-diffusion Reactions	K_M (μM) Control	+ BM	+ NEM	K_i (μM) BM
$Malate_{in} \longrightarrow P_{i\ out}$	980	980	440	6
$Malate_{in} \longrightarrow Succinate_{out}$	2440	2440	1180	1240
$Malate_{in} \longrightarrow Malate_{out}$	340	340	150	10 800
$Succinate_{in} \longrightarrow Malate_{out}$	430	430	240	9000
$Succinate_{in} \longrightarrow P_{i\ out}$	1670	1670	–	3
$P_{i\ in} \longrightarrow Malate_{out}$	600	600	260	2
$P_{i\ in} \longrightarrow Succinate_{out}$	1670	1670	–	1
$P_{i\ in} \longrightarrow P_{i\ out}$	910	910	440	3200

Experimental conditions as described under Materials and methods and fig.1. The data reported, mean value of four separate mitochondrial preparations, were derived from intercept of lines using the method of least squares. Dixon plot was used for K_i determination.

in a purely non-competitive manner with K_i of 160 μM and the inhibition is released by NEM.

K_Ms of various exchange–diffusion reactions involving P_i and dicarboxylates together with the K_i values of BM, are reported in table 1. BM behaves for all the reactions studied as a non-competitive inhibitor and NEM as an activator. The K_i value of BM inhibition changes from 2 μM to 10.8 mM with malate as external substrate, from 1 μM to 1.2 mM with succinate, and from 3 μM to 3.2 mM with P_i. Similarly the efflux of malate, succinate and P_i have a differential sensitivity to BM inhibition with K_is ranging respectively from 6 μM to 10.8 mM, from 3 μM to 9 mM and from 1 μM to 3.2 mM. The relevance of these last results will be better realized if one consider that, due to the experimental procedure used, loaded mitochondria were in exactly the same conditions before coming in contact with different external substrates. The stimulatory effect of NEM appears to be independent of the nature of both internal and external substrates in that, for all the reactions studied, it promotes a one half reduction in the K_M value. Table 3 illustrates that also the K_Ms of externally added malate, succinate and P_i are influenced, in a lesser degree compared to the K_i values of BM, by the nature of internal counter-anions. The sensitivity of P_i-OH^-exchange to NEM and BM is reported in fig.3. Since the reaction rate [13] as well as NEM and BM effect [14,15] are pH dependent, the P_i-OH^- exchange was promoted by the presence of X-464 in the

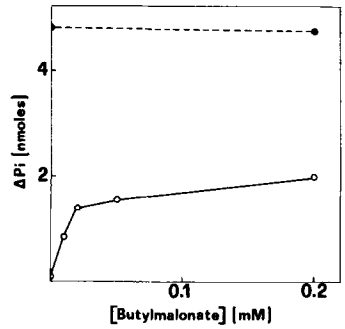

Fig.3. Effect of BM on the inhibition of Pi-OH^- exchange by NEM. The efflux of P_i from preloaded mitochondria was followed as described under Materials and methods with 1.4 μM X-464 added to the incubation layer. Pre-incubation time and additions: (●——●) at zero time mitochondria, at 5 min BM at the concentrations indicated and centrifugation at 10 min; (○——○) at 5 min 0.1 mM NEM, at 10 min BM and centrifugation at 15 min. In the absence of X-464 the P_i content of matrix space relative to 3.0 mg protein, was 27 nmol.

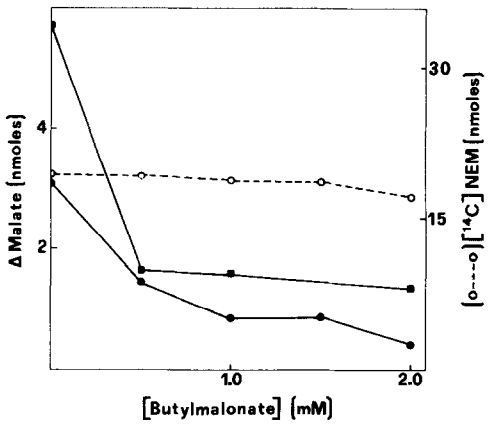

Fig.4. Effect of BM on the stimulation of malate$_{in}$→ succinate$_{out}$ exchange by NEM and on mitochondrial content of [^{14}C]NEM. The efflux of malate from preloaded mitochondria was followed as described under Materials and methods and fig.1, with 0.8 mM succinate added to the incubation layer. Preincubation time and additions: (•——•) zero time mitochondria, at 5 min BM at the concentrations indicated and centrifugation at 10 min; (▪——▪) at 5 min 0.5 mM NEM, at 10 min BM and centrifugation at 15 min; (○- - - -○) zero time mitochondria loaded with cold malate, at 5 min 0.5 mM [^{14}C]NEM, at 10 min BM and centrifugation at 15 min.

