Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 205-211

RECONSTITUTION OF THE DICARBOXYLATE EXCHANGE ACTIVITY BY INCORPORATION INTO LIPOSOMES OF A TRITON-EXTRACT OF MITOCHONDRIAL RAT-LIVER INNER MEMBRANES

Mireille SAINT-MACARY and Bernard FOUCHER

Laboratoire de Biochimie, Laboratoire Associé au CNRS n° 203
Faculté des Sciences, Université de Rouen Haute-Normandie
76130, Mont-Saint-Aignan, France

Received March 29, 1983

The exchange between external [^{14}C] malonate and internal malate or phosphate was reconstituted in liposomes prepared by incorporation of a Triton-extract of mitochondrial rat-liver inner membranes. The conditions of transport were investigated and the kinetic parameters of malonate-malate and malonate-phosphate exchanges were determined. The exchange was sensitive to butylmalonate and to organomercurials. Sulfate and phosphate decreased the rate of malonate-malate exchange and phosphate inhibition was found to be competitive. This report demonstrates the possibility to reconstitute a functional dicarboxylate transport and to use the system for further purification and for studies at the molecular level.

Among the ten distinct translocating systems demonstrated in mitochondria, (for a recent review, see 1 and 2), only three of them have been purified or partially purified and their transport activity reconstituted in artificial vesicles. These three transport systems are : the phosphate carrier (3), the ADP-ATP carrier (4,5) and the citrate carrier (6). The reconstitution of a transport activity by using liposomes is very often the only efficient means to restore the activity of isolated carriers which almost exclusively work in an hydrophobic environment.

The experiments presented in this report deal with the reconstitution in lipid vesicles of the dicarboxylate transport system of rat-liver mitochondria. Mitochondrial inner membranes were solubilized with Triton X-100 and the extract was incorporated into liposomes. The exchange activity of malonate-malate and malonate-phosphate mediated by the dicarboxylate carrier was studied and the kinetic parameters were determined. The exchange was sensitive to the usual inhibitors of dicarboxylate transport of mitochondria and the inhibitory effect of phosphate was particularly studied and shown to be competitive.

0006-291X/83 $1.50

The results obtained settle the first step of the purification of the dicarboxylate carrier and point out the interest of the reconstituted system to study further the mechanism of the dicarboxylate transport.

METHODS

Preparation of liposomes : 12.5 mg of cardiolipin were dried under a stream of nitrogen, supplemented with 237.5 mg of phosphatidylcholine and 2 ml of a medium made of 50 mM NaCl, 15 mM MPS, 20 mM malate or phosphate pH 7.2 then left to stand overnight at 4°C. This mixture was submitted to sonication under nitrogen at 4°C for 15 minutes by alterned periods : 30 seconds on, 30 seconds off with a type 20-200 Alcatel Sonifier fitted with a microtip and used at 25 W.

Preparation of the Triton-extract : inner membrane vesicles were prepared by sonication of mitoplasts obtained from rat-liver mitochondria as described in a previous report (7). 7 mg of inner membrane proteins were solubilized in 0.360 ml of the preceding buffer containing 3% Triton X-100 and let to incubate for 15 minutes at 4°C before being centrifuged at 105,000 x g for 8 minutes in a air-driven Beckman ultracentrifuge. Proteins were determined by the biuret method in presence of cholate.

Preparation of proteoliposomes : a 80 µl fraction of the Triton-extract containing 1 mg protein was mixed with 2 ml of liposomes by gentle stirring at 4°C for 2 minutes. Proteoliposomes were quickly frozen and thawed then sonicated by brief pulses for 10 seconds. Finally external malate or phosphate was removed by filtration through a 0.6 x 15 cm Dowex-acetate column equilibrated with 0.140 M sucrose ; the final pH value was adjusted to 7.2.

The exchange activity was initiated by adding [2 $^{-14}$C] malonate (specific activity : 6.3 mCi x mmol.$^{-1}$) to proteoliposomes and stopped 30 seconds later by addition of 20 mM butylmalonate, the temperature was 22°C ; after incubation, proteoliposomes were filtered through a cold 0.5 x 6 cm Dowex-acetate column to eliminate external malonate and the radioactivity of the filtrate was counted by liquid scintillation. For each experiment, a control was performed in which proteoliposomes were preincubated with 20 mM butylmalonate for 30 seconds before adding malonate. Control value was soustracted from the corresponding experiment.

