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BINDING OF MALONATE TO THE INNER MEMBRANE OF RAT LIVER MITOCHONDRIA

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SUMMARY

The binding of malonate to the external face of the mitochondrial inner membrane has been investigated by using mitoplasts and on the opposite face, by using inside-out oriented vesicles prepared from sonicated mitoplasts. The external face of the inner membrane displays a single class of binding sites whereas two classes are observed in vesicles. In trypsin treated vesicles, only high-affinity sites are evidenced. The disappearance of the low affinity sites is correlatively related with the loss of succinate dehydrogenase activity. Both high-affinity sites of mitoplasts and vesicles are sensitive to the presence of malate ; they are also masked by 2-butylmalonate, phosphate, citrate and mercurials. Our results suggest that the internal and external high-affinity sites of the inner membrane are involved in the dicarboxylate transport system.

Many reports deal with the existence of a dicarboxylate transport system localized in the inner membrane of mitochondria which is able to mediate the exchange-diffusion of phosphate and certain dicarboxylates against phosphate or the same or another dicarboxylate (1,2). Except a few data relative to kinetics of dicarboxylate exchange in intact mitochondria (3,4) or mitoplasts (5), very little is known about molecular mechanisms and the chemical structure of the dicarboxylate carrier.

The comparison of the specific dicarboxylate binding sites on both sides of the inner membrane is very interesting to study forward the molecular mechanisms of the transport system. Unfortunately because of the impossibility to obtain a homogenous population of membrane vesicles oriented like mitochondria, we studied separately the outer binding sites on mitoplasts and the inner ones on the inside-out oriented vesicles.

This paper gives the results of Scatchard plots of malonate binding experiments carried out in the presence of various effec-

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tors. It is concluded that high-affinity malonate binding sites belong to the dicarboxylate transport system.

METHODS

Mitoplasts for the preparation of vesicles were obtained by the digitonin fractionation procedure of Schnaitman et al (6). The release of the outer membrane was followed by electron microscopy and by the disappearance of kynurenine hydroxylase activity (7). Mitoplasts for binding experiments were specially prepared to eliminate the dicarboxylate exchange. This was achieved by a 15 min incubation of mitochondria at 15°C in a medium containing 170 mM mannitol, 70 mM sucrose, 20 mM KCl, 10 mM HEPES at pH 7.0 supplemented with 1 mM phosphate to eliminate exchangeable dicarboxylates. After sedimentation, mitochondria were incubated in the same medium in the presence of hexokinase, glucose and 2 mM $MgCl_2$ at 18°C for 15 min to obtain a very low phosphate level before being converted to mitoplasts. These were kept overnight at 4°C and washed again in a large volume of the same medium before use.

Inner membrane vesicles were prepared by sonication of mitoplasts adjusted to 20 mg of protein per ml, in a hypotonic medium containing 120 mM sucrose, 5 mM HEPES pH 7.2. Unbroken mitoplasts were spun down at 15,000 x g for 10 min. The supernatant was centrifuged at 105,000 x g for 45 min and the pellet resuspended in 240 mM sucrose, 10 mM HEPES pH 7.2. The polarity of the inner membrane vesicles was assessed by three different tests : (i) retention of vesicles on a Sepharose cytochrome c column (8), (ii) determination of the rate of inhibition by antimycin of the reduction of ferricyanide by succinate (9), (iii) ratio of the ATPase activity of the vesicles prepared by sonication in a medium containing 1 mM ADP, measured in the presence or absence of atractyloside. The results indicate a turning inside-out yield of about 90 %. All results were expressed with respect to the cytochrome a content of the inner membrane, determined according to the method of Rieske (10). [2-^{14}C] malonic acid was obtained from Amersham.

RESULTS

It is important for the study of dicarboxylate binding in mitoplasts that the translocation of this molecule into the matrix space must be kept as low as possible. This was realized by the special treatment described in methods section. In these conditions, the measurement of bound malonate was made by displacing it with butylmalonate, a competitive and non penetrating inhibitor (11). The results are given in Fig. 1. The Scatchard plot shows a single class of sites sensitive to 2-butylmalonate (0.20 nmol sites $\cdot$ nmol^{-1} cytochrome a, K_D : 3.8 μM).

