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Purification of the rat-liver mitochondrial dicarboxylate carrier by affinity chromatography on immobilized malate dehydrogenase

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Abstract

The dicarboxylate carrier of rat-liver mitochondria, extracted by Triton X-100 and partially purified by hydroxylapatite chromatography, was retained by malate dehydrogenase immobilized on Sepharose gel, and eluted with 0.4 M NaCl. SDS-polyacrylamide gel electrophoresis of the eluate showed a predominant peptide band with an M_r of 28000. The purified protein, incorporated into liposomes, mediated a butylmalonate sensitive $malonate_{out}/malate_{in}$ exchange that was inhibited by *p*-chloromercuriphenylsulfonate. Sulfate, malate and phosphate decreased the rate of exchange. The highly purified protein displayed all the properties of the dicarboxylate carrier. Moreover, the results suggest a possible functional interaction between mitochondrial carrier protein and malate dehydrogenase.

Key words: Mitochondrion; Dicarboxylate carrier; Purification; Affinity chromatography; Malate dehydrogenase; (Rat liver)

1. Introduction

For the last few years the topics related to the studies of mitochondrial inner-membrane carriers have deviated towards problems of structure determinations, membrane topology and molecular mechanism of the transport. Difficulties to obtain highly purified transport proteins in mitochondria have two origins: (i) transporters share the same physicochemical properties such as hydrophobicity and their very similar molecular masses of their subunits, (ii) they exist in very small amounts. Among mitochondrial transport systems (for a review, see La Noue et al. [1]), the ADP/ATP [2], the P_i [3] and the 2-oxoglutarate [4] carriers are at present the most completely studied.

The dicarboxylate carrier has been partially purified and reconstituted from rat liver mitochondria by Saint-Macary et al. [5] and Kaplan et al. [6]. It has been also obtained in a more highly purified and active form and completely purified but in inactive form by Bisaccia et al. [7]. On the other hand, the dicarboxylate carrier has been purified from beef-liver mitochondria on immobilized *p*-aminophenylsuccinate by Szewczyk et al. [8] and by Nalecz et al. on Cibacron-blue gel [9]. This latter method was unsuccessful with rat-liver mitochondria in our laboratory.

Brent and Srere [10] have previously reported that citrate synthase and several other enzymes of the mitochondrial matrix share the property of binding to the inner membrane. Using these results, they have partially purified the citrate transporter by affinity chromatography on immobilized citrate synthase [11]. One of the enzymes bound to the inner membrane is malate dehydrogenase. This report deals with a new method of purification of the mitochondrial rat-liver dicarboxylate carrier to homogeneity, in a fully active form by means of affinity chromatography on immobilized malate dehydrogenase.

2. Materials and methods

0.5 g of CNBr-activated Sepharose CL-4B (Pharmacia) was washed with 50 ml of 1 mM HCl on a sintered-glass filter. The gel was suspended into 1.7 ml of 0.1 M $NaHCO_3$ (pH 9) in the presence of 3 ml of malate dehydrogenase in ammonium sulfate (Boehringer, 6000 $U \cdot ml^{-1}$). The mixture was gently swung for 2 h at room temperature and washed with 50 ml of

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0.1 M $NaHCO_3$. The gel was incubated for 2 h in 8 ml of 0.1 M Tris (pH 8). Unbound malate dehydrogenase was eliminated by washing three times respectively with 10 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8) and 10 ml of 0.1 M acetate, 0.5 M NaCl (pH 4.0). The gel was finally equilibrated in 5 mM Mops (pH 7.0) containing 2% Triton X-100.

Rat-liver mitochondria were solubilized at 4°C in 3% Triton X-100, 15 mM Mops, 10 mM malate, 50 mM NaCl (pH 7.0) at a protein concentration of 20 mg · ml^{-1}. After centrifugation at 105000 × g for 8 min to discard insoluble material, 0.6 ml of supernatant was added on top of a dry hydroxylapatite column and eluted with 0.6 ml of 2% Triton X-100, 5 mM Mops, 5 mM malate (pH 7.0). The eluates of five extractions were pooled and loaded on top of a column (0.5 cm, inner diameter) containing the affinity gel (1.7 ml). The column was washed with 10 ml of equilibration buffer and eluted with 7.2 ml of the same buffer supplemented with 2.5% ethylene glycol, 1 mg · ml^{-1} phosphatidylcholine and increasing concentrations of NaCl. The purified carrier protein was incorporated into liposomes made up as in [5]; exchange activity was carried out as in Ref. 5 and protein concentration was determined by the Lowry procedure in the presence of SDS [12].

