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Purification and reconstitution of two anion carriers from rat liver mitochondria: the dicarboxylate and the 2-oxoglutarate carrier

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Two anion-transporting systems, i.e., the dicarboxylate carrier and the 2-oxoglutarate carrier, have been purified from rat liver mitochondria and functionally identified. The dicarboxylate carrier has been isolated in active form by hydroxyapatite chromatography after partial removal of the solubilizing detergent Triton X-114 from the mitochondrial extract. The SDS gel electrophoresis of this preparation consists mainly of one protein band with an apparent M_r of 28000, identified as the dicarboxylate carrier. Complete purification of the 28 kDa protein in inactive form has been achieved by sequential chromatography on hydroxyapatite and Celite followed by SDS extraction of the retained protein. The 2-oxoglutarate carrier has been purified by hydroxyapatite chromatography after extensive removal of Triton X-114 from the detergent extract. SDS gel electrophoresis of the purified fraction shows a single band with an apparent M_r of 32500. When reconstituted into liposomes, the functional properties of the two isolated carrier proteins resemble closely those of the dicarboxylate and the 2-oxoglutarate transport systems characterized in mitochondria.

Introduction

The inner mitochondrial membrane is equipped with anion-transporting systems which catalyze net flux or exchange of physiologically important metabolites between the cytosol and the matrix of mitochondria (for a review, see Ref. 1).

The properties of these carriers have been extensively investigated in intact mitochondria. Thus, at least eight anion carriers have been well characterized and functionally differentiated. On this basis, the existence of two separate transport systems for dicarboxylic acids in the mitochondrial membrane has been proposed, i.e., the dicarboxylate carrier and the 2-oxoglutarate carrier. Both systems catalyze an electroneutral exchange of substrate anions. While the 2-oxoglutarate carrier transports only 2-oxoglutarate and some other dicarboxylates [2–4], the dicarboxylate carrier accepts not only dicarboxylates (but not 2-oxoglutarate), but also phosphate and some sulphur-containing compounds, e.g., sulphate and thiosulphate [2,5–7]. Both carriers are inhibited by sulphydryl reagents (but not by *N*-ethylmaleimide) and by some dicarboxylate analogues like butylmalonate [2–12]. The 2-oxoglutarate carrier is strongly inhibited by phthalonate, whereas the dicarboxylate carrier is only slightly affected [13]. Furthermore, the 2-oxoglutarate carrier is active

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

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both in liver and heart [3,14–15], whereas the dicarboxylate carrier has low activity in heart mitochondria [16].

The final proof for the existence of a particular carrier protein is its isolation. We have recently reported the purification of the 2-oxoglutarate carrier from heart mitochondria [17]. On SDS gels, it consists of a single protein of M_r 31500 and corresponds to band 4 of the five protein bands present in the hydroxyapatite pass-through of Triton-extracted heart mitochondria. The dicarboxylate carrier, on the other hand, has been only partially purified from liver [18–19]. The enriched fraction contained at least five to seven polypeptides of M_r 27000–36000 which is the size of most of the mitochondrial anion carriers identified so far [17,20–23]. It is not yet known which of these bands represents the dicarboxylate carrier.

The isolation of mitochondrial anion carriers from liver appears to be more difficult as compared to heart. So far, only the phosphate carrier has been obtained in a pure state from liver [23]. Its isolation needs a much more elaborate procedure than the corresponding heart carrier. Similarly for the 2-oxoglutarate carrier, the procedure developed for its isolation from heart [17] does not result in a pure preparation when applied to liver (unpublished data).

In this paper, we describe the purification of both the dicarboxylate and the 2-oxoglutarate carrier from rat liver mitochondria using functional reconstitution as a monitor of the carrier activity during isolation.

Materials and Methods

Materials

Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, 2-[1-^{14}C]oxoglutarate, L-[U-^{14}C]malate, [^{32}P]phosphate, [2-^{3}H]ADP, L-[U-^{14}C]aspartate, [1,5-^{14}C]citrate and [1-^{14}C]pyruvate from Amersham International, U.K., phospholipids (phosphatidylcholine from fresh turkey egg yolk), Pipes and Triton X-114 from Sigma, cardiolipin from Avanti-Polar Lipids, α-cyanocinnamate from R. Emmanuel Wembley. Phthalonic acid was a gift from Drs. G. Randazzo and A. Evidente. Other reagents were obtained as reported [3,5]. All other reagents were of the highest purity commercially available.

Isolation of the dicarboxylate carrier

Rat liver mitochondria prepared as described in Ref. 11 were solubilized in 3% Triton X-114 (w/v)/20 mM Na_2SO_4/1 mM EDTA/10 mM Pipes (pH 7) at a final concentration of 20 mg protein/ml. After 2 min at 0°C, the mixture was centrifuged at 138000 × *g* for 10 min.

The dicarboxylate carrier was purified in active form by the Amberlite/hydroxyapatite procedure as follows: 1 ml of supernatant, supplemented with cardiolipin (4 mg/ml), was passed four times though an Amberlite column XAD-2 (pasteur pipettes, 0.3 g of dry material) preequilibrated with 10 mM Pipes (pH 7). The eluate (1 ml) was applied to a hydroxyapatite column (0.7 cm diameter containing 2.0 g of dry material) and eluted at 4°C with the solubilization buffer containing 0.1% Triton X-114 instead of 3%. The first 0.5 ml was collected.