MPS : 3-Morpholinopropanesulfonic acid was purchased from Merck ; Cardiolipin from Biochemical Co, Dowex AG 1X8 (50-100 mesh) and Triton X-100 from Serva, Phosphatidylcholine (type II-S from Soy Beans) was obtained from Sigma Chemical Co and [2 $^{-14}$C] malonate from Amersham.

RESULTS

EXPERIMENTAL CONDITIONS FOR THE RECONSTITUTION OF THE DICARBOXYLATE-CARRIER

Mitochondrial inner membranes were solubilized in Triton X-100 and the extract was incorporated into liposomes according to the technique described under methods. Attempts to solubilize and to reconstitute a functional dicarboxylate carrier with other detergents such as lubrol or octylglucoside were unsuccessful. The solubilization of membrane proteins needs the presence of a sufficient amount of detergent, however a too high concentration migh have a deleterous effect on liposomes. Result presented in Fig. 1 show both the variations of specific activity of malonate-malate exchange in proteoliposomes and the proportion

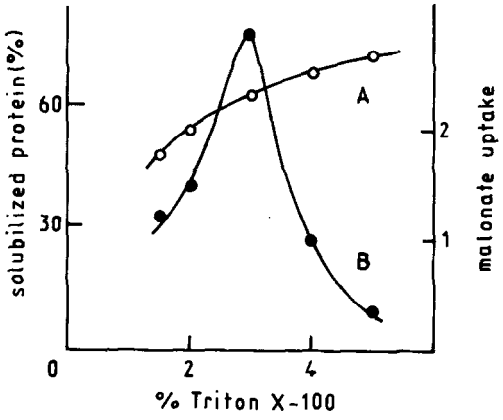

Fig.1. Effect of Triton X-100 concentration on the solubilization of inner membrane-proteins and on malonate uptake by proteoliposomes.
Curve A shows the proportions of solubilized proteins versus detergent concentration. Curve B gives specific activity of malonate-malate exchange as a function of the concentration of Triton used to solubilize inner membrane vesicles. The specific activity is expressed as nmoles of malonate exchanged per minute and per mg of added proteins. [^{14}C] malonate concentration was 0.19 mM.

of solubilized membrane-proteins as a function of detergent concentration. Maximum exchange activity is observed with a buffer containing 3% Triton X-100 whereas for higher concentrations the activity falls drastically. On the other hand, at this concentration of Triton X-100, 65% of membrane-proteins were solubilized and this amount was not very much increased by higher concentrations of detergent.

Exchange activity of malonate was proportional to the amount of solubilized proteins added to the liposomes (up to 0.7 mg of proteins per ml of liposomes, which was the maximum concentration used).

The rate of exchange of externally added [^{14}C] malonate was studied as a function of the concentration of malate entrapped inside the liposomes. The highest activity was observed with 20 mM malate and no exchange activity occured in malate free liposomes.

In liposomes prepared with various concentrations of cardiolipin, the rate of exchange increased about forty percent when the amount of cardiolipin was varied from 0 to 5% (W/W) of total phospholipids.

The effect of pH was also investigated ; the exchange activity did not change significantly in the range of pH 6.7 to 7.3.

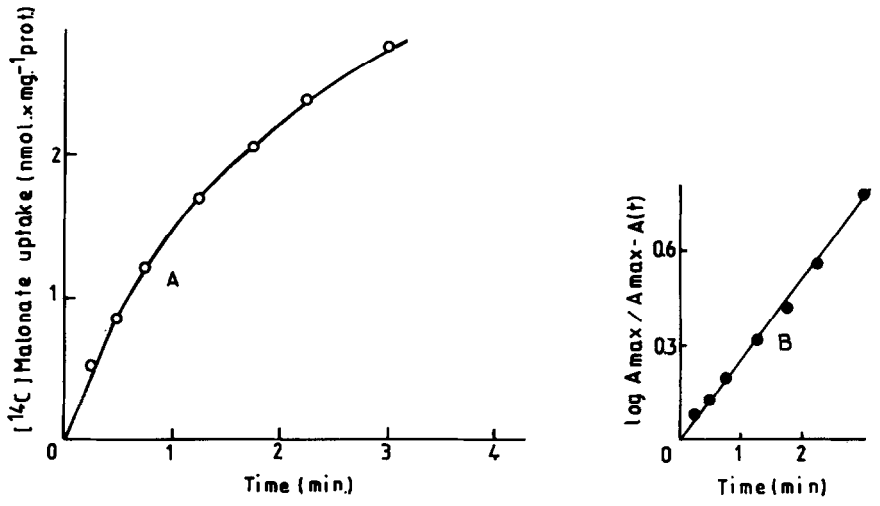

Fig.2A. Time-course of malonate uptake during malonate-malate exchange in reconstituted proteoliposomes.
Assays were performed as described under methods with a concentration of 0.077 mM [^{14}C] malonate and incubated for the indicated periods ; the activity was corrected for the butylmalonate insensitive exchange.
Fig.2B. Logarithmic plot of malonate uptake versus time assuming first order kinetics. A max. is the extent of maximum malonate uptake ; A (t) the extent uptake at time t.

