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CITRATE TRANSPORT IN LIPOSOMES RECONSTITUTED WITH TRITON EXTRACTS FROM MITOCHONDRIA

I. Stipani[1], R. Krämer[2], F. Palmieri[1] and M. Klingenberg[2]

[1]Institute of Biochemistry, University of Bari, 70126 Bari, Italy, and [2]Institute of Physical Biochemistry, University of Munich, 8000 Munich 2, F.R.G.

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SUMMARY

The exchange between external [^{14}C] citrate and internal citrate, malate or phosphoenolpyruvate can be reconstituted with a Triton extract of submitochondrial particles from rat liver. The reconstituted activity is dependent on the phospholipid composition of the liposomes and is influenced by the simultaneously incorporated Triton. The kinetic properties, the substrate and tissue specificity, and the inhibitor sensitivity of citrate transport in liposomes are similar to those described for the tricarboxylate transport in mitochondria. The maximal rate of citrate exchange in the reconstituted system (13.5 μmol x min^{-1} x g^{-1} at 25°C and pH 7.5) accounts for 12% of the original mitochondrial activity.

Liver mitochondria contain a specific transport system, known as the tricarboxylate carrier, which catalyzes a 1:1 exchange between citrate or other tricarboxylates, some dicarboxylates and PEP (1). The exchange is inhibited by 1,2,3-benzenetricarboxylate, and less specifically by p-iodobenzylmalonate (2-4).

A 94-fold purification of a citrate-binding protein from Triton-solubilized SMP was achieved by affinity chromatography on a Sepharose-aminobenzene-1,2,3-tricarboxylate column

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Abbreviations: SMP, submitochondrial particles; EYPL, egg yolk phospholipids; MPL, mitochondrial phospholipids; BTA, benzenetricarboxylic acid; PEP, phosphoenolpyruvate; MOPS, morpholinopropane sulfonic acid.

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followed by exclusion chromatography on Sephadex G-200 (5). The purified protein (subunit M.W. 20,000) bound the substrates of the tricarboxylate carrier specifically and with high affinity (K_D = 2 µM for citrate) in a 1,2,3-benzenetricarboxylate-sensitive and 1,3,5-benzenetricarboxylate-insensitive manner. The isolated citrate binding protein was unable to reconstitute citrate transport in liposomes (5).

In this report it is shown that a Triton extract from submitochondrial particles of rat liver added to liposomes catalyzes an exchange of citrate. This transport activity exhibits most of the properties of the mitochondrial tricarboxylate carrier. The maximal rate of the reconstituted transport accounts for 12% of the original mitochondrial activity.

MATERIALS AND METHODS

EYPL were obtained from Merck or prepared according to (6). Pure lecithin was prepared from EYPL (7). MPL were isolated from rat liver mitochondria as described (8). Phosphatidylethanolamine and cardiolipin were obtained from Sigma, p-iodobenzylmalonate from K & K and [1.5-^{14}C] citric acid from the Radiochemical Center (Amersham). Other reagents and chemicals were obtained as described (4).

The reconstitution followed largely the procedures as described for the ADP,ATP carrier (10). Liposomes were prepared by sonicating phospholipids (125 mg/ml) in 50 mM NaCl, 10 mM citrate and 10 mM MOPS, pH 7.5, under N_2 until a clear suspension was obtained. SMP from rat liver, beef heart or rat brain were extracted with 3% Triton, 50 mM NaCl and 10 MOPS pH 7.5, at a final concentration of 10-15 mg protein/ml, for 20 min at 0°C and then centrifuged at 147,000 x g. The Triton-insoluble residue accounting for about 30% of the total protein was discarded. The supernatant (300-400 µg protein and 1.2 mg Triton in 40 µl) was mixed with 1.25 ml liposomes. After 2 min at 0° reconstitution was achieved by the freeze-thaw-sonication procedure (9,10). The sonication step exhibits a distinct optimum between 12-17 sec of sonication, probably due to protein inactivation after longer sonication. After removal of the external citrate by a Dowex-acetate column, preequilibrated with 100 mM sucrose, the exchange was initiated by adding 0.15 mM [^{14}C] citrate to the reconstituted liposomes and terminated with 10 mM 1,2,3-BTA. The assay was carried out at 25°C. The reaction mixtures were passed through small Dowex columns and the radio-