For complete purification of the dicarboxylate carrier in inactive form, the hydroxyapatite/Celite procedure was used: 1 ml of supernatant supplemented with cardiolipin (4 mg/ml) was applied on hydroxyapatite columns (0.7 cm diameter, 2.0 g of dry material) and eluted as described above. The first 500 μl of the eluate from two hydroxyapatite columns was pooled and applied to Celite columns (pasteur pipettes, 0.4 g of dry material). Elution was performed at 4°C with the solubilization buffer containing 200 mM Na_2HPO_4 (2 ml), and then with 2% SDS. The 1st ml of the SDS eluate was collected.

Isolation of the 2-oxoglutarate carrier

1 ml of supernatant, obtained by solubilization of the mitochondria and subsequent centrifugation (see above), was passed six times through an Amberlite column (pasteur pipettes, 0.75 g). By this procedure, the concentration of Triton X-114 was decreased from 3% to approx. 0.4%. The eluate (1 ml) was applied to a hydroxyapatite column (0.7 cm diameter, containing 2.0 g of dry material). Elution was performed at 4°C with 1.5

ml 0.1% Triton X-114, followed by 1% Triton plus 4 mg/ml cardiolipin. Fractions of 0.5 ml were collected. The 2-oxoglutarate carrier was specifically eluted by the addition of 1% Triton plus cardiolipin (fractions 6–8).

Reconstitution of the protein eluates

The hydroxyapatite eluates containing the dicarboxylate or the 2-oxoglutarate carrier (see above) were passed through Dowex AG1-X8 columns, 100–200 mesh, acetate form (0.5 × 5 cm equilibrated with H_2O), in order to remove the anions present, i.e., phosphate arising from the dry hydroxyapatite, and sulphate from the solubilization buffer. Liposomes were prepared as described previously [17] 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 [24–26]. In this procedure, the mixed micelles containing detergent, proteins and phospholipids were repeatedly passed through Amberlite columns. The composition of the mixture used for reconstitution was: 200 μl of hydroxyapatite or Dowex AG1-X8 eluate (2–15 μg protein), 100 μl of 10% Triton X-114, 8 mg of phospholipids in the form of sonicated liposomes, 20 mM phosphate or other substrates as indicated in the legends to tables and figures, 10 mM Pipes (pH 7), in a final volume of 0.68 ml. After vortexing, this mixture was passed 15 times through an Amberlite column (0.5 × 4.5 cm) preequilibrated with a buffer containing 10 mM Pipes and 20 mM of the substrate present in the starting mixture. All the operations were performed at 4° C, except the passage through Amberlite, which was carried out at room temperature.

Transport measurements

The substrate present outside the proteoliposomes was removed by passing 600 μl of the liposomal suspension through a Sephadex G-75 column preequilibrated with 50 mM NaCl/10 mM Pipes (pH 7.0). The first 750 μl of the slightly turbid eluate from the Sephadex column, containing the proteoliposomes, 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 [27]. Transport was initiated by adding the labelled substrate and stopped after the desired time interval by the addition of the appropriate inhibitor. The activity of the dicarboxylate carrier was assayed using 0.1 mM [^{14}C]malate or 0.1–0.2 mM [^{32}P]phosphate as external substrates and 10 mM butylmalonate as inhibitor, the activity of the 2-oxoglutarate carrier using 0.1 mM [^{14}C]2-oxoglutarate as external substrate and 10 mM phthalonate as inhibitor. The intraliposomal substrate was present at a concentration of 20 mM. In some experiments performed with fractions containing both the dicarboxylate and the 2-oxoglutarate carriers, 10 mM pyridoxal phosphate was used as inhibitor, since this is a rapid and effective inhibitor of the 2-oxoglutarate carrier [26] and of the dicarboxylate carrier (unpublished data). In control samples, the inhibitor was added together with the labelled substrate at zero time. In order to remove the external radioactivity, each sample was passed through a Dowex AG1-X8 column, 100–200 mesh, acetate 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, and vortexed and counted. The transport activity was calculated by subtracting the control from the experimental values.

Other methods

Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [28]. The separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150 to give a high resolution of polypeptides of an M_r value close to 30000 [21]. Staining was performed by the Silver nitrate method [29]. The molecular weights were determined by comparison to Pharmacia low-molecular-weight standards. Protein was determined by the method of Lowry et al. modified for the presence of Triton [30]. The activity of other transport systems was assayed as described above using the following stop inhibitors: *N*-ethylmaleimide (phosphate carrier), benzene-1,2,3-tricarboxylate (tricarboxylate carrier), carboxyatractyloside (ADP/ATP carrier), pyridoxal phosphate (aspartate/glutamate carrier) and α-cyanocinnamate (pyruvate carrier).

