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Identification and purification of the tricarboxylate carrier from rat liver mitochondria

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The tricarboxylate carrier from rat liver mitochondria was solubilized with Triton X-100 and purified by chromatography on hydroxyapatite and celite. SDS-gel electrophoresis of the purified fraction showed a single polypeptide band with an apparent M_r of 30000. When reconstituted into liposomes, the tricarboxylate transport protein catalyzed a 1,2,3-benzenetricarboxylate-sensitive citrate/citrate exchange. We obtained a 1070-fold purification with respect to the mitochondrial extract, the recovery was 22% and the protein yield 0.02%. The properties of the reconstituted carrier, i.e., requirement for a counteranion, substrate specificity and inhibitor sensitivity, were similar to those of the tricarboxylate transport system as characterized in intact mitochondria.

Introduction

The inner membrane of liver mitochondria contains a specific carrier system for the transport of tricarboxylates which is important for fatty acid synthesis, gluconeogenesis and for the transfer of reducing equivalents across the membrane [1,2]. Since 1967, when the existence of a specific carrier for tricarboxylates has been proposed [3], the properties of this carrier have been extensively investigated in intact mitochondria [1]. Besides tricarboxylates, also phospho*enol*pyruvate, L-malate and other dicarboxylates are transported by a strict antiport mechanism. The impermeable specific inhibitor 1,2,3-benzenetricarboxylate has a higher affinity for the carrier than any substrate. The tricarboxylate carrier is also inhibited by sulphydryl reagents, but not by *N*-ethylmaleimide, and by some dicarboxylate analogues such as *p*-iodobenzylmalonate.

In contrast to other mitochondrial anion carriers except the dicarboxylate carrier, the tricarboxylate carrier is present in liver but virtually absent in heart and brain [4,5]. Although the existence of this carrier is well documented in intact mitochondria, this protein has not yet been isolated.

We have previously reported a partial purification of the tricarboxylate carrier by hydroxyapatite chromatography after solubilization of the mitochondria with Triton X-100 [6]. On SDS gels, the enriched fraction consisted of 6–7 bands of molecular mass between 27 and 36 kDa. A correlation of one of these polypeptides with the tricarboxylate carrier has not yet been achieved.

In this paper we describe the identification and the purification of the tricarboxylate carrier from rat liver mitochondria using functional reconstitution as a monitor of the carrier activity during isolation. Upon SDS gel electrophoresis the purified tricarboxylate transport protein appears to be a single polypeptide with an apparent molecular mass of 30 kDa. When incorporated into liposomes it exhibits transport properties which are very similar to those described for the tricarboxylate transport system in mitochondria.

Materials and Methods

Materials

Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad; Celite 535 from Serva; Amberlite XAD-2 from Fluka; [^{14}C]citrate from Amersham International (Amersham, U.K.); egg yolk phospholipids (phosphatidylcholine from eggs) from Fluka; Pipes and Triton X-100 from Sigma; and Sephadex G-75 from Pharmacia. Other reagents were obtained as reported [6,7].

Abbreviations: Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecyl sulphate.

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Isolation of the tricarboxylate carrier

Rat liver mitochondria prepared as described in Ref. 8 were solubilized in 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA and 10 mM Pipes (pH 7.0) at a final concentration of 20 mg protein per ml and centrifuged at $100000 \times g$ for 10 min. The tricarboxylate carrier was purified by hydroxyapatite and celite chromatography as follows: 600 μl of ultracentrifuge supernatant (extract), supplemented with cardiolipin (2 mg/ml), were applied to a hydroxyapatite column (2 cm diameter containing 6.0 g of dry material) and eluted with a buffer containing 0.5% Triton X-100 and 5 mM citrate (pH 7.0). The first 2 ml were collected. 600 μl of hydroxyapatite eluate were applied to a celite column (pasteur pipette, 0.25 g of dry material) and eluted with 2 ml of a buffer consisting of 0.5% Triton X-100, 5 mM citrate and 20 mM Na_2SO_4 (pH 7.0), followed by 2 ml of hydroxyapatite-treated buffer (see below) supplemented with 4 mg/ml cardiolipin and 20 mM Na_2SO_4. Pure tricarboxylate carrier is collected in the 1st ml of the latter buffer. The hydroxyapatite-treated buffer was prepared by passing a solution of 0.5% Triton X-100 and 5 mM citrate (pH 7.0) through a hydroxyapatite column containing 4.0 g of dry material. The first 10 ml were collected. All the operations were performed at 4° C.