SDS-polyacrylamide gel electrophoresis was performed on slabs (140 × 180 × 1 mm) according to Laemmli [13], the acrylamide concentration was 12.5% and the acrylamide/bisacrylamide ratio 30:0.8. The gels were stained for proteins with silver nitrate [14].

3. Results and discussion

The purification of the dicarboxylate carrier from rat liver mitochondria was achieved in several steps. For each step, the results were controlled by SDS-polyacrylamide gel electrophoresis and by assaying the exchange activity of the carrier after reconstitution. Fig. 1 shows the electrophoresis results. The pass-through of the hydroxylapatite column, having lost more than 90 percent of proteins (lane 1), was loaded on top of the affinity gel column. Proteins that were not retained were collected with the filtrate Fig. 1 (lane 2) whereas bound proteins were displaced by increasing NaCl concentration. Very few proteins were present in the fractions eluted with 0.01 and 0.1 M NaCl (lane 3). The fraction eluted with 0.4 M NaCl (lanes 4a and 4b) contained essentially a single band protein that displayed an apparent M_r of 28000.

The proteins of the various fractions were incorporated into liposomes and assayed for transport activity. Only the fraction eluted with 0.4 M NaCl was active. Table 1 shows the specific and total activities of the reconstituted transport activity, measured at each purification step. The specific activity of the pass-through of hydroxylapatite was increased 10-fold compared to the activity of the starting material and was 40-fold more active in the fraction eluted by 0.4 M NaCl. 40% of the total activity were recovered after this step.

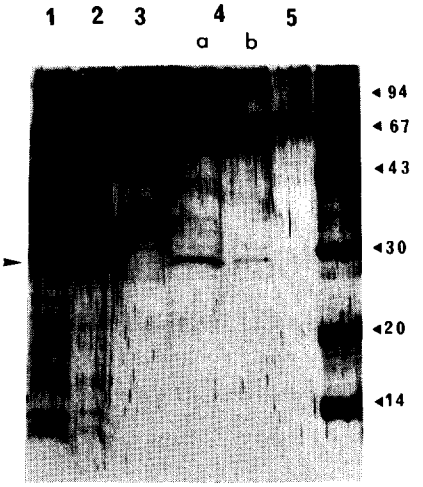

Fig. 1. Sodium dodecylsulfate/polyacrylamide gel electrophoresis pattern of the material collected during the various purification steps of the dicarboxylate carrier. Hydroxylapatite filtrate (lane 1); unbound material recovered in the filtrate of the affinity column (lane 2); material eluted by 0.1 M (lane 3), 0.4 M (lanes 4a, 4b) and 1 M NaCl (lane 5). The molecular weight markers were phosphorylase *b* (94000) bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400). Experimental conditions are described in Materials and methods.

The purified transporter was characterized after incorporation into liposomes. Fig. 2 shows the butylmalonate-sensitive kinetics of malonate uptake in exchange with malate contained inside the proteo-

Table 1
Purification of the dicarboxylate carrier
Fractions, collected at each purification step, were incorporated into liposomes loaded with 20 mM malate. The activity of $malonate_{out}$/$malate_{in}$ exchange was initiated with 0.5 mM [^{14}C]malonate (specific activity 12 mCi mM^{-1}) and stopped with 20 mM butylmalonate. The other experimental conditions are indicated in Materials and methods.

Purification step	Specific activity of exchange (nmol/min per mg protein)	Purification factor	Total activity (nmol/min)	Yield (%)
Extract of solubilized mitochondria	3.4	1	122	100
Hydroxylapatite pass-through	30	9	55	45
Eluate of affinity column (0.4 M NaCl)	1250	368	37	30

liposomes. The reaction order of the malonate/malate exchange was investigated by plotting the logarithm of $(A_{max} - A_t)$ versus time. As shown in Fig. 2B a straight line was obtained, demonstrating that the reconstituted exchange followed first order kinetics similar to the results observed with whole mitochondria. The rate constant of the reaction was 0.64 min^{-1}. Kinetic data of the exchange have been determined by varying malonate concentration. The results, calculated by Lineweaver-Burk plots (not shown) gave a K_m value of 2 mM, a value slightly higher than in whole mitochondria [15] and a V value of 1.25 μmol/min per mg protein.