Results

Purification of the dicarboxylate and the 2-oxoglutarate carrier

Many of the substrate carriers from the inner mitochondrial membrane have been isolated by application of mitochondrial protein solubilized by Triton in media of low ionic strength onto small hydroxyapatite columns [17–19,21,23,31–35]. Fig. 1 (lanes A_1 and A_2) shows that the hydroxyapatite eluate obtained from rat liver mitochondria contains many more protein bands than the eluate obtained from heart mitochondria under similar conditions (cf. Ref. 21). This was the case even in presence of cardiolipin, a phospholipid known to decrease the number of elutable proteins [21,23,32,34]. As can be seen in lanes A_1 and A_2 (Fig. 1), two main protein bands are evident in the M_r region around 30000 with an apparent M_r of 32500 and 28000, respectively. Also present are bands in the range 35–45 kDa and others around 68 kDa. The first fraction of the hydroxyapatite eluate from rat liver mitochondria (lane A_1 of Fig. 1) was tested for reconstitutable activity of the dicarboxylate and the 2-oxoglutarate carrier, respectively. The presence of both carriers is indicated by the fact that the reconstituted malate–phosphate exchange showed a specific activity of 1680 nmol/10 min per mg protein and the reconstituted 2-oxoglutarate–2-oxoglutarate exchange showed a specific activity of 1220 nmol/10 min per mg protein. Elution of the hydroxyapatite column with 0.1% instead of 3% Triton X-114 led to an increase in the specific activity of both the dicarboxylate (18%) and the 2-oxoglutarate carrier (35%). Analysis on SDS gels of the 0.1% Triton X-114 hydroxyapatite eluate revealed the disappearance of some polypeptides in comparison to the 3% Triton X-114 eluate (cf. lanes B_1 and B_2 with A_1 and A_2).

In order to purify the two carriers specific for dicarboxylic acids further, the amount of hydroxyapatite per column was increased from 0.6 to 2.0 g. Lane C_1 shows that the polypeptide pattern of

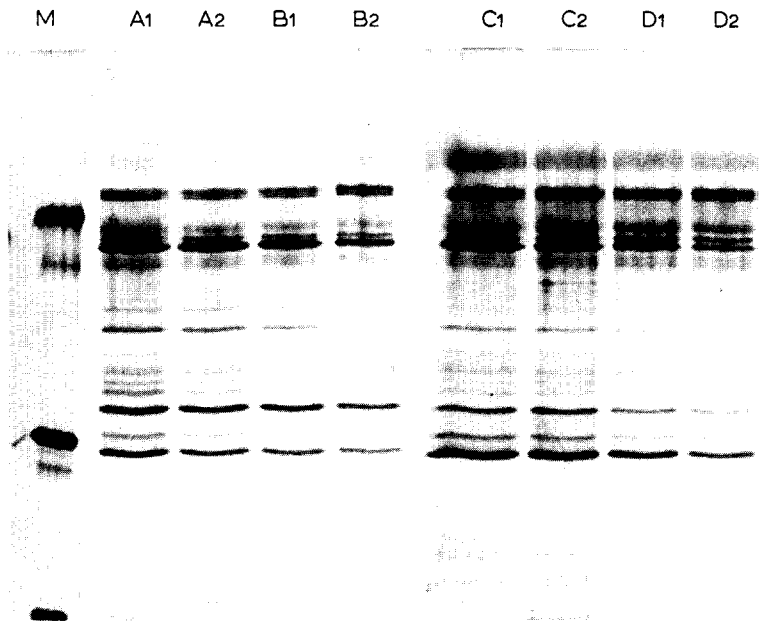

Fig. 1. Influence of the amount of hydroxyapatite and the Triton X-114 concentration on the polypeptide pattern obtained after hydroxyapatite chromatography of solubilized rat liver mitochondria. The mitochondrial extract supplemented with cardiolipin was applied to columns containing 0.6 g (A, B) or 2.0 g (C, D) hydroxyapatite, as described in Materials and Methods. The columns were eluted with the solubilization buffer containing either 3% (A, C) or 0.1% (B, D) Triton X-114. Subscripts 1 and 2 indicate the first and the second fraction of the eluate, respectively. Lane M, molecular weight markers: from the top to the bottom, bovine serum albumin (68000), carbonic anhydrase (30000) and cytochrome *c* (12500).

the first fraction of the eluate obtained from the larger column does not differ markedly from that obtained with the smaller column (lane A_1). However, the ratio between the bands with Mr 32 500 and 28 000 was changed significantly; using 2.0 g of hydroxyapatite, the band of 28 kDa increased in the eluate with respect to the 32.5 kDa band. Concomitantly, these conditions increased the ratio between the activities of the dicarboxylate and the 2-oxoglutarate carrier. Elution of the larger hydroxyapatite column with 0.1% instead of 3% Triton X-114 further increased the activity of the dicarboxylate carrier to 2460 nmol/10 min per mg protein, whereas the 2-oxoglutarate carrier activity diminished slightly to 740 nmol/10 min per mg protein. The corresponding polypeptide pattern is shown in lane D_1 of Fig. 1.