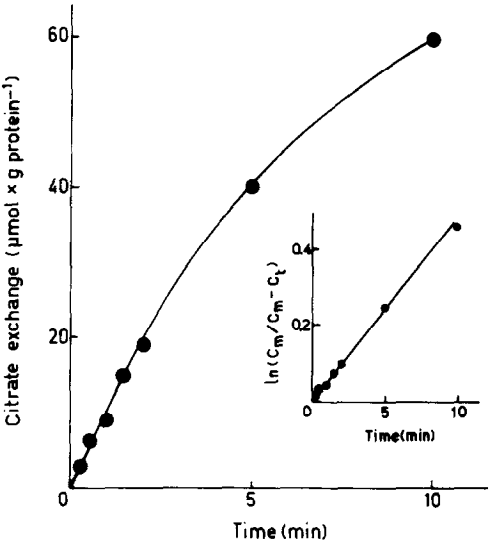

Fig. 1: Time course of citrate exchange in reconstituted liposomes (internal citrate, external [^{14}C] citrate, 25°C). The insert is a logarithmic plot of ln $C_m/(C_m-C_t)$ (C_m, maximum exchange 92 µmol citrate/g; C_t, exchange at time t) according to the relation ln $C_m/(C_m-C_t)$ = Kt (cf. ref. 4). Liposomes prepared from a mixture of EYPL and 25% of MPL.

activity eluted together with the liposomes was counted. Each determination was accompanied by a control in which the inhibitor was added before the labelled substrate. The results were obtained by subtracting the control values from the experimental samples. The protein was measured by the method of Lowry in the presence of 1% sodium lauryl sulfate.

RESULTS

Kinetics. Fig. 1 shows the time course of citrate exchange in reconstituted liposomes at 25°C. Citrate uptake increases linearly with the time for about 90 sec at a rate of 9 µmol x min^{-1} x g $protein^{-1}$. A total amount of 92 µmol citrate/g protein is taken up with t1/2 of 6 min. The exchange of citrate appears to follow a first order type of reaction (insert of Fig. 1) similarly as found in intact mitochondria (4).

The rate of citrate exchange increases about ten times from 10 to 40°C. Between 10 and 30°C a straight line is obtained

Table I

Dependence of citrate exchange on internal anions

Internal anion	Citrate exchange (μmol/g x 7 min)
-	0.2
Phosphate	0.6
Oxoglutarate	1.6
1,2,3-BTA	1.4
Citrate	16.3
Malate	13.0
PEP	12.1
Citrate + 1,2,3-BTA	6.0

Exchange between external [^{14}C] citrate and the indicated internal anions. These were present, at a concentration of 10 mM, during the preparation of the liposomes from EYPL.

in an Arrhenius plot with an E_A varying from 14.6 to 21.0 Kcal/mol in three experiments. A pH optimum of 7-7.5 has been observed with a decrease to about 50% activity at pH's 6.0 and 8.5 respectively. The dependence of the rate of citrate exchange on the externally added [^{14}C] citrate concentration gives linear reciprocal plots. In four experiments conducted at 25°C, pH 7.5 and with liposomes prepared from a mixture of EPYL and 25% of MPL, the V_{max} was 13.5 ± 1.4 mol x min^{-1} x g^{-1} and the K_m was 63 ± 9 μM.

Dependence on internal anion and tissue specificity. The data reported in Table I show that the citrate uptake is virtually absent when the liposomes do not contain anions which are substrates of the citrate carrier, like P_i, oxoglutarate and 1,2,3-BTA. Externally added [^{14}C] citrate exchanges not only with citrate but also with internal malate or PEP. The [^{14}C] citrate/citrate exchange is inhibited by internal 1,2,3,-BTA, which is a specific inhibitor of the transport of citrate in mitochondria. The citrate exchange is very low when Triton extract of SMP from heart or brain is used (1.2 and 1.5 μmol/g x 7 min) in agreement with the