In vesicles, the saturation curve for the binding of malonate presents an intermediary plateau and a maximum (not shown). When the results are expressed according to a Scatchard plot, a bipha-

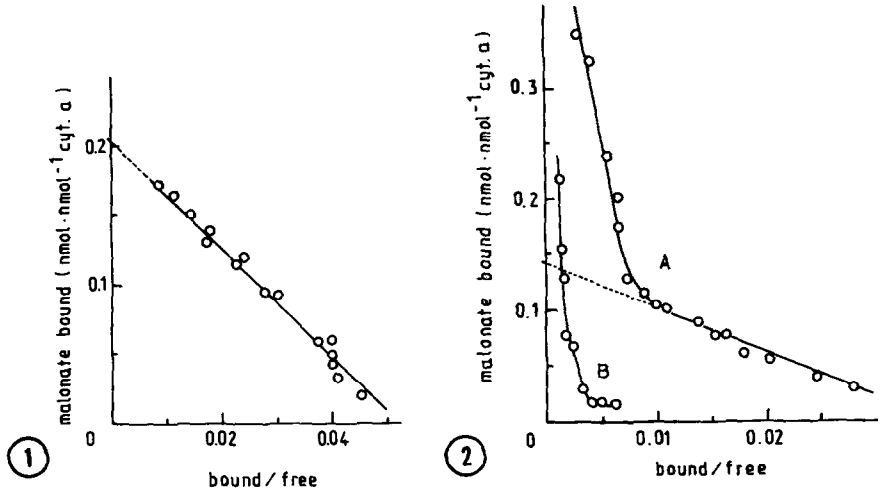

Fig. 1 Scatchard plot of the binding of malonate to mitoplasts. Mitoplasts (3 mg of protein), were incubated in 0.63 ml of a medium containing 170 mM mannitol, 70 mM sucrose, 20 mM KCl, 10 mM HEPES at pH 7.0 and 18°C in the presence of 0.3 µg antimycin, 2 µg rotenone and 1.5 µg oligomycin with increasing concentrations of [^{14}C] malonate (specific activity : 19.5 mCi · mmol^{-1}). After the equilibrium state was reached (4 min), bound malonate was measured by the difference of the incorporated radioactivity between two assays supplemented either with 50 µl of buffer or with 50 µl of 2-butylmalonate (13 mM final concentration). Mitoplasts were centrifuged in a microcentrifuge and the radioactivity was counted in the pellet and in the supernatant. Controls were made to verify that the level of dicarboxylate exchange kept low.

Fig. 2 Scatchard plots of the binding of malonate to sonic inner membrane vesicles in the absence (A) or in presence (B) of 13 mM 2-butylmalonate. Vesicles (1.3 mg of protein) were equilibrated in 0.15 ml of 190 mM sucrose, 10 mM HEPES, 3 mM $MgCl_2$, 20 mM KCl, pH 7.0 at 18°C for 4 min, in the presence of 0.3 µg antimycin, 2 µg rotenone and 1.5 µg oligomycin, with various concentrations of [^{14}C] malonate, then centrifuged at 105,000 x g for 8 min in a air-driven Beckman Ultracentrifuge and the radioactivity was counted in the pellet and the supernatant. Correction was made for the radioactivity contained in sediment space available to [^{14}C] sucrose.

sic curve is observed indicating two types of sites, with different affinities, Fig. 2A. The high-affinity binding sites have a dissociation constant of 3.9 µM and are present at approximately 0.14 nmol per nmol cytochrome a. The low-affinity sites are present in a large excess and have a dissociation constant of 90 µM. In the presence of 13 mM 2-butylmalonate, we observe (Fig. 2B) the disappearance of the high-affinity binding sites.

As indicated under methods, the vesicles are essentially oriented inside-out and consequently succinate dehydrogenase which is found on the external side of the membrane can fix malonate. By a

mild treatment with trypsin, the vesicles lose about 85 % of their succinate dehydrogenase activity ; however they can still bind malonate. In these conditions, only high-affinity sites are observed as shown in Fig. 3A with 0.14 nmol sites · $nmol^{-1}$ cytochrome a and a K_D value of 12.4 µM. The addition of 2-butylmalonate before the ligand as well as at the end of the incubation period, bring the labelling back to a very low level (Fig. 3, B curves). This shows that butylmalonate displaces the ligand from its membrane site and that no accumulation of malonate occurs into the vesicles.