Reconstitution of the tricarboxylate carrier in the liposomes

Liposomes were prepared as described previously [9] by sonication of 100 mg/ml egg yolk phospholipids in water for 60 min. Reconstitution of the protein eluates into liposomes was performed by removing the detergent with a hydrophobic ion-exchange column [10–11]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through Amberlite XAD-2 columns. The composition of the reconstitution mixture was: 10–400 μl of extract, hydroxyapatite or celite eluates (up to 100 μg protein in the case of mitochondrial extract, not more than 2 μg in the case of celite eluates), 90 μl of 10% Triton X-114, 20 μl of 20 mg/ml cardiolipin, 90 μl of 10% phospholipids in the form of sonicated liposomes, 70 μl of 100 mM Pipes (pH 7) and 35 μl of 200 mM citrate or other substrates, as indicated in the legends to tables and figures, in a final volume of 700 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5×3.6 cm) preequilibrated with a buffer containing 10 mM Pipes and 10 mM of the substrate present in the starting mixture. All the operations were performed at 4° C, except for the passage through Amberlite, which was carried out at room temperature.

Transport measurements

The substrate present outside the proteoliposomes was removed by passage through a Sephadex G-75 column preequilibrated with 50 mM NaCl and 10 mM Pipes (pH 7.0). The first 600 μl of the turbid eluate from the Sephadex column were collected, transferred to reaction vessels (150 μl each), incubated at 25° C (for 4 min), and then used for transport measurements by the inhibitor stop method [12]. Transport was initiated by adding 0.1 or 0.5 mM [^{14}C]citrate (80000 cpm) and stopped, after the desired time interval, by the addition of 10 mM 1,2,3-benzenetricarboxylate. The intraliposomal substrate was present at a concentration of 10 mM. In control samples, 1,2,3-benzenetricarboxylate was added together with the labelled substrate at time zero. In order to remove the external radioactivity, each sample was passed through a Dowex AG1-X8 column, 100–200 mesh, chloride form (0.5×4 cm equilibrated with 50 mM NaCl). The liposomes eluted with 1 ml of 50 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. The transport activity was calculated by subtracting the control from the experimental values.

Electrotransfer and immunodecoration

The proteins separated on 17.5% acrylamide slab gels were transferred to nitrocellulose [13], incubated with an antiserum raised against the ADP/ATP carrier purified from rat liver and then incubated with an anti-rabbit Ig horseradish peroxidase-linked antibody. The peroxidase reaction was performed by 20 ml of a mixture of 0.05% 4-chloro-1-naphthol, 16% methanol, 0.5% bovine serum albumin in 0.14 M NaCl, 0.01 M phosphate (pH 7.0) with the final addition of 12 μl of 30% H_2O_2.

Other methods

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [14]. The separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150 to give a high resolution of polypeptides of M_r close to 30000 [15]. Staining was performed by the silver nitrate method [16]. The molecular weights were determined by comparison to standards obtained from Sigma (Dalton mark VII-L). Protein was determined by the method of Lowry et al., modified for the presence of Triton [17]. The activity of other transport systems was assayed as described above using the following stop inhibitors: *N*-ethylmaleimide (phosphate carrier), butylmalonate (dicarboxylate carrier), phthalonate (2-oxoglutarate carrier), carboxyatractyloside (ADP/ATP carrier), pyridoxal phosphate (aspartate/glutamate carrier) and α-cyanocinnamate (pyruvate carrier).

Results and Discussion

Purification of the tricarboxylate carrier

Our standard procedures for solubilization and hydroxyapatite chromatography have been described in

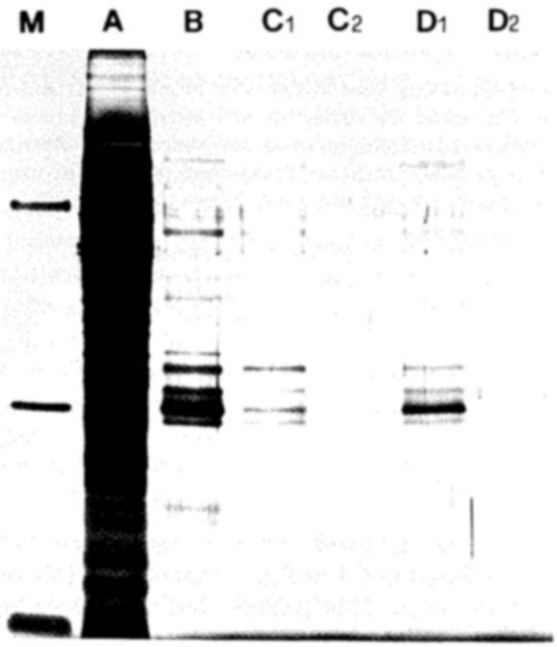

Fig. 1. Influence of cardiolipin added during celite chromatography on the elution of the citrate carrier. SDS-gel electrophoresis of hydroxyapatite and celite eluates. M, protein markers (bovine serum albumin, carbonic anhydrase and cytochrome *c*); A, mitochondrial extract; B, hydroxyapatite eluate; C1 and C2, first and second fractions of celite eluate without added cardiolipin; D1 and D2, first and second fraction of celite eluate with added cardiolipin.

Ref. 15 and in other publications for the isolation of mitochondrial metabolite carriers (for review see Ref. 18). When applying these conditions to rat liver mitochondria, the activity of the tricarboxylate carrier was significantly enriched, but still several different proteins were present. In preliminary experiments we have therefore optimized the conditions for the purification of the tricarboxylate carrier from rat liver mitochondria by hydroxyapatite chromatography. It was found that the specific activity of the reconstituted citrate/citrate exchange in the hydroxyapatite eluate increased by decreasing the ionic strength and the detergent concentration of the elution buffer as well as by decreasing the protein/hydroxyapatite ratio. Under the conditions described in Materials and Methods the eluate from hydroxyapatite contained five protein bands in the region 28–37 kDa (see Fig. 1, lane B) and several transport activities corresponding to the tricarboxylate carrier, the ADP/ATP carrier, the dicarboxylate carrier, the oxoglutarate carrier and the voltage-dependent anion channel of the outer mitochondrial membrane or porin.

In an attempt to improve the purification of the tricarboxylate carrier the eluate from hydroxyapatite was applied to a second hydroxyapatite column in the presence and in the absence of 4 mg/ml cardiolipin. Without added cardiolipin the eluate from the second hydroxyapatite did not differ at all from the starting material with respect to both specific activity and polypeptide pattern as revealed by SDS-gel electrophoresis. In the presence of cardiolipin, on the other hand, all the protein bands from 28 to 37 kDa were retained and could be eluted by a gradient of phosphate, citrate or NaCl. However, the ionic strength necessary for eluting the tricarboxylate carrier activity was identical to that required for eluting the other proteins. The results indicated that these proteins behaved similarly on hydroxyapatite and presumably also on other ion-exchangers. Thus for further purification, chromatography on celite was used. In contrast to hydroxyapatite chromatography, the eluate from celite contained citrate transport activity virtually only in the presence of cardiolipin, i.e., the reconstituted citrate/citrate exchange was 560 μmol/10 min per g protein after elution with cardiolipin and 20 μmol/10 min per g protein without cardiolipin. Both measurements were performed in the presence of 0.1 mM [^{14}C]citrate. Also the polypeptide pattern was different in the absence and in the presence of cardiolipin. As shown in Fig. 1, lanes C1 and D1, a polypeptide band of 30 kDa was much more prominent in the presence of cardiolipin. Under these conditions no protein was present in the second fraction either in the presence or in the absence of cardiolipin. Since cardiolipin proved to be necessary for the elution of the tricarboxylate carrier from celite, we subsequently tried to elute the tricarboxylate carrier bound to celite by adding cardiolipin after elution of the other proteins without added cardiolipin. We found, however, that after the elution of the protein bands in the first fraction as shown in Fig. 1, lane C1, no further protein could be eluted by adding cardiolipin (data not shown). The same result was obtained when 200 mM salt (NaCl, Na_2SO_4, phosphate or citrate) and/or 5% Triton X-100 was added together with cardiolipin. These observations suggested that not only cardiolipin but also other factors present in the hydroxyapatite pass-through (and therefore in the first fraction of celite) were necessary for eluting the tricarboxylate carrier from celite. For this reason we applied a new experimental approach for elution of this particular carrier protein from celite by addition of cardiolipin-supplemented hydroxyapatite-treated buffer (i.e., buffer passed through hydroxyapatite). Such an experiment is shown in Fig. 2. 600 μl of the hydroxyapatite pass-through were applied to celite, followed by 2 ml of buffer (0.5% Triton X-100, 5 mM citrate, 20 mM Na_2SO_4 (pH 7.0)) without cardiolipin and then by 2 ml of hydroxyapatite-treated buffer containing 4 mg/ml cardiolipin and 20 mM Na_2SO_4 (pH 7.0). The eluted fractions (600 μl the first and 1 ml the following fractions) were tested both for transport activity and polypeptide pattern by SDS gel electrophoresis. Fractions C2 and C3 did not exhibit any activity and did not contain protein. Fraction C4 had an activity of 13 500 μmol/10 min per g protein (at 0.5 mM citrate) and upon SDS-gel electrophoresis

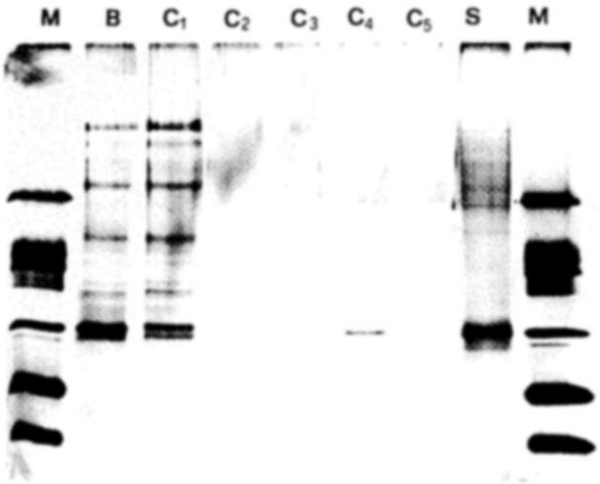

Fig. 2. Purification of the tricarboxylate carrier. SDS gel electrophoresis of fractions obtained by hydroxyapatite and celite chromatography. M, protein markers (Dalton mark VII-L from Sigma); B, hydroxyapatite eluate (50 μl); C1, first fraction of celite eluate (50 μl); C2 and C3, second and third fractions of the celite column eluted with 0.5% Triton X-100, 5 mM citrate and 20 mM Na_2SO_4 (pH 7.0) (1 ml); C4 and C5, fourth and fifth fractions eluted with hydroxyapatite-treated buffer containing 4 mg/ml cardiolipin and 20 mM Na_2SO_4 (pH 7.0) (1 ml); fraction S was eluted with 1 ml 1% SDS (200 μl).

showed a single protein band of 30 kDa. It should be noted that the samples applied to lanes B and C1 of Fig. 2 corresponded to 50 μl eluate, while those applied to lanes C4 and C5 corresponded to 1 ml eluate. This means that the 30 kDa protein eluted in C4 of Fig. 2, to which the tricarboxylate carrier activity was associated, was only a small fraction of the total amount of the 30 kDa protein band applied to celite. In fact, the major part of this band was retained by celite and could be extracted only by addition of 1% SDS (Fig. 2, lane S).

TABLE I

Purification of the tricarboxylate carrier

The proteoliposomes were loaded with 10 mM citrate and the exchange was started by adding 0.5 mM external [^{14}C]citrate. Other conditions as described in Materials and Methods. The activity of the reconstituted citrate exchange is expressed in μmol/10 min per g protein (specific activity) and μmol/10 min (total activity).

	Protein (μg)	Citrate exchange		
		specific activity	total activity	purification (fold)
Mitochondrial extract	11200	12.6	141	1
Hydroxyapatite eluate	200	630	126	50
Celite eluate	2.3	13500	31	1071

The total reconstituted tricarboxylate carrier activity present in fraction C4 of Fig. 2 represented 25% of that applied to celite. This finding may be due to partial inactivation and/or incomplete elution from celite.

The complete purification procedure for isolating the tricarboxylate carrier from rat liver mitochondria is presented in Table I. The specific activity was increased 1071-fold with respect to that of the mitochondrial extract. Approx. 22% of the total transport activity was recovered with a protein yield of 0.02%.

So far, the 30 kDa protein band from mitochondria which passes through hydroxyapatite has always been ascribed solely to the ADP/ATP carrier [15,18–20]. Since the fraction isolated above containing a high activity of reconstituted citrate/citrate exchange consisted of a single protein band of 30 kDa, it was important to test whether ADP/ATP carrier was still present in this fraction. Since ADP/ADP exchange activity could not be found in this fraction (see below),

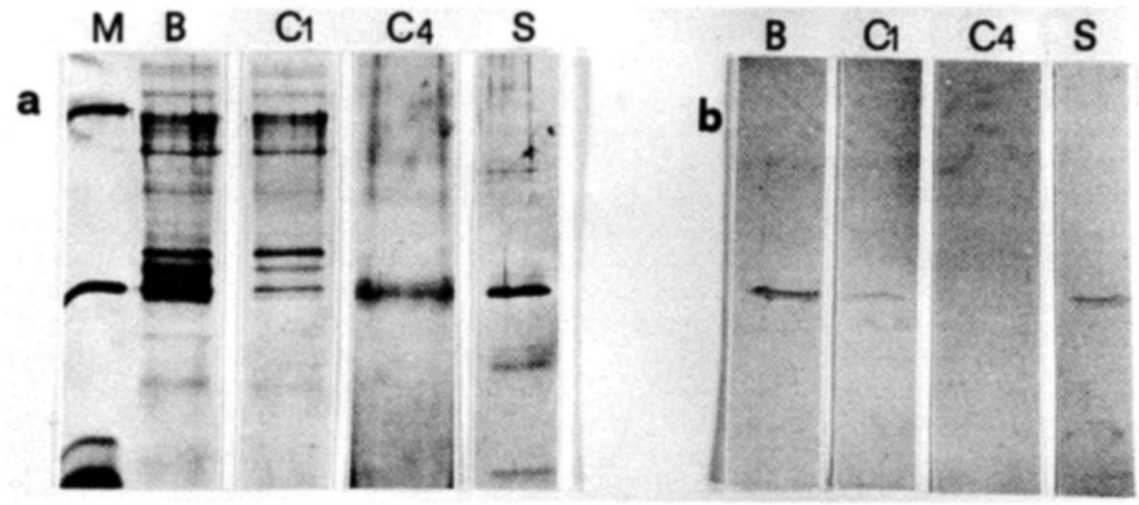

Fig. 3. Immunoblotting of different chromatographic fractions with an antiserum specific for the ADP/ATP carrier. (a) SDS-polyacrylamide gel electrophoresis; (b) immunoblotting. Lanes: M, protein markers (bovine serum albumin, carbonic anhydrase and cytochrome *c*); B, hydroxyapatite eluate (50 μl); C1, first fraction of celite eluate (50 μl); C4, purified tricarboxylate carrier obtained as in Fig. 2 (4 ml); S, SDS extract from celite (200 μl).

the presence of functionally active ADP/ATP carrier could be excluded. In order to test whether an ADP/ATP carrier in denaturated form still represented by the 30 kDa protein was present, the reactivity of this band with an antiserum raised against the ADP/ATP carrier purified from rat liver mitochondria was investigated (Fig. 3). The immunoblotting shown in Fig. 3b demonstrated that there was no reactivity of the specific antiserum against the ADP/ATP carrier with the pure preparation of the tricarboxylate carrier (lane C4). In contrast, the antiserum in fact reacted with the 30 kDa band from the hydroxyapatite eluate (lane B), from the first fraction of celite eluate (lane C1) and from the SDS extract obtained from celite (lane S). It should be noted that even the small amount of ADP/ATP carrier as present in lane C1 was sufficient to be recognized by the antibody.

Properties of the reconstituted tricarboxylate carrier

In all the experiments described in the following, the fraction shown in Fig. 2, lane C4, consisting of a single protein band with an apparent molecular weight of 30000 was used for reconstitution.

In Fig. 4, the time-course of the 1,2,3-benzenetricarboxylate-sensitive [^{14}C]citrate uptake by proteoliposomes which have been loaded with unlabelled citrate is reported. Citrate uptake increased linearly with time for about 1 min at a rate of 0.84 mmol/min per g protein at 25°C (at 0.1 mM citrate). A total amount of 11.9 mmol citrate/g protein was taken up into the proteoliposomes. In the absence of internal substrate, uptake of citrate could not be observed. Both the absolute dependence on an appropriate counteranion and the inhibition by the specific inhibitor 1,2,3-benzenetricarboxylate clearly indicate that the purified tricarboxylate carrier has been reconstituted.

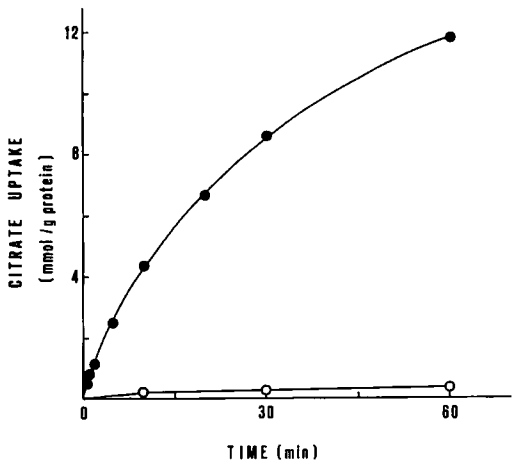

Fig. 4. Time-course of citrate uptake in reconstituted liposomes. 0.1 mM [^{14}C]citrate was added at time zero to reconstituted liposomes with 10 mM citrate as internal substate (●), or to reconstituted liposomes without internal substrate (○).

TABLE II

Dependence of citrate transport in reconstituted liposomes on internal substrate

The proteoliposomes were loaded with the indicated substrates. Transport was initiated by the addition of 0.1 mM [^{14}C]citrate.

Internal substrate (10 mM)	Citrate transport (μmol/10 min per g protein)
−(Cl^-)	120
Citrate	4500
cis-Aconitate	4620
threo-D_S-Isocitrate	3960
1,2,3-Propanetricarboxylate	4260
Phospho*enol*pyruvate	3750
L-Malate	3140
trans-Aconitate	510
ATP	90
2-Oxoglutarate	270
Phosphate	30

The dependence of the tricarboxylate carrier activity on intraliposomal counteranions was further investigated in proteoliposomes loaded with a variety of substrates. The intraliposomal concentration of the anions used was 10 mM and the exchange time was 10 min. The data reported in Table II show that labelled citrate could be exchanged not only against citrate but also against internal *cis*-aconitate, isocitrate, 1,2,3-propanetricarboxylate, phospho*enol*pyruvate and L-malate which are also known to be substrates of the tricarboxylate carrier in mitochondria [1,7,21]. In contrast, no significant exchange activity was found using *trans*-aconitate or substrates of other mitochondrial carriers, like ATP, 2-oxoglutarate and phosphate.

Also the sensitivity of the reconstituted citrate/citrate exchange to externally added substrates and inhibitors was investigated. Table III, Expt. 1, shows that citrate exchange was inhibited strongly by *cis*-aconitate, isocitrate, phospho*enol*pyruvate and L-malate, and less efficiently by succinate, malonate, phenylsuccinate and butylmalonate. In contrast, *trans*-aconitate, D-malate, fumarate, ATP, oxoglutarate, glutamate, aspartate, phosphate and pyruvate had very little effect (inhibition < 14%) (not shown). In addition, citrate exchange was inhibited by 1,2,3-benzenetricarboxylate, *p*-iodobenzylmalonate, mersalyl and *p*-hydroxymercuribenzoate, but only slightly by 1,2,4-benzenetricarboxylate, 1,3,5-pentanetricarboxylate and *N*-ethylmaleimide (Table III, Expt. 2). Also 1,2,5-benzenetricarboxylate, carboxyatractyloside, phthalonate and α-cyanocinnamate were found to be almost ineffective (not shown). The same inhibition pattern was observed in mitochondria [1,7].

In further experiments (not shown), we found that the pure preparation of the 30 kDa protein as shown in Fig. 2, lane C4, when reconstituted into liposomes did not catalyze the exchange reactions ADP-ADP (adenine

TABLE III

Sensitivity of citrate/citrate exchange in reconstituted liposomes to externally added substrates and inhibitors

The proteoliposomes were loaded with 10 mM citrate and the exchange was started by adding 0.1 mM external [^{14}C]citrate. The SH reagents were added 2 min before the labelled substrate at 1 mM concentration. The other inhibitors and external anions were added together with [^{14}C]citrate at a concentration of 1 mM. The control values of uninhibited citrate exchange were 4420 and 4560 μmol/10 min per g protein in Expts. 1 and 2, respectively.

Addition	% Inhibition
Expt. 1	
cis-Aconitate	88
threo-D_S-Isocitrate	81
Phospho*enol*pyruvate	85
L-Malate	73
Succinate	50
Malonate	42
Phenylsuccinate	48
Butylmalonate	39
Expt. 2	
1,2,3-Benzenetricarboxylate	96
1,2,4-Benzenetricarboxylate	18
1,3,5-Pentanetricarboxylate	7
p-Iodobenzylmalonate	54
Mersalyl	96
p-Hydroxymercuribenzoate	93
N-Ethylmaleimide	16

nucleotide carrier), 2-oxoglutarate-2-oxoglutarate (2-oxoglutarate carrier), malate-phosphate (dicarboxylate carrier), aspartate-aspartate (aspartate-glutamate carrier), pyruvate-pyruvate (pyruvate carrier) and phosphate-phosphate (phosphate carrier). Thus, the purified tricarboxylate carrier obviously is not contaminated by other mitochondrial anion-transporting systems.

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