DETERMINATION OF KINETIC PARAMETERS OF MALONATE UPTAKE AND STUDY OF VARIOUS EFFECTORS OF THE MALONATE-MALATE AND MALONATE-PHOSPHATE EXCHANGES.

The time-course of the exchange between external [^{14}C] malonate and internal malate in proteoliposomes is shown in Fig. 2A. The reaction proceeds linearly with time for about 40 seconds. At low concentrations, malonate uptake follows a first order reaction as can be seen in Fig. 2B. The rate constant k of this reaction is 0.57 min^{-1} with a half-time of 73 seconds.

The rate of malonate uptake in malonate-malate exchange was studied as a function of concentration of added malonate. K_m and V values were calculated from Lineweaver-Burk plots and are reported in Table I.

The dicarboxylate carrier being able to mediate a dicarboxylate-dicarboxylate exchange as well as a dicarboxylate-phosphate exchange, this reaction was assayed under the same experimental conditions, by replacing malate by phosphate inside the liposomes. This exchange also occured and the results can be seen in Table I.

Table I

Data relative to kinetic parameters of [^{14}C] malonate uptake during malonate-malate and malonate-phosphate exchanges by proteoliposomes and effect of various effectors of the dicarboxylate carrier on malonate-malate exchange.

Exchange		Rate of malonate uptake (nmol.x min^{-1}x mg^{-1}protein)	K_m (mM)
-[^{14}C] malonate-malate		**(V) *3.37 ± 0.28 (8)	*0.15 ± 0.03 (8)
Additions			
p-chloromercuriphenyl-sulfonate (0.4 mM)	0	1.20	
	+	0.08	
phosphate (1.5 mM)	0	1.39	
	+	0.65	
sulfate (1.5 mM)	+	0.71	
Mg^{2+} (2 mM)	0	1.99	
	+	2.01	
-[^{14}C] malonate-phosphate		**(V) *2.20 ± 0.10 (4)	*0.17 ± 0.02 (4)

*The values given are the means ± S.E. ; between brackets : number of experiments. ** Maximum rate. Proteoliposomes were incubated for 30 seconds with p-chloromercuriphenylsulfonate or Mg^{2+} before addition of malonate. Phosphate and sulfate were added simultaneously with malonate. Other experimental conditions are described under methods.

The same Table summarizes the effects on malonate-malate exchange of several effectors known to modify the activity of the dicarboxylate transporter in whole mitochondria. Preincubation with Mg^{2+} did not modify the rate exchange. p-chloromercuriphenylsulfonate, a thiol group reagent, completely inhibited the exchange activity which was also strongly decreased by phosphate and sulfate.

A more occurate study of the inhibition by phosphate of the exchange was carried out by varying the concentration of malonate. The results can be seen in Fig. 3. The double reciprocal plots display the characteristic features of a competitive inhibition.

DISCUSSION

The exchange activity of the dicarboxylate carrier of rat-liver mitochondria was reconstituted into lipid vesicles. It can be pointed out that the highest activity of malonate-malate exchange was obtained in liposomes containing cardiolipin ; the same observations have been already made for the phosphate (8) and the citrate carriers (6).

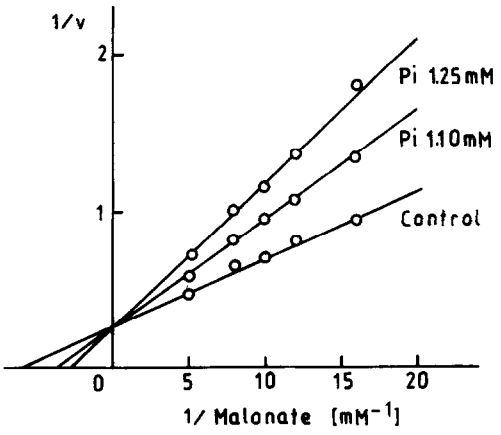

Fig.3. Lineweaver-Burk plots of phosphate inhibition on malonate-malate exchange in reconstituted proteoliposomes.
v is expressed in nmoles per minute and per mg protein. Exchange was started by addition of various concentrations of [^{14}C] malonate or by simultaneous addition of malonate plus 1.1 and 1.25 mM phosphate. The reaction was stopped after 30 seconds by addition of 20 mM butylmalonate. Experiments were completed and results calculated as indicated under methods. The inhibition by phosphate was repeatedly found to be competitive with five different preparations of proteoliposomes.