The finding that the amount of Triton X-114 present in the elution medium is a critical parameter suggested that a decreased detergent concentration after extraction might be advantageous. The Triton concentration was decreased to 0.43% by passage of the 3% Triton X-114 extract through Amberlite. This procedure, i.e., lowering the Triton concentration to 0.43%, does not cause any decrease in the activity of the dicarboxylate carrier or of the oxoglutarate carrier present in the mitochondrial extract. The Triton-depleted extract was then applied to hydroxyapatite and eluted with 0.1% Triton X-114. As shown in Fig. 2, the first two fractions of the eluate contained only proteins of high M_r (lanes 1 and 2). These proteins, when reconstituted into liposomes, showed no dicarboxylate or 2-oxoglutarate carrier activity. 0.1% Triton X-114 failed to elute further proteins (fractions 3–5). On the other hand, when 1% Triton X-114 plus cardiolipin were added to the elution buffer, only one single protein band with a M_r of 32 500 appeared in the eluate (see lanes 6–8 of Fig. 2a). This protein has been functionally identified as the 2-oxoglutarate carrier (see below). Starting with 20 mg mitochondrial protein, 6.3 μg of pure 2-oxoglutarate carrier were obtained. The specific activity of the reconstituted 2-oxoglutarate–2-oxoglutarate exchange was 2510 nmol/10 min per mg protein, i.e., 165-fold higher than that of the mitochondrial extract. This purification factor gives an estimation for the enrichment of

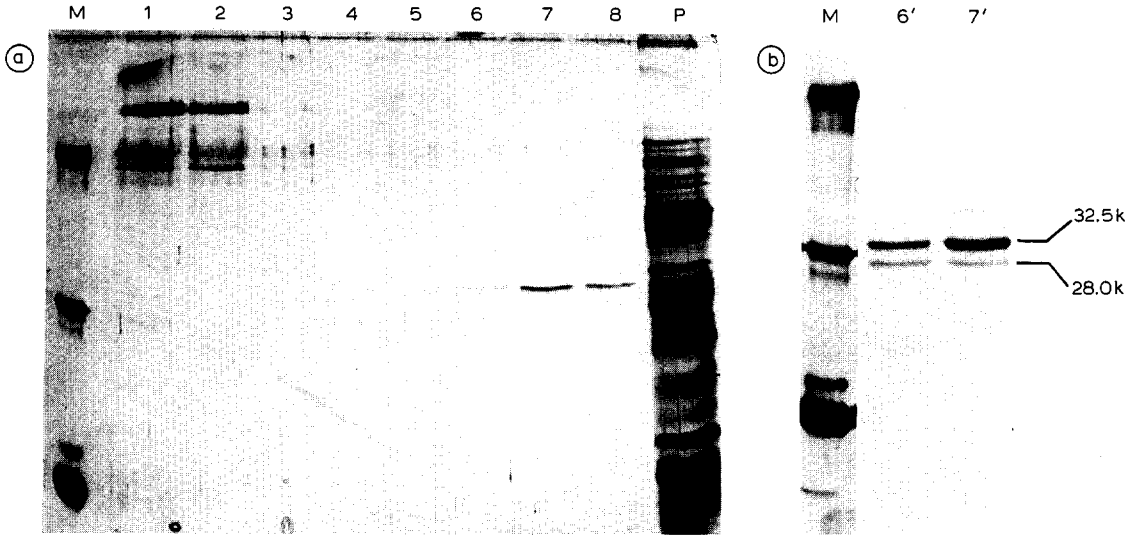

Fig. 2. (a) Purification of the 2-oxoglutarate carrier and (b) co-purification of the dicarboxylate and the 2-oxoglutarate carriers. SDS gel electrophoresis of the fractions obtained by hydroxyapatite chromatography of the detergent-depleted (0.43%) Triton X-114 extract. In (a), fractions 1–5 were eluted with 0.1% Triton X-114, fractions 6–8 containing the purified 2-oxoglutarate transport protein were eluted with 1% Triton X-114 plus 4 mg/ml cardiolipin. In (b) the conditions were the same as in (a), except that fractions 6′ and 7′, containing the dicarboxylate and the 2-oxoglutarate carriers, were eluted with 1% Triton X-114/20 mM Na_2SO_4/10 mM Pipes (pH 7.0)/4 mg/ml cardiolipin. Fraction P was eluted with 200 mM Na_2HPO_4/1.5% Triton X-114/10 mM Na_2SO_4/5 mM Pipes (pH 7.0). Lanes M, marker proteins (bovine serum albumin, carbonic anhydrase and cytochrome *c*).

the carrier protein. However, purification factors based on reconstituted activities are in general not very accurate due to possible inactivation during purification and incorporation into liposomes.

No selective elution of other bound proteins could be observed with 1% (or higher) Triton X-114, either with or without added salts. However, under defined conditions (Fig. 2b, lanes 6′ and 7′), only the protein bands with M_r 32500 and 28000 were eluted. The fractions containing these two bands, when reconstituted into liposomes, exhibited the activities of both the 2-oxoglutarate and the dicarboxylate carrier. In fractions 6′ and 7′, the activity of the malate–phosphate exchange was 2360 and 2140 nmol/10 min per mg protein and that of the 2-oxoglutarate–2-oxoglutarate exchange, 1330 and 1850 nmol/10 min per mg protein, respectively. Since the 32.5 kDa protein is the oxoglutarate carrier, this experiment provides strong evidence for the identification of the dicarboxylate carrier with the 28 kDa protein. Unfortunately, it was not possible to elute pure 2-oxoglutarate carrier and dicarboxylate carrier one after the other, since the elution of the former by 1% Triton plus cardiolipin (lanes 6–8, Fig. 2a) was not complete.

