Purification of the active mitochondrial tricarboxylate carrier by hydroxylapatite chromatography

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Received 1 August 1983

The mitochondrial tricarboxylate carrier has been extracted from rat liver mitochondria or SMP with Triton X-100, in the presence of 1,2,3-BTA and DPG, and partially purified by chromatography on HTP. The purified fraction, which also contains the ADP/ATP carrier and the phosphate carrier, after incorporation into liposomes catalyzes a 1,2,3-BTA-sensitive [^{14}C]citrate/citrate exchange. The tissue and substrate specificity, the inhibitor sensitivity and the kinetic properties of citrate transport in liposomes are similar to those described for the citrate transport in mitochondria. The maximal rate of citrate exchange in the reconstituted system is 338 μmol · min^{-1} · g protein^{-1}, at 30°C and pH 7.0.

Tricarboxylate carrier *Mitochondria* *Hydroxylapatite* *Reconstitution* *Membrane transport*

1. INTRODUCTION

The inner membrane of liver mitochondria contains a specific carrier system for the transport of tricarboxylates and PEP which may have important physiological functions in fatty acid synthesis, gluconeogenesis and transfer of reducing equivalents across the membrane (reviews) [1,2]). The properties of the tricarboxylate carrier have been extensively investigated in intact mitochondria [1]. It transports citrate, *cis*-aconitate, isocitrate, PEP and malate by a strictly 1:1 exchange reaction inhibited by 1,2,3-BTA, *p*-iodobenzylmalonate and some SH reagents. Its activity varies greatly in mitochondria from different sources. The kinetic parameters have been determined in liver mitochondria [3,4]. The specific impermeable inhibitor 1,2,3-BTA has a higher affinity for the carrier than any substrate. Despite an attempt based on the binding capacity for citrate [5], purification of the tricarboxylate carrier has been hindered by the lack of an assay of its function after solubilization of the mitochondrial membrane. We have recently reported, however, that the Triton extract from SMP of rat liver is able to reconstitute citrate transport in liposomes [6].

Here, we partially purified the tricarboxylate carrier from rat liver mitochondria by chromatography on HTP. The purified tricarboxylate carrier fraction incorporated into liposomes exhibits transport properties which closely resemble those observed in intact mitochondria. Part of this work has been communicated [7].

Abbreviations: BTA, benzenetricarboxylic acid; DPG, cardiolipin; HTP, hydroxylapatite; NEM, *N*-ethylmaleimide; PEP, phosphoenolpyruvate; SDS, sodium dodecylsulfate; SMP, submitochondrial particles

2. MATERIALS AND METHODS

Hydroxylapatite (Bio-Gel HTP) and AG1 × 8 (100–200 mesh) were purchased from Bio-Rad, 1,5-[^{14}C]citric acid from the Radiochemical Centre (Amersham), cardiolipin from Serdary, egg yolk phospholipids (L-α-phosphatidylcholine, type X-E) from Sigma, octylglucoside from Calbiochem, Genapol X-80 from Hoechst. Other phospholipids, detergents and reagents were obtained as reported [4,6–9].

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001457593/83/$3.00

2.1. *Isolation of the citrate transport protein*

Mitochondria and submitochondrial particles (SMP) from rat liver, beef heart or rat brain, isolated with standard procedures, were extracted with 4% Triton X-100, 50 mM NaCl, 10 mM morpholinopropane sulfonic acid (pH 7.0), 2.5 mM 1,2,3-BTA, 2 mg/ml DPG at a final concentration of 10–12 mg protein/ml. Deviations from this procedure are indicated in the legends of the tables and figures. After 20 min at 0°C the extract was centrifuged at 147000 × *g* for 45 min; 600 μl supernant (3.5–5.0 mg protein) were applied to cold HTP columns (Pasteur pipettes containing 600 mg of dry material) and the elution was performed with the solubilization buffer.