Since malate, phosphate, citrate and mercurials are respectively substrates and inhibitors of the dicarboxylate transport (12), they should affect the binding of malonate on both sides of the inner membrane. This hypothesis is confirmed by the results shown in Table I.

DISCUSSION

The study of the binding of malonate to inside-out oriented vesicles reveals two types of sites which can be essentially attributed to the matrix side of the inner membrane. The low-affinity sites have been identified as those of succinate dehydrogenase and

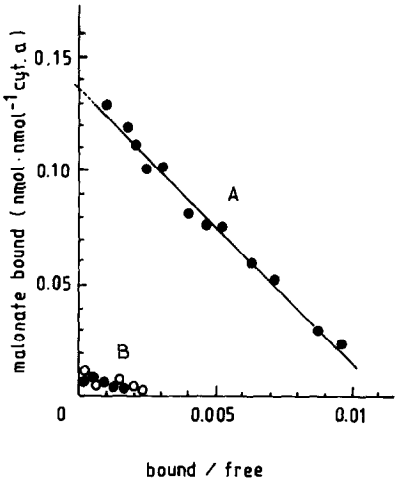

Fig. 3 (A) Scatchard plot of the binding of malonate to trypsin treated vesicles. Inner membrane particles (6 mg of protein per ml) were incubated in 240 mM sucrose, 10 mM HEPES pH 7.2 with 0.1 mg trypsin per mg of protein for 15 min at 20°C, then centrifuged at 105,000 x g for 45 min. Treated trypsin vesicles were incubated with [^{14}C] malonate as in the legend to Fig. 2. (B) the same experiments were carried out in the presence of 13 mM 2-butylmalonate added prior to the addition of malonate (●-●) or 3 min after (o-o).

Table I. Effect of various metabolites and mercuriphenylsulfonate on the binding of [^{14}C] malonate to inner membrane. Experimental conditions were the same as in Fig. 1 for mitoplasts and as Fig. 2 for vesicles.

Additions	Inhibition (%) of the maximum number of the high-affinity binding sites	
	Mitoplasts	Vesicles
Citrate 0.8 mM	20	50
Phosphate 0.8mM	40	55
Malate 0.8 mM	100	90
p-chloromercuriphenyl-sulfonate 0.4 mM	95	95

can be removed by a mild trypsin treatment. The comparison of the high-affinity sites of vesicles with those of mitoplasts show that they exist in approximately by the same amount and have very close dissociation constant values. Moreover, high-affinity sites of both mitoplasts and vesicles are modified in presence of the effectors which inhibit or are competitive with dicarboxylate transport in intact mitochondria. Malonate was preferentially used in this study for its weak affinity with the citrate and oxoglutarate carriers (13,14) and because it is not readily metabolized.

In order to eliminate possibilities of breakage or leakage of mitoplast membranes and therefore that the titrated sites were not those of the inner face, several controls were made. The conclusions are the following : (i) the mitoplasts as well as the vesicles showed an inaccessible sucrose space (ii) more than 75 % of succinate dehydrogenase activity of the mitoplasts measured in the presence of ferricyanide were inhibited by antimycin (9) (iii) the specific activity of malate dehydrogenase in the mitoplasts is about 3/4 of that in intact mitochondria. If we take into account these results, assuming that a maximum of 30 % of the mitoplasts are open or leaky, the amount of sites titrated on the external face of the membrane become exactly equal to those titrated in inverted vesicles i.e. 14 nmol sites $\cdot$ nmol^{-1} cytochrome a. These are, of course, the results expected for a transport system. However, other findings will be needed to specify whether the sites titrated on each side of the inner membrane are the same or are independent.

In conclusion, our results support evidence that the titrated sites which we find to be in equal amount on both faces of the

inner membrane, belong to the dicarboxylate transport system. They show that butylmalonate and mercurials are effective on both sides and as far as the carrier is actually a protein it is deeply embedded in the membrane since the binding of malonate is not affected after treatment by a protease.

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