In order to purify the dicarboxylate carrier, we examined the effect of partial removal of detergent from the extract on specific carrier elution. Passage of the 3% Triton X-114 extract through 0.3 g Amberlite 2, 4 and 6 times decreased the detergent concentration to approx. 2.2, 1.5 and 1.0%, respectively: these extracts were applied to 2.0 g hydroxyapatite and eluted with 0.1% Triton. As shown in Fig. 3 (lanes A–C), the first fraction of these eluates contained the 28 kDa protein, together with a few bands of high M_r. These bands were all present in lanes 1 and 2 of Fig. 2; their behaviour on SDS gels was identical, especially as regards molecular weight. With the extract containing 2.2% Triton, but not with those containing 1.5 or 1.0% Triton, a faint band of M_r slightly higher than 30000 was also present. The amount of the 28 kDa protein which was re-

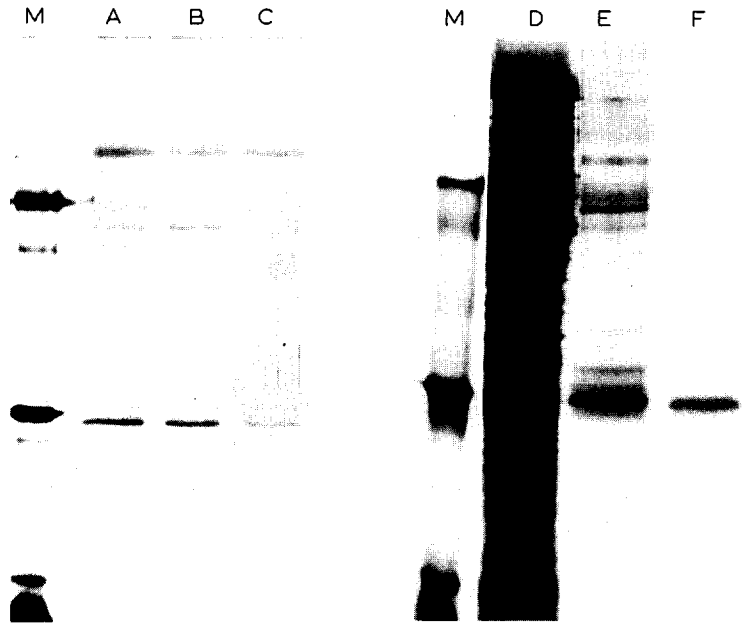

Fig. 3. Purification of the dicarboxylate carrier by the Amberlite/hydroxyapatite and the hydroxyapatite/celite procedures. SDS gel electrophoresis of the eluates (first fractions) obtained using the Amberlite/hydroxyapatite procedure (A, B and C) described in Materials and Methods. The passage through Amberlite was repeated two times (A), four times (B) and six times (C). F shows the purified dicarboxylate carrier prepared by the hydroxyapatite/celite procedure (see Materials and Methods). E, hydroxyapatite eluate applied to celite column. D, mitochondrial extract in 3% Triton X-114; M, marker proteins (bovine serum albumin, carbonic anhydrase and cytochrome *c*).

covered from the column diminished as the Triton concentration in the extract decreased. Therefore, the best preparation of the 28 kDa protein was obtained by hydroxyapatite chromatography of the extract in which the Triton X-114 was decreased to 1.5% (Fig. 3, lane B). This preparation, when reconstituted into liposomes, exhibited a high dicarboxylate carrier activity (see below). The specific activity was increased 226-fold by the entire purification procedure with respect to that of the mitochondrial extract.

Purification of inactive dicarboxylate carrier

Further attempts at complete purification of the 28 kDa protein were made starting from the first fraction of the hydroxyapatite eluate obtained from the partially detergent-depleted (1.5%) Triton X-114 extract. First, after further removal of the detergent down to about 0.4%, the eluate was rechromatographed on hydroxyapatite. The proteins of high M_r passed through, whereas the 28 kDa protein was retained. However, it was no longer possible to elute the protein of 28 kDa by increasing the concentration of Triton and/or the ionic strength. Even SDS (2%) failed to elute this protein. It should be mentioned that the non-retarded high M_r proteins did not show any transport activity of the anion carriers assayed in this study.

Second, the hydroxyapatite eluate was chromatographed on celite. In this case, too, the 28 kDa protein was retained, but it was easily eluted in pure form by SDS (lane F of Fig. 3). This protein, however, could not be functionally reconstituted in liposomes, presumably because of the use of SDS for elution. The high M_r proteins were not retained by celite nor did they exhibit any malate–phosphate exchange activity when reconstituted in liposomes.

Properties of the reconstituted dicarboxylate carrier

In all experiments described in this section, the fraction shown in Fig. 3, lane B (containing mainly the 28 kDa protein band) was used for reconstitution.

Table I shows that there was a considerable amount of butylmalonate-sensitive uptake of malate and phosphate in proteoliposomes prepared by incorporating the hydroxyapatite eluate,

TABLE I

EFFECT OF REMOVING PHOSPHATE AND SULPHATE IONS FROM THE HYDROXYAPATITE ELUATE BEFORE THE RECONSTITUTION OF THE PURIFIED DICARBOXYLATE CARRIER

The dicarboxylate carrier was purified by the Amberlite/hydroxyapatite procedure as described in Materials and Methods. Where indicated, before incorporation into liposomes, the hydroxyapatite eluate was passed through Dowex in order to remove phosphate arising from the hydroxyapatite chromatography and sulphate from the solubilization buffer. Transport was initiated by adding 0.1 mM [^{14}C]malate or 0.2 mM [^{32}P]phosphate to proteoliposomes loaded with or without 20 mM phosphate. After 10 min, the reaction was stopped by adding 10 mM butylmalonate.