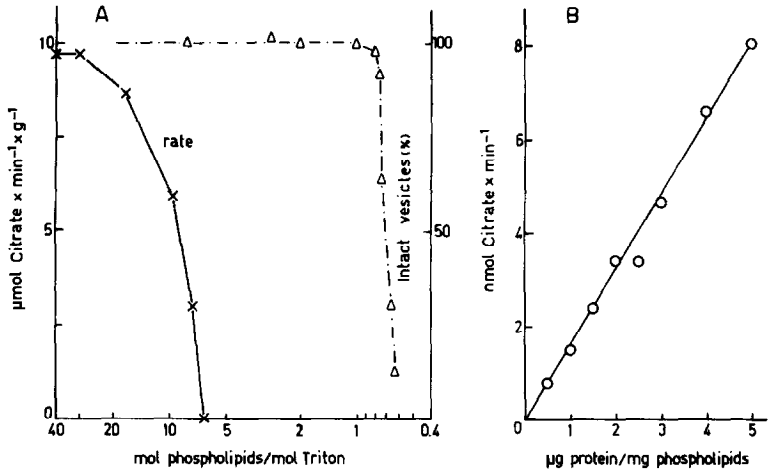

Fig. 2: A) Dependence of the rate of citrate exchange on the amount of Triton. Increasing concentrations of Triton are added to the reconstituted system. In the assay (0.33 ml) the Triton varied from 0.31 to 1.98 mg, protein and PL were 62 µg and 16.4 mg respectively. The M.W. of Triton was taken as 620, that of PL as 800. B) Effect of protein concentration on citrate exchange. Increasing amounts of protein (79-790 µg in a volume of 100 µl containing 3.0 mg Triton) were added to 1.25 ml liposomes (125 mg PL/ml). In the assay (0.33 ml) protein varied from 10.5 to 105 µg, PL and Triton were 20.8 mg and 0.4 mg respectively. Liposomes were preapred from a mixture of EYPL and 25% of MPL.

observation that the citrate carrier is virtually absent in heart and in brain mitochondria (11,12).

Dependence on detergent and protein concentration. In the experiment reported in Fig. 2A the concentration of Triton was varied, keeping the protein/phospholipid ratio constant. The activity of the carrier was diminished on increasing the detergent concentration, reaching zero at a molar ratio of 0.15 Triton/phospholipids. Breakage of the vesicles occurs at a much higher concentration of Triton.

Fig. 2B shows that at constant detergent and phospholipid concentration, the amount of citrate taken up per min by the liposomes increases linearly with the protein concentration. The highest concentration of protein used in this experiment corresponds to 315 µg/ml of assay. This value was not exceeded

Table II

Dependence of the rate of citrate exchange on the phospholipid composition

Phospholipids (weight ratio)	Exchange rate (μmol citrate/g x min)
PC	1.0
PC + PE (80:20)	1.9
PC + PE (50:50)	1.7
PC + DPG (95:5)	10.1
PC + MPL (75:25)	8.1
EYPL	2.5
EYPL + DPG (95:5)	11.5
EYPL + MPL (75:25)	9.1

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, cardiolipin; MPL, mitochondrial phospholipids; EYPL, egg yolk phospholipids.

in order to keep the Triton concentration sufficiently low to measure nearly optimal rates of exchange.

Phospholipid specificity. The rate of citrate exchange is dependent on the type of phospholipids used to prepare the liposomes (Table II). Addition of cardiolipin or of MPL to either pure lecithin or EYPL increases the transport activity several fold.

Substrate specificity and inhibitor sensitivity. The specificity of citrate transport in reconstituted liposomes was investigated by studying the ability of a variety of anions to inhibit the [^{14}C] citrate exchange. The results (Table III) show that the rate of citrate exchange is strongly inhibited by known substrates of the mitochondrial tricarboxylate carrier. In contrast, the *trans*-isomers (*trans*-aconitate and fumarate), and the di- and tricarboxylates with carboxylic groups not at an appropriate distance (malonate and 1,3,5-pentanetricarboxylate) do not significantly affect the rate of citrate exchange. Other anionic substrates (not shown) which are transported in mitochondria by different transport systems (P_i, oxoglutarate, aspartate, glutamate, sulphate and pyruvate) have no significant effect (less than 10% inhibition). Table

Table III

Effect of externally added anions and thiol reagents on the rate of [^{14}C] citrate/citrate exchange.