The effect of specific effectors of the dicarboxylate carrier, externally added to the incubation medium, are reported in Table 2. The reconstituted dicarboxylate carrier was inhibited by the thiol group reagent *p*-chloromercuriphenylsulfonate but not by *N*-ethylmaleimide as in whole mitochondria. The substrates of the dicarboxylate carrier, malate, phosphate and sulfate added simultaneously with radioactive malonate decreased the exchange rate. These last results, namely the inhibition of malonate transport by phosphate and sulfate, confirm that the purified protein is the dicarboxylate carrier and rule out a possible confusion with the 2-oxoglutarate carrier.

As mentioned above, the purification of mitochondrial carrier proteins is a particularly critical problem especially after hydroxylapatite chromatography, since most of the transporters are still present in the column pass-through. Therefore, only an arduous screening of specific techniques experimented for each carrier, could allow to obtain positive results. Dicarboxylate-carrier

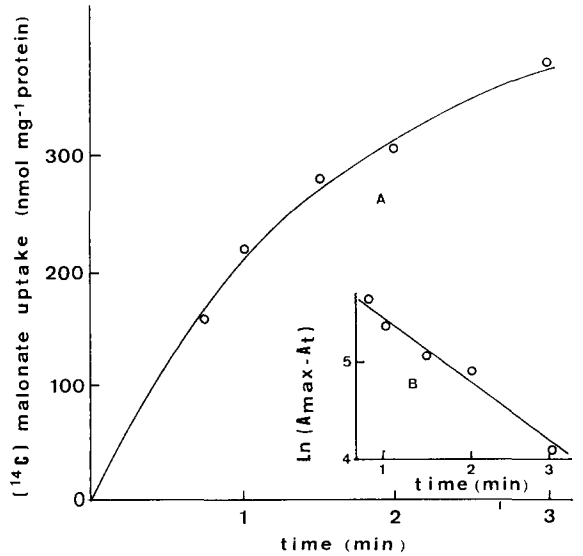

Fig. 2. (A) Kinetics of malonate uptake. 0.5 mM [^{14}C]malonate was added to proteoliposomes loaded with 20 mM malate. The reaction was stopped by 25 mM butylmalonate at various times. (B) Logarithmic plot of malonate exchange versus time assuming first order type kinetics. A_{max} is the extent of maximal malonate uptake. A_t is the extent of malonate uptake at time t.

Table 2
Effect of inhibitors and of externally added substrates on the reconstituted malonate/malate exchange of the purified dicarboxylate carrier
The substrates were added simultaneously with 0.5 mM [^{14}C]malonate. The inhibitors were preincubated 30 s with the proteoliposomes before adding malonate. Experimental conditions are the same as in Table 1.

Effector	Rate of malonate uptake (nmol/min per mg protein)	Inhibition (%)
None	376	
0.8 mM *p*-Chloromercuriphenylsulfonate	26	93
0.8 mM *N*-Ethylmaleimide	380	0
3 mM Malate	31	92
3 mM Phosphate	0	100
3 mM Sulfate	44	88

purification has been realized by using various adsorption methods but which need several steps. The method reported here is based on an apparent specific binding between mitochondrial malate dehydrogenase and the dicarboxylate carrier. It has allowed to obtain in a straight way with an appreciable yield a highly purified and fully active protein that displays all the functional properties of the dicarboxylate carrier as observed in whole mitochondria: substrate and inhibitor specificities and substrate saturation. The protein has an apparent M_r of 28000 as determined by SDS-polyacrylamide gel electrophoresis, this result is in perfect agreement with those reported by other groups [7,9].

This successfull affinity chromatography was based on the property according to which some matrix enzymes seem to be tightly bound to the inner membrane. The starting hypothesis assuming that mitochondrial transport proteins and enzymes which use the same substrates could be specifically associated is again strengthened. These results also agree with the experiments previously mentioned of Person and Srere [11]; they need however further research to study the molecular mechanisms of this association. It is particularly interesting to postulate once more a supramolecular structure of mitochondrial matrix proteins and to compute the possibilities that proceed from this property.

The obtention of the mitochondrial dicarboxylate carrier in a highly purified state, could be the starting point to study its structure, to determine its membranar topology and to display possible analogies with other mitochondrial transporters.

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