Liposomes loaded with	Uptake (nmol/10 min per mg protein)			
	hydroxyapatite eluate-reconstituted liposomes		Dowex eluate-reconstituted liposomes	
	malate	phosphate	malate	phosphate
Phosphate	3480	2850	1670	1330
–	1810	1470	71	68

even without addition of phosphate during the preparation of the vesicles. Both malate and phosphate uptake were virtually abolished when the hydroxyapatite eluate was passed through an anion exchange column (Dowex AG1-X8) before the incorporation. These results could be accounted for by the fact that the hydroxyapatite eluate contains 20 mM sulphate (from the solubilization buffer) and 2–6 mM phosphate (from the dry hydroxyapatite), and that these two anions are able to exchange with malate or phosphate in a reaction catalyzed by the dicarboxylate carrier [6]. Thus, in order to measure transport activities in the absence of internal substrates or in the presence of substrates different from sulphate and phosphate, the hydroxyapatite eluate had to be passed through anion-exchange columns before incorporation. This procedure may lead to partial inactivation of the dicarboxylate carrier, as indicated by the decrease of the butylmalonate-sensitive malate–phosphate and phosphate–phosphate exchanges observed in Table I. A similar inactivation caused by passage through Dowex has been reported for the 2-oxoglutarate carrier purified from heart mitochondria [36].

In Fig. 4, the time-course of two exchange

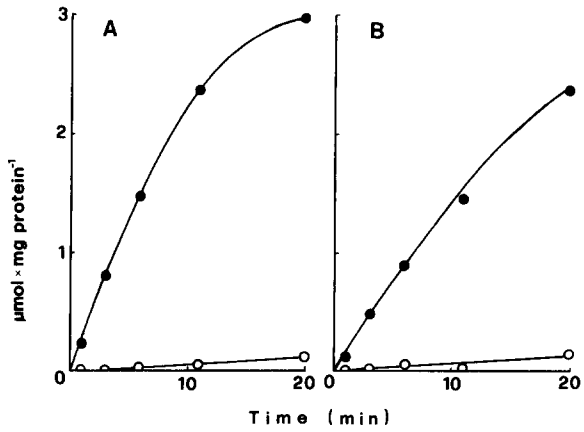

Fig. 4. Time-course of [^{14}C]malate or [^{32}P]phosphate uptake into reconstituted liposomes. The dicarboxylate carrier was purified by the Amberlite/hydroxyapatite procedure as described in Materials and Methods. Before incorporation into liposomes the hydroxyapatite eluate was passed through Dowex. (A) 0.1 mM [^{14}C]malate was added to proteoliposomes loaded with 20 mM phosphate (●———●) or with 20 mM NaCl (○———○). (B) 0.2 mM [^{32}P]phosphate was added to proteoliposomes loaded with 20 mM malate (●———●) or with 20 mM NaCl (○———○). Transport was stopped at the indicated time by adding 10 mM butylmalonate. Similar results were obtained when fumarate or oxoglutarate were substituted for NaCl in the controls.

activities is reported: [^{14}C]malate against internal phosphate (Fig. 4A), and [^{32}P]phosphate against internal malate (Fig. 4B). The initial transport rates were 225 ([^{14}C]malate–phosphate exchange) and 150 ([^{32}P]phosphate–malate exchange) nmol/min per mg protein at 25 °C. In the absence of internal substrate, the uptake of both malate and phosphate was negligible. In intact mitochondria, the dicarboxylate carrier catalyzes an obligatory counterexchange of anions [37–38]. The exchange activities catalyzed by the reconstituted protein, i.e., malate–phosphate and phosphate–phosphate exchange, but not unidirectional transport of these anions (Table I and Fig. 4), and the observed inhibition by butylmalonate, clearly indicate that the purified dicarboxylate carrier has been reconstituted.

The dependence of the dicarboxylate carrier activity on intraliposomal counteranions was further investigated in proteoliposomes loaded with a variety of substrates. The intraliposomal concentration of the anions used was 20 mM and the exchange time was 10 min. The data reported in Table II show that labelled malate and phosphate could be exchanged with malate, malonate, succinate, phosphate, sulphate and thiosulphate, which are known to be substrates of the dicarboxylate carrier in mitochondria [2,5–7]. In contrast, no significant exchange was observed with fumarate and substrates of other mitochondrial carriers, e.g., 2-oxoglutarate, aspartate, ADP, citrate and pyruvate. The residual low activity in the presence of these anions was approximately the same as the obviously nonspecific activity observed in the presence of Cl^- (control value).

Table III reports the sensitivity of the malate–phosphate exchange in reconstituted liposomes to inhibitors of various anion-transporting systems. The malate–phosphate exchange activity was strongly inhibited by butylmalonate, benzylmalonate, *p*-I-benzylmalonate, phthalate and bathophenanthroline, which are known inhibitors of the dicarboxylate carrier in mitochondria [9–10,39–40]. Interestingly, butylmalonate was a more

TABLE II

DEPENDENCE OF [^{14}C]MALATE AND [^{32}P]PHOSPHATE TRANSPORT IN RECONSTITUTED LIPOSOMES ON INTERNAL SUBSTRATE

The dicarboxylate carrier was purified by the amberlite/hydroxyapatite procedure as described in Materials and Methods. Before incorporation into liposomes, the hydroxyapatite eluate was passed through Dowex. The proteoliposomes were loaded with 20 mM of the indicated substrate. Transport was started by adding 0.1 mM [^{14}C]malate or 0.1 mM [^{32}P]phosphate and stopped after 10 min by adding 10 mM butylmalonate.