2.2. *Reconstitution of the citrate–citrate exchange activity*

Liposomes were prepared by sonication of egg yolk phospholipids and 25% mitochondrial phospholipids as in [6]; 1.25 ml liposomes were mixed with 40–60 μl extracts or HTP eluates. After 2 min at 0°C the mixture was frozen in liquid N_2, thawed in a water bath at 10–15°C and then pulse-sonicated (0.2 s sonication, 0.8 s intermission) for 12 s at 0°C. After removal of external citrate by an AG1 × 8 column acetate form (0.5 × 8 cm equilibrated with 170 mM sucrose), the proteoliposomes were diluted with 250 μl 50 mM NaCl and 10 mM morpholinopropane sulfonic acid, pH 7.0. The proteoliposomes were distributed in 4 Eppendorf cups (320 μl each) and tempered at 30°C for 4 min. The exchange was started by the addition of 0.3 mM [^{14}C]citrate and stopped with 10 mM 1,2,3-BTA. In control samples 1,2,3-BTA was added 2 min before [^{14}C]citrate, 320 μl of each sample were applied to an AG1 × 8 column chloride-form (0.5 × 4 cm equilibrated with 170 mM sucrose), eluted with 1.7 ml 170 mM sucrose, collected in a scintillation vial, vortexed with 4 ml of scintillation mixture (Backer) and counted. The citrate exchange activity was calculated by subtracting the control values from the experimental samples. The purified citrate transport protein was rapidly inactivated at 30°C with a half-time of 5 min. In contrast, after incorporation into liposomes the transport activity decreased only 24% at 30°C for 1 h.

Table 1

Effect of various detergents, 1,2,3-benzenetricarboxylate and cardiolipin on the solubilization of the active citrate carrier protein

Detergents	Citrate exchange (μmol.10 min^{-1}.g protein^{-1})	Solubilized protein (%)
Genapol X-80	30	46
Emulphogen BC720	33	63
LAPAO	6	80
Octylglucoside	4	69
Cholate	2	57
Brij 58	3	25
Lubrol WX	2	35
Tween 20	5	16
Triton X-100	73	73
Triton X-100 – 1,2,3-BTA	56	76
Triton X-100 – DPG	40	75
Triton X-100 – 1,2,3-BTA and DPG	28	75

SMP from liver mitochondria were extracted as described in section 2 by the indicated detergents at a 4% concentration. When indicated 1,2,3-BTA and/or DPG were omitted from the extraction medium

2.3. *Other methods*

Protein was determined in the presence of 1% SDS as in [10]. SDS-gel electrophoresis was performed as in [11].

3. RESULTS

The conditions for optimal solubilization of the tricarboxylate carrier protein in undenaturated state were thoroughly investigated by measuring the reconstituted citrate exchange activity in SMP extracts of rat liver. The effects of a number of detergents on the solubilization of the active carrier protein are compared in table 1. Among all the detergents tested, Triton X-100 is the most effective solubilizer of the active citrate carrier protein. Genapol X-80 and Emulphogen BC 720 are considerably less effective. In contrast, there is virtually no citrate exchange activity in the extracts obtained by using other non-ionic detergents like LAPAO and octylglucoside and the ionic detergent cholate, although they solubilize a large portion of the mitochondrial membrane proteins. The polyoxyethyleneglycol homologues of Triton Brij

58, Lubrol WX and Tween 20, which solubilize a smaller fraction of the total proteins, are also unable to solubilize the active citrate carrier.

Since it is likely that all detergents denaturate the carrier protein to varying extents, the specific inhibitor, 1,2,3-BTA and the phospholipid DPG were included in the solubilization buffer to preserve the native state of the protein. Table 1 shows that omission of 1,2,3-BTA from the extraction medium containing Triton X-100 leads to a decrease of the reconstituted citrate exchange activity. Omission of DPG has a more pronounced effect and omission of both is even more harmful, showing that both agents are needed for maximal protection. If citrate, instead of 1,2,3-BTA, is added to the solubilization buffer, the reconstituted citrate exchange activity is somewhat lower (not shown), indicating that the higher affinity of 1,2,3-BTA is required for a better protection of the carrier.

In other experiments (not shown) the concentration of Triton X-100 was varied in an attempt to improve the extraction of the carrier. It was found that, in the absence of DPG, both the total and the specific activities increase on increasing the concentration of Triton up to 4% and decrease at higher detergent concentrations. From 4–8% Triton the total activity decreases by 70% while the protein solubilized increases from 74–80%. In the presence of DPG, the total and the specific activities are higher, at 4% Triton and nearly remain constant at higher detergent concentrations up to 10%, showing that DPG prevents the inactivation of the citrate carrier by Triton. The influence of the salt concentration and of the pH of the extraction medium were also studied on the reconstituted citrate exchange activity. We found a pH optimum of 7.0 and an optimal salt concentration between 50–100 mM.

