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Purification by affinity chromatography of the dicarboxylate carrier from bovine heart mitochondria

Adam Szewczyk [a], Maciej J. Nałęcz [a], Clemens Broger [b], Lech Wojtczak [a] and Angelo Azzi [b]

[a] *Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw (Poland)* and [b] *Institute for Biochemistry and Molecular Biology, University of Bern, Bern (Switzerland)*

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Submitochondrial particles were prepared from bovine heart mitochondria, solubilized with Triton X-114 in the presence of lipids and submitted to hydroxylapatite chromatography. The eluate obtained, containing a mixture of mitochondrial carriers, was processed further by affinity chromatography using as ligand *p*-aminophenylsuccinate coupled via a diazo bond to aminohexyl-Sepharose 4B. The activity of the dicarboxylate exchanger was measured after reconstitution into asolectin vesicles at each step of the purification procedure. All samples studied were found to display substrate and inhibitor specificity similar to those described for the dicarboxylate carrier in mitochondria. The specific activity of the final material eluted from the affinity column was found to be about 1000-times higher than that of the Triton X-114 extract of submitochondrial particles. SDS-polyacrylamide gel electrophoresis analysis of the affinity chromatography eluate showed the presence of only two polypeptides.

Introduction

The outer mitochondrial membrane does not form a true permeability barrier for small molecules due to the presence of a pore-forming protein similar to bacterial porins [1]. The permeability of the inner mitochondrial membrane, instead, is highly selective for compounds with a hydration diameter larger than 0.6 nm. Thus only a limited number of molecules, like O_2, H_2O and CO_2, is able to pass this barrier, while the substrates of the Krebs cycle, of the respiratory chain and of the ATP synthase cannot penetrate the membrane without the help of specialized translocating systems (see Refs. 2–5 for reviews). The existence and specificity of such systems were first demonstrated by the observation that mitochondria swell when suspended in a solution of ammonium salts of certain substrates [6] and subsequently by studying the accumulation of radioactive substrates [7]. It is now well established that the inner mitochondrial membrane contains at least 12 functionally different carriers [2–5]. Recently, attention has been focused on the molecular mechanism of the transport and the isolation of mitochondrial carrier proteins. Among many others, also the dicarboxylate carrier has been extracted and partially purified from liver, kidney

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEMED, *N,N,N′,N′*-tetramethylenediamine; pCMB, *p*-chloromercuribenzoic acid; pCMBs, *p*-chloromercuriphenyl sulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PPO, 2,5-diphenyloxazol; POPOP, 1,4-bis(2-(5-phenyloxazolyl)benzene.

Correspondence: A. Azzi, Institute for Biochemistry and Molecular Biology, University of Bern, Bern, Switzerland.

and heart mitochondria, and its activity has been measured after reconstitution into liposomes [8,9]. Here we report a further progress in the purification of this protein, namely the application of affinity chromatography using a phenylsuccinate-Sepharose column. Part of this work has been presented as a preliminary report [10].

Experimental Procedure

Preparation of bovine heart mitochondria and submitochondrial particles, solubilization of the membranes and hydroxylapatite chromatography were performed as described [11,12]. The only modifications introduced were that in some of the experiments the addition of pure cardiolipin to the buffers for solubilization and hydroxylapatite chromatography was omitted, and that asolectin at a final concentration of 10 mg/ml was used.

Reconstitution and activity measurements

The activity of the dicarboxylate carrier was studied after reconstitution of the fractions obtained at each step of the purification procedure by measuring phenylsuccinate-sensitive malonate/malonate exchange (0.2 mM radioactive malonate outside vs. 10 mM malonate inside the liposomes). The reconstitution procedure (freeze-thaw sonication) as well as the activity measurements were performed as described in Refs. 11–13.