Additions	% Inhibition	Additions	% Inhibition
cis-Aconitate	96	PEP	69
trans-Aconitate	18	1,2,3-BTA	100
threo-D$_s$-Isocitrate	87	1,2,4-BTA	10
1,2,3-PTA	100	1,3,5-BTA	3
1,3,5-PTA	2	p-IBM	80
Malate	79	Mersalyl (0.2 mM)	93
Malonate	6	p-HMB (0.2 mM)	92
Maleate	67	NEM (0.2 mM)	21
Fumarate	0	NEM (1 mM)	30

2.5 mM of the indicated anions were added simultaneously with [^{14}C] citrate. The SH-blocking reagents were added 2 min before the labeled substrate at the concentrations indicated. In the control samples, the rate of citrate exchange was 8.7 µmol/g x min. EYPL and 25% MPL were used to prepare the liposomes. Abbreviations: 1,2,3-PTA, 1,2,3-propanetricarboxylate; 1,3,5-PTA, 1,3,5-pentanetricarboxylate; BTA, benzenetricarboxylate; p-IBM, p-iodobenzylmalonate; p-HMB, p-hydroxymercuribenzoate.

III also reports the inhibitor sensitivity of citrate transport in reconstituted liposomes. As in intact mitochondria, the exchange of citrate is inhibited by 1,2,3-BTA and p-iodobenzylmalonate, which are known to interact with the carrier without being transported (see also Table I). No inhibition is observed with 1,2,4- and 1,3,5-benzenetricarboxylate. In addition, as found in mitochondria, the citrate exchange catalyzed by the liposomes is strongly inhibited by 0.2 mM mersalyl and p-hydroxymercuribenzoate, but only slightly by NEM (2,4,13).

DISCUSSION

The data presented in this paper show that the activity of citrate exchange can be reconstituted from a Triton extract of submitochondrial particles following the two stage sonication method developed for the reconstitution of the ADP,ATP exchange activity (10). The absolute requirement for an appropriate counteranion, the substrate and tissue specificity and the

inhibitor sensitivity of the citrate exchange in reconstituted liposomes are distinctive features of the tricarboxylate transport in mitochondria (1). Further similarities are shown by the kinetic properties, e.g. the K_m of the citrate exchange in liposomes is very close to that found in mitochondria (4).

The rate of citrate exchange in the reconstituted system is influenced by several factors. For example the presence of acidic phospholipids in the liposomes increases the rate of transport possibly by favoring the extent of incorporation. In the experimental conditions used in the present paper the maximal rate of citrate exchange in proteoliposomes is 13.5 $\mu mol \times min^{-1} \times g^{-1}$. Compared to the activity of rat liver mitochondria extrapolated to 25°C (4), the extent of the reconstituted transport amounts to 12% of the original mitochondrial exchange activity.

Since the partially purified citrate-binding protein (5) could not be functionally reconstituted in liposomes, it may be considered that the transport activity is lost during the purification steps employed, although the binding capacity for its substrates is retained. It is hoped that the apparent ability of the solubilized mitochondrial tricarboxylate carrier reconstituted from detergent extracts can be used as an assay during isolation and purification.

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REFERENCES

1. LaNoue, K.F. and Schoolwerth, A.C. (1979) Ann. Rev. Biochem. 48, 871-922.
2. Quagliariello, E. and Palmieri, F. (1972) Biochemistry and Biophysics of Mitochondrial Membrane, pp. 659-680, Academic Press, New York.

3. Robinson, B.H., Williams, G.R., Halperin, M.L. and Leznoff, C.C. (1971) Eur. J. Biochem. 20, 65-71.
4. Palmieri, F., Stipani, I., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 26, 587-594.
5. Palmieri, F., Stipani, I., Genchi, G. and Quagliariello, E. (1978) Mechanisms of Proton and Calcium Pumps, pp. 239-250, Elsevier, Amsterdam.
6. Pangborn, M.C. (1951) J. Biol. Chem. 188, 471-476.
7. Beyer, K. and Klingenberg, M. (1978) Biochemistry 17, 1424-1431.
8. Rouser, G. and Fleischer, S. (1967) Methods Enzymol. X (Estabrook, R.W. and Pullman, M.E., eds.) pp. 325-339, Academic Press, New York.
9. Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384-7390.
10. Krämer, R. and Klingenberg, M. (1977) FEBS Lett. 82, 363-367.
11. Chappell, J. B. (1968) Brit. Med. Bull. 24, 150-157.
12. Sluse, F.E., Meijer, A.J. and Tager, J.M. (1971) FEBS Lett. 18, 149-151.
13. Robinson, B.H. and Oei, J. (1970) FEBS Lett. 11, 200-204.