In order to isolate the tricarboxylate carrier protein, the Triton X-100 extract of liver SMP was applied to various adsorbents and ion exchangers of liver SMP was applied to various adsorbents and ion exchangers and the eluates were analyzed for activity and SDS-gel electrophoresis. The use of CM-Sephadex, DEAE-Sephadex, alumina, celite and cellulose, for example, resulted in no, or very little, purification. Phenyl-Sepharose and octyl-Sepharose, however, completely bound the citrate carrier. A strikingly efficient purification is obtained by applying the crude extract of liver SMP to HTP (table 2). The citrate transport protein passes through HTP whereas >90% of the other proteins are retained. By chromatography on HTP the specific activity of the reconstituted citrate exchange increases 5.7-fold and 7-fold in the absence and in the presence of DPG, respectively. The total activity in the eluate after HTP in the presence of

Table 2

Purification of the tricarboxylate carrier protein by chromatography on hydroxylapatite

	Protein applied or eluted (mg)	Citrate exchange	
		Specific activity	Total activity
Experiment 1:			
SMP extract (−DPG)	4.20	40	168
HTP eluate from SMP extract (−DPG)	0.31	228	71
SMP extract (+DPG)	4.50	69	311
HTP eluate from SMP extract (+DPG)	0.31	486	151
Experiment 2:			
Mitochondrial extract (+DPG)	4.50	35	158
HTP eluate from mitochondrial extract (+DPG)	0.36	280	101

The activity of the reconstituted citrate-citrate exchange is expressed as $\mu mol \cdot 10\ min^{-1} \cdot g\ protein^{-1}$ (spec. act.) and $\mu mol \cdot 10\ min^{-1}$ (total activity)

Fig.1. SDS-gel electrophoretic pattern of the pass-through of Triton X-100-extracted liver SMP after chromatography on HTP. (A) Marker proteins: cytochrome *c* (12500, Boehringer), carbonic anhydrase (30000, Boehringer); bovine serum albumin (67000, Serva); (B) HTP eluate from beef heart SMP extract; (C) rat liver SMP extracted with Triton X-100; (D) HTP eluate from rat liver SMP extract (gel stained with Coomassie blue).

DPG accounts for 48% of that applied to the column. The SDS–PAGE of the HTP eluate derived from Triton-extracted liver SMP shows 6–7 bands (fig.1), two of which, with M_r 30000 and 33000, represent the ADP/ATP carrier and the phosphate carrier, respectively. Some bands are not present in the HTP eluate from the heart SMP extract (cf. lane D,B). In particular, the purified protein fraction containing the reconstitutively active citrate carrier (lane D) shows a peptide of M_r 27000, which has never been observed in the HTP eluate from heart both in the presence and in the absence of DPG.

Rat liver mitochondria solubilized under optimal conditions, in the presence of 1,2,3-BTA and DPG, exhibit a specific activity of reconstituted citrate exchange of 30–35 μmol.10 min^{-1}.g $protein^{-1}$. The citrate carrier protein can also be purified by directly applying the mitochondrial extract (instead of the SMP extract) to HTP (table 2). However, in this case, the activity of the pass-through is lower and the SDS-gel electrophoresis shows more protein bands. This result is in agreement with the observation [12] that the phosphate carrier protein purified by means of HTP is consistently contaminated with peptides of a M_r of about 68000 when rat liver mitochondria, instead of inner membrane vesciles, are used as starting material.