Dicarboxylate transport presents in liposomes the same properties as in mitochondria : internal dicarboxylate or phosphate is needed as exchangeable substrate and the exchange is sensitive to the same effectors.

Apparent K_m values for malonate uptake in malonate-malate and malonate-phosphate exchanges are similar and are slightly higher than the K_m values observed in mitochondria (9). V was smaller for malonate-phosphate exchange than for malonate-malate ; this difference may be explained by different requirements for phosphate compared to malate in the exchange with external malonate, according to experimental conditions. In contrast to the results obtained with mitoplasts (9) and mitochondria (9,10), we have observed no modification of activity either by varying the pH or by addition of Mg^{2+}.

Part of the malonate-malate exchange could be also attributed to the oxoglutarate carrier ; however the following observations support evidence for an essential participation of the dicarboxylate carrier : (i) The malonate uptake measured is the one which is sensitive to butylmalonate, the most specific inhibitor of the dicarboxylate carrier. (ii) In our experiments malonate was used at low concentrations and its affinity is much higher for the dicarboxylate

carrier than for the oxoglutarate carrier (2). (iii) Malonate uptake is completely inhibited by organomercurials and is prevented by phosphate and sulfate which are both specific effectors of the dicarboxylate carrier in mitochondria and have no effect on the oxoglutarate carrier.

The study of the inhibition by phosphate of malonate uptake in the malonate-malate exchange suggests that dicarboxylate and phosphate might be mutually exclusive and have the same binding site. This result is surprising indeed because it is in disagreement with previous proposals, where mixed type inhibition was evidenced in mitochondria (11,12). However this result could be explained either by a modification of protein conformation during the process of solubilization and reconstitution or by a greater simplification of the system used in which other transport systems, regulations and conformational changes might not interfere. Further experiments will be necessary to go more deeply into this problem.

In conclusion this report points out the possibility to reconstituted in liposomes the activity of the dicarboxylate transporter from rat-liver mitochondria and to use this system to pursue the purification of the carrier-protein in order to study further the molecular mechanism of this transport.

REFERENCES

1. Klingenberg, M. (1979) Methods Enzymol. 56, 245-252.
2. La Noue, K.F. and Schoolwerth, A.C. (1979) in Annual Review of Biochemistry, 48, (Snell, E.E., Boyer, P.D., Meister, A. and Richardson, C.C. eds) pp. 871-922.
3. Banerjee, R.K., Shertzer, B.G., Kanner, B.I. and Racker, E. (1977) Biochem. Biophys. Res. Comm. 75, 772-778.
4. Krämer, R. and Klingenberg, M. (1977) FEBS Lett. 82, 363-367.
5. Brandolin, G., Doussière, J., Gulik, A., Gulik-Krzywicki, T., Lauquin, G.J. M. and Vignais, P.V. (1980) Biochim. Biophys. Acta 592, 592-614.
6. Stipani, I., Krämer, R., Palmieri, F. and Klingenberg, M. (1980) Biochem. Biophys. Res. Comm. 97, 1206-1214.
7. Saint-Macary, M. and Foucher, B. (1980) Biochem. Biophys. Res. Comm. 96, 457-462.
8. Kadenbach, B., Mende, P., Kolbe, H.V.J., Stipani, I. and Palmieri, F. (1982) FEBS Lett. 139, 109-112.
9. Saint-Macary, M., Lainé, M. and Foucher, B. (1979) Biochim. Biophys. Acta 547, 170-174.
10. Meisner, H., Palmieri, F. and Quagliariello, E. (1972) Biochemistry 11, 949-955.
11. Crompton, M., Palmieri, F., Capano, M. and Quagliariello, E. (1975) Biochem. J. 146, 667-673.
12. Palmieri, F., Prezioso, G. Quagliariello, E. and Klingenberg, M. (1971) Eur. J. Biochem. 22, 66-74.