incubation layer. On the bases of physico-chemical properties Stempel et al [16] have presented evidences that X-464 and nigericin are identical. In control experiments, not reported here, we have indeed found that the two antibiotics behave identically in promoting the efflux of P_i and anionic substrates from mitochondria [3,5]. NEM at 100 μM fully inhibited the P_i-OH^-exchange and the inhibition could be removed by very low concentrations of BM added after NEM. BM per se had no effect on this reaction. Fig.4 shows that while the mitochondrial content of [^{14}C]NEM is not affected by increasing concentrations of BM up to 200 μM, the stimulatory effect of NEM on malate$_{in}$→ succinate$_{out}$ exchange, is completely removed. Results identical to those reported in figs.3 and 4 were obtained when BM was added before NEM (see also fig.1).

4. Discussion

The data reported give conclusive evidences that the differential effect of NEM and BM, described by many authors on P_i transport cannot be accounted for the existence of two catalytically separated components of mitochondrial membrane. This is mainly based on the findings that both in equilibrium [10] and kinetic conditions, the inhibition of P_i-OH^- exchange brought about by NEM is removed or prevented by very low concentrations of BM and that BM inhibition of malate–succinate exchange, as well as of all the reactions reported in table 1, is removed or prevented by NEM. The non-competitive inhibition of BM and the activation of NEM in that they are common to all the possible exchange–diffusion reactions involving P_i and dicarboxylates, give further support to the hypothesis that the translocation of these substrates is mediated by only one system. On the other hand the stimulatory effect of NEM and the purely non-competitive inhibition brought about by BM and BP together with the finding that externally added substrates may have different K_M values, according to the nature of the counter-anion, give substantial support to the mechanism proposed earlier of a conformative response of the translocating system to inhibitors and substrates (see [10]). Experiments on the product inhibition of the malate–malonate and malate–oxoglutarate exchange reactions in rat heart mitochondria [17], have given direct information on the decisive role, for the activity of the translocator, worked out by the relative concentrations of the two substrates on one side of the membrane. Recently reported additional studies have prompted Sluse and co-workers [18] to favour the possibility of the existence of one oxoglutarate translocator with 'local configuration changes' induced by the substrates in alternative to the possibility of 'several translocator species'. The inhibitory effect of NEM on the P_i-OH^- exchange may be considered as the expression of an inactive conformational state of the translocator which can be converted into an active state by the subsequent binding of P_i or malate on the external side of the membrane (compare fig.3 and table 1). In line with the model proposed is also the finding that the K_i of BM is directly related to the nature of complementary substrates involved in the transport process as well as to their relative position on the two sides of the mitochondrial membrane. The net contrast between the results here reported and that

showing the competitive nature of BM and BP inhibition [19,20] could be ascribed to a different response of the translocator to substantial different experimental conditions and analytical procedure. Indeed it has been found that the cation composition and the pH value of incubation mixture may affect the translocation of anionic substrates [21]. Mersalyl inhibition of malonate uptake can be shifted from a competitive to a non-competitive one, preincubating the mitochondrial suspension with the inhibitor [22]. The experiment of fig.4 give direct evidence that the effect of NEM consists in promoting a positive modification of the translocating unit which can be removed without affecting the binding of [^{14}C]NEM to the membrane. Related to this is the finding that the inhibitory effect of BM and BP which would likely react respectively with different binding sites, is removed or prevented by a thiol reagent. These observations would suggest that the SH groups are not directly involved in the transport process and could favour the possibility that NEM, as well as BM and BP may interact with other components of the membrane in which the P_i-dicarboxylate translocator is embedded. The data reported may have relevant implications for further investigations to clarify the molecular aspects of P_i and dicarboxylate transport in mitochondria.

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