Synthesis of the affinity resin

Coupling of a six carbon spacer to Sepharose 4B

100 ml of the Sepharose 4B was washed with 1 l 0.1 M NaCl and then with H_2O. The gel was suspended 1 : 1 in water and stirred continuously. 5 g CNBr (50 mg/ml gel) were added and the pH was kept at 11 with additions of 2 M NaOH. After 15 min the gel was filtered and quickly washed with 1 l ice-cold H_2O. The obtained CNBr-activated Sepharose was mixed with 200 ml diaminohexane solution (58 mg/ml, total 100 mmol) and the mixture was stirred by rolling for about 10 h at 4°C. The gel was filtered, washed quickly with 1 l H_2O and incubated with 400 ml 1 M ethanolamine (2 h, rolling, 4°C) to block not reacted groups on the resin. The final aminohexane-Sepharose thus obtained was washed with 1 l 1 M NaCl and 3 l H_2O. The reaction of the gel with 2,4,6-trinitrobenzenesulfonate (see Ref. 14 for details) gave a positive test for the presence of amino groups.

Synthesis of the ligand, p-amino-phenylsuccinate

Nitration. Commercially available phenylsuccinate (20 g) was added in small portions over about 1 h to 50 ml of fuming nitric acid at 0°C. After 10 more minutes, ice cubes were added to the mixture until they did not melt immediately. The mixture was filtered and the white precipitate was collected on a filter. The precipitate was dissolved in boiling water and the solution was filtered again. The white product of this step was crystallized from hot water overnight at 4°C, with the final yield of 14.5 g. The NMR spectrum of the product dissolved in ^2H_2O confirmed its structure as *p*-nitrophenylsuccinate.

Reduction of the nitro group. 1 g of the product from the previous step was dissolved in 20 ml methanol and mixed with 0.1 g 5% palladium/charcoal. The mixture was placed in a round-bottom flask and exposed to hydrogen gas under atmospheric pressure. The mixture was continuously vigorously mixed. A rapid consumption of hydrogen was observed during the first 30 min of incubation, which slowed down later. When no further consumption of H_2 was observed (approx. after 2 h), the reaction mixture was removed, diluted with 200 ml of ethanol and filtered. The filtrate was concentrated to about 5 ml on a rotary evaporator, diluted to about 20 ml with boiling water and left for crystallization overnight at 4°C. The resulting white crystals were filtered and subsequently washed with ice-cold methanol. The procedure yielded about 400 mg of the product, identified by the NMR spectrum as *p*-aminophenylsuccinate.

Coupling of the ligand to the resin

Coupling of the *p*-aminophenylsuccinate to the aminohexyl-Sepharose 4B was obtained via a diazo bond, as described by Cuatrecasas [14]. 7.5 ml of the sedimented Sepharose derivative was suspended in 20 ml of the solution containing 200 mM sodium borate (pH 9.3) and 40% (v/v) dimethylformamide. 10 ml of 200 mM *p*-nitrobenzoylazide (freshly prepared solution in 50%,

v/v, dimethylformamide) was added to the suspension and mixed for 1 h at room temperature. The mixture was filtered and the resin was washed on a funnel with 1 l of 50% (v/v) dimethylformamide. Subsequently, the resin was suspended in 40 ml of 0.5 M sodium dithionite (freshly prepared solution in 0.5 M sodium hydrocarbonate), pH 8.5. The suspension was mixed for 40 min at 40 °C, then filtered and washed extensively with water (about 2 l). The last washing (about 0.5 l) was performed with 0.5 M HCl. At this point the resin was resuspended in 40 ml of 0.5 M HCl and 280 mg of powdered sodium nitrite were added. Diazotation was allowed to proceed for 10 min at 4 °C, followed by quick filtration and brief washing (about 100 ml) of the resin with saturated solution of sodium borate. The gel was then suspended in about 30 ml of the saturated sodium borate solution (pH 8.5) and 75 mg of the *p*-aminophenylsuccinate (0.35 mmol) were added to the suspension. The coupling reaction was allowed to proceed for 8 h at 4 °C, with continuous stirring. Already during the first minutes of incubation the resin became yellow-orange, the color being changed later into orange-red and, finally, into dark red. The resin was separated from the solution on a filter and subsequently washed with about 2 l of water (slightly acidified with HCl), about 1 l of 1 M NaCl and about 2 l of water again. The resin was stored at 4 °C in water in the presence of sodium azide. Scheme I shows the structure of the affinity chromatography resin synthesized as described above.