It has been reported that the tricarboxylate carrier is virtually absent in heart and brain mitochondria [13,14]. In agreement with these observations, the reconstituted [^{14}C]citrate–citrate exchange activity of the HTP eluates is very low (<26 μmol.10 min^{-1}.g $protein^{-1}$) when crude extracts of mitochondria or SMP from heart and brain (instead of liver) are applied to HTP. The substrate specificity of the purified citrate transport protein fraction was investigated by changing the anion trapped into the liposomes and by studying the effect of externally added anions on the [^{14}C]citrate–citrate exchange. The data reported in table 3 show that [^{14}C]citrate exchanges not only with internal citrate but also with *cis*-aconitrate, PEP and malate. On the other hand, virtually no [^{14}C]citrate is taken up by the proteoliposomes when they contain no anion, or anions which are not substrates of the tricarboxylate carrier like phosphate, ADP or 2-oxoglutarate (uptake <24 μmol.10 min^{-1}.g $protein^{-1}$). Among the externally added anions, *cis*-aconitrate, isocitrate, PEP and malate strongly inhibit the exchange of citrate. In contrast, *trans*-aconitrate, ADP, 2-oxoglutarate, malonate, fumarate, phosphate,

Table 3

Substrate specificity and inhibitor sensitivity of the citrate transport protein in reconstituted liposomes

Internal anion	External anions and inhibitors	Citrate exchange (μmol · 10 min^{-1} · g protein^{-1})
cis-Aconitate	[^{14}C]Citrate	389
PEP	[^{14}C]Citrate	362
Malate	[^{14}C]Citrate	353
Citrate	[^{14}C]Citrate	516
Citrate	[^{14}C]Citrate + *cis*-Aconitate	114
Citrate	[^{14}C]Citrate + *threo*-Ds-isocitrate	130
Citrate	[^{14}C]Citrate + PEP	174
Citrate	[^{14}C]Citrate + Malate	165
Citrate	[^{14}C]Citrate + 1,2,3-BTA	51
Citrate	[^{14}C]Citrate + *p*-Iodobenzylmalonate	139
Citrate	[^{14}C]Citrate + NEM 0.2 mM	425
Citrate	[^{14}C]Citrate + NEM 2 mM	310
Citrate	[^{14}C]Citrate + Mersalyl 0.2 mM	42
Citrate	[^{14}C]Citrate + *p*-Hydroxymercuribenzoate 0.2 mM	68

The HTP eluate was incorporated into liposomes containing 20 mM of the indicated anions; 2.7 mM external anions and the inhibitors 1,2,3-BTA and *p*-iodobenzylmalonate were added simultaneously with [^{14}C]citrate. The SH reagents were added 2 min before the labeled substrate at the concentrations indicated

pyruvate and aspartate have very little effect (inhibition <15%). The inhibitor sensitivity of the purified citrate transport protein is also shown in table 3. As in mitochondria, the reconstituted citrate exchange is strongly inhibited by 1,2,3-BTA, *p*-iodobenzylmalonate, 0.2 mM mersalyl and *p*-hydroxymercuribenzoate, but only slightly by NEM. Furthermore, 1,2,4-BTA, 1,2,5-BTA and known inhibitors of other mitochondrial carriers (carboxyatractyloside, butylmalonate, phthalonate and α-cyanocynnamate) have no significant effect.

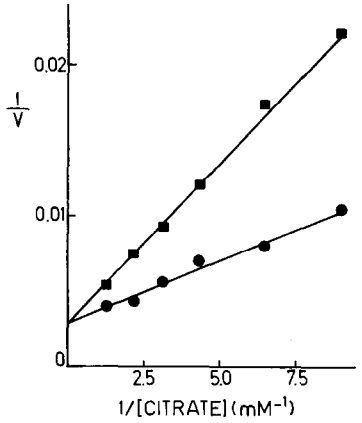

Fig.2. The dependence of the rate of citrate exchange on the external citrate concentration and the competitive inhibition by 1,2,3-BTA in liposomes reconstituted with the HTP eluate. [^{14}C]Citrate was added at the concentrations indicated. When present, 0.2 mM 1,2,3-BTA was added simultaneously with [^{14}C]citrate. (●) Control; (■) with 1,2,3-BTA; v is expressed in μmol.min^{-1}.g protein^{-1}.