Internal substrate (20 mM)	Substrate uptake (nmol/mg protein per 10 min)	
	[^{14}C]malate	[^{32}P]phosphate
None (Cl^- present)	53	75
L-Malate	1430	700
Malonate	800	550
Succinate	1180	650
Phosphate	1120	1050
Sulphate	430	410
Thiosulphate	920	720
Fumarate	68	60
2-Oxoglutarate	130	90
Aspartate	75	65
ADP	53	57
Citrate	39	41
Pyruvate	80	80

TABLE III

SENSITIVITY OF THE MALATE–PHOSPHATE EXCHANGE IN RECONSTITUTED LIPOSOMES TO INHIBITORS

The dicarboxylate carrier was purified by the amberlite/hydroxyapatite procedure, as described in Materials and Methods, and incorporated into liposomes. Exchange was started by adding 0.1 mM [^{14}C]malate to proteoliposomes loaded with 20 mM phosphate and stopped after 10 min by adding 10 mM butylmalonate. The sulphydryl reagents were added 2 min before the labelled substrate, at 1 mM concentration. The other inhibitors were added together with [^{14}C]malate at a concentration of 2 mM, except carboxyatractyloside (0.15 mM), α-cyanocinnamate (0.15 mM) and glisoxepide (5 mM). The control value of [^{14}C]malate transport was 3580 nmol/mg protein per 10 min.

Inhibitor	% Inhibition
Butylmalonate	86
Phenylsuccinate	51
Benzylmalonate	76
p-I-Benzylmalonate	91
Phthalate	84
Bathophenanthroline	95
Mersalyl	97
p-Hydroxymercuribenzoate	99
N-Ethylmaleimide	16
α-Cyanocinnamate	10
Glisoxepide	8
Benzene-1,2,3-tricarboxylate	24
Phthalonate	27
Carboxyatractyloside	18

effective inhibitor of the malate–phosphate exchange than phenylsuccinate. This finding is in agreement with the observation that the dicarboxylate carrier in intact mitochondria is more sensitive to butylmalonate than to phenylsuccinate [6]. In contrast, the 2-oxoglutarate carrier is more sensitive to the latter than to the former inhibitor [3,36]. The reconstituted malate–phosphate exchange was also inhibited by the sulphydryl-blocking reagents mersalyl and *p*-hydroxymercuribenzoate but not by *N*-ethylmaleimide, as found in mitochondria [8]. In contrast, α-cyanocinnamate, glisoxepide, benzene-1,2,3-tricarboxylate, phthalonate and carboxyatractyloside, which inhibit other mitochondrial transport systems more or less specifically [1], had little effect on the malate–phosphate exchange.

In further experiments (not shown), it was found that the purified preparation of the 28 kDa protein band reconstituted into liposomes does not catalyze the exchange reactions 2-oxoglutarate–2-oxoglutarate (2-oxoglutarate carrier), citrate–citrate (tricarboxylate carrier), ADP–ADP (adenine nucleotide carrier), aspartate–aspartate (aspartate-glutamate carrier), pyruvate–pyruvate (pyruvate carrier) and phosphate–phosphate (phosphate carrier) when measured with the appropriate stop inhibitors reported in Materials and Methods. Thus, the purified dicarboxylate carrier fraction is not contaminated by the other mitochondrial anion-transporting systems.

Properties of the reconstituted 2-oxoglutarate carrier

When reconstituted into liposomes, the purified protein identified as a band of 32.5 kDa in SDS gels (Fig. 2, lanes 7–8) catalyzed a phthalonate-sensitive 2-oxoglutarate–2-oxoglutarate exchange (Table IV). Uptake of 2-oxoglutarate was negligible when unloaded liposomes were used. Besides 2-oxoglutarate, also malate and (not shown) malonate, succinate and oxaloacetate, which are

TABLE IV

FUNCTIONAL PROPERTIES OF THE RECONSTITUTED 2-OXOGLUTARATE CARRIER

The 2-oxoglutarate carrier was purified as described in Materials and Methods and incorporated into liposomes. Transport was started by adding 0.1 mM 2-[^{14}C]oxoglutarate to proteoliposomes loaded with the indicated substrate (20 mM) and stopped after 10 min by adding 10 mM phthalonate. Where present, 1 mM sulphydryl reagents were added 2 min before the labelled substrate. The other inhibitors were added together with 2-[^{14}C]oxoglutarate at a concentration of 2 mM.

Internal substrate	Inhibitor	2-Oxoglutarate transport (nmol/mg protein per 10 min)
None (Cl^- present)	–	85
2-Oxoglutarate	–	2560
L-Malate	–	1420
Phosphate	–	190
Citrate	–	70
2-Oxoglutarate	phthalonate	100
2-Oxoglutarate	butylmalonate	890
2-Oxoglutarate	*p*-hydroxymercuribenzoate	180
2-Oxoglutarate	mersalyl	195
2-Oxoglutarate	*N*-ethylmaleimide	2110

known substrates of the 2-oxoglutarate carrier in mitochondria [3–4], could be used as counteranions. In contrast, substrates of other mitochondrial carriers, like phosphate, citrate and (not shown) sulphate, ADP and aspartate, were not accepted by the purified protein. Table IV shows further that the reconstituted 2-oxoglutarate–2-oxoglutarate exchange was inhibited by phthalonate and, less efficiently, by dicarboxylate analogues like butylmalonate. The exchange was also inhibited by *p*-hydroxymercuribenzoate and mersalyl, but not by *N*-ethylmaleimide. All these properties are in agreement with those described for 2-oxoglutarate transport in mitochondria [3] and for the 2-oxoglutarate carrier purified from heart [17].

Discussion

Since the method of functional reconstitution has to be used for identification of both the 2-oxoglutarate and the dicarboxylate carrier, it was very important to discriminate clearly the two activities in the transport assays. It should be emphasized that this is only possible by choosing the appropriate pairs of exchange substrates, because the two carriers show overlapping substrate specificity. Thus, for identification of the dicarboxylate carrier on the one hand, the malate–phosphate and not the malate–malate or the malate–malonate exchange was used, since both malate and malonate are also accepted by the 2-oxoglutarate carrier. The activity of the 2-oxoglutarate carrier on the other hand has been specifically identified by using the 2-oxoglutarate–2-oxoglutarate exchange.

Although our standard procedure of hydroxyapatite chromatography, as described in Fig. 1 and in numerous publications for the isolation of mitochondrial carrier proteins, already results in a considerable enrichment of the two carrier activities, these proteins are by no means pure. Therefore, we systematically varied all the parameters during solubilization and purification which may influence both the activity and the purity of the two carrier proteins under study during the isolation procedure, i.e., pH, type and concentration of salts, presence of phospholipids, detergent concentration and dimension of the hydroxyapatite columns.

Among these parameters, an important factor was found to be the presence of cardiolipin during the chromatography on hydroxyapatite. In fact, cardiolipin had to be present during all stages of the isolation procedure, since a drastic decrease of transport activity is observed when cardiolipin was omitted. Furthermore, out of the list of parameters mentioned above, in particular the concentration of detergent and the ratio of solubilized membranes/dry hydroxyapatite were found to be important for the purification.

The first step of improvement was achieved by varying the amount of hydroxyapatite and the concentration of Triton X-114 in the elution buffer. This leads to a simultaneous enrichment of both the dicarboxylate and the 2-oxoglutarate carrier in comparison to all other contaminants. The breakthrough in the purification of the single carrier was achieved by extensive removal of the detergent by hydrophobic chromatography before application onto the hydroxyapatite columns. This led to adsorption of the carrier proteins to the hydroxyapatite and made possible the specific elution of these translocators.

Once bound to the column according to this procedure, the 2-oxoglutarate carrier could be easily eluted in pure form by application of the appropriate buffer and detergent concentration. The isolated protein consists of one single band in SDS gel chromatography, showing a slightly higher molecular weight as compared to that of the 2-oxoglutarate carrier isolated from heart mitochondria [17].

The same procedure does not lead to purification of the dicarboxylate carrier, since this protein binds more strongly to hydroxyapatite and could be eluted from the column only in small amounts and in the presence of the 2-oxoglutarate carrier. The experimental consequence was to optimize the concentration of detergent which should be high enough on the one hand to avoid strong interaction of the dicarboxylate carrier with the hydroxyapatite during column chromatography and low enough on the other hand to retain the contaminating proteins. Although we achieved a high enrichment of the carrier protein by this procedure as documented in Fig. 3, this preparation of the dicarboxylate carrier still contained minor contaminations of proteins with high molecular

weight. These contaminants, however, (a) lack any transport activity when reconstituted separately into proteoliposomes and (b) are absent in fractions which exhibit high dicarboxylate carrier activity (Fig. 2b, lanes 6′, 7′), demonstrating that none of them is the dicarboxylate carrier.

By further variation of the isolation procedure, we finally obtained a pure preparation also of the dicarboxylate carrier. However, it was necessary to use celite columns and elution by the denaturating detergent SDS. Thus, the eluted protein, although showing a single protein band of 28 kDa on SDS gels, was no longer active when reconstituted into phospholipid liposomes.

In this paper, the aim was to achieve optimal purification and not optimal reconstitutive activity. Therefore, activities much higher than those reported can be obtained by optimizing the conditions, e.g., the substrate concentration and the buffer composition.

It is interesting to note that the procedure for the isolation of carrier proteins from liver seems to be more difficult in general as compared to the isolation from heart mitochondria. This becomes obvious here also in the case of the 2-oxoglutarate carrier. Several factors could be responsible for this observation. First, there is presumably an intrinsic difference between the proteins from different sources, as revealed by differences in their behaviour in SDS gel chromatography. Second, the protein/lipid and the membrane protein/soluble protein ratios are different in the mitochondria from the two organs. Third, the lipid composition in heart and liver mitochondria are different, which may influence the elution behaviour on hydroxyapatite columns via the residual amount of lipid bound to the solubilized proteins.

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