By studying the time course of the reconstituted citrate–citrate exchange at 30°C, it was observed that citrate uptake increases linearly with time for about 60 s and the curve represents an exponential approach to isotopic equilibrium. The exchange of citrate therefore follows first order kinetics, as in mitochondria [4]. The dependence of the rate of citrate exchange in proteoliposomes on the externally added [^{14}C]citrate concentration, in the presence of 20 mM internal citrate, gives linear reciprocal plots (fig. 2). The K_m and V_{max}-values for citrate uptake are 0.28 mM and 338 μmol citrate.min^{-1}.g protein^{-1}, respectively, at 30°C and pH 7.0. As found in mitochondria [4], fig. 2 shows that 1,2,3-BTA is a competitive inhibitor with respect to citrate with a K_i of 0.13 mM.

4. DISCUSSION

In this paper the citrate transport protein has been purified by a simple and fast method 14-fold relative to the rat liver mitochondrial extract (cf. lines 4 and 5 of table 2). Most important for the purification of the citrate carrier protein is its property of not being adsorbed to HTP in Triton extract. This property is shared with other transport proteins, such as the ADP/ATP carrier [8] and the phosphate carrier [15,16], and other integral membrane proteins like porine [17]. The non-adsorptivity to HTP points to a very hydrophobic protein nature of the citrate carrier, as also indicated by the high concentration of Triton required for solubilization. Similarly to other transport proteins [8,18], the solubilized citrate carrier is very labile, since it is highly susceptible to the denaturating action of detergents and is rapidly inactivated unless incorporated into liposomes. Interestingly, the specific inhibitor 1,2,3-BTA and DPG can be used to preserve the protein in the native state.

The data presented also show that the citrate carrier fraction incorporated into liposomes exhibits transport properties which are distinctive features of the tricarboxylate carrier in mitochondria, like the absolute requirement for an appropriate counteranion, the tissue and the substrate specificity, and the inhibitor sensitivity. Further similarities are shown by the kinetic parameters; e.g., the K_m for citrate and the K_i for 1,2,3-BTA in liposomes are close to those found in mitochondria. The purified tricarboxylate carrier catalyzes a rate of citrate exchange in proteoliposomes of 338 μmol.min^{-1}.g protein^{-1} at 30°C, which is in the same order of that found for the isolated and reconstituted ADP/ATP carrier [19].

REFERENCES

[1] LaNoue, K.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922.
[2] Meijer, A.J. and Van Dam, K. (1981) in: Membrane Transport (Bonting, S. and De Pont J. eds) pp. 235–255, Elsevier, Amsterdam, 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., Genchi, G., Stipani, I., Riccio, P. and Quagliariello, E. (1977) Biochem. Soc. Trans. 5, 527–531.
[6] Stipani, I., Krämer, R., Palmieri, F. and Klingenberg, M. (1980) Biochem. Biophys. Res. Commun. 97, 1206–1214.
[7] Stipani, I., Barbero, M.C. and Palmieri, F. (1983) in: Symp. Structure and Function of Membrane Proteins (Fasano, May 23–26, 1983) Elsevier, Amsterdam, New York, in press.
[8] Klingenberg, M., Riccio, P. and Aquila, H. (1978) Biochim. Biophys. Acta 503, 193–210.
[9] Krämer, R. and Klingenberg, M. (1977) Biochemistry 16, 4954–4961.
[10] Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661.
[11] De Pinto, V., Tommasino, M., Palmieri, F. and Kadenbach, B. (1982) FEBS Lett. 148, 103–106.
[12] Wehrle, J.P. and Pedersen, P.L. (1983) Arch. Biochem. Biophys. 223, 477–483.
[13] Chappell, J.B. (1968) Brit. Med. Bull. 24, 150–157.
[14] Sluse, F.E., Meijer, A.J. and Tager, J.M. (1971) FEBS Lett. 18, 149–151.
[15] Wohlrab, H. (1980) J. Biol. Chem. 255, 8170–8173.
[16] Kolbe, H.V.J., Böttrich, J., Genchi, G., Palmieri, F. and Kadenbach, B. (1981) FEBS Lett. 124, 265–269.
[17] Freitag, H., Genchi, G., Benz, R., Palmieri, F. and Neupert, W. (1982) FEBS Lett. 145, 72–76.
[18] Mende, P., Kolbe, H.V.J., Kadenbach, B., Stipani, I. and Palmieri, F. (1982) Eur. J. Biochem. 128, 91–95.
[19] Krämer, R. and Klingenberg, M. (1980) FEBS Lett. 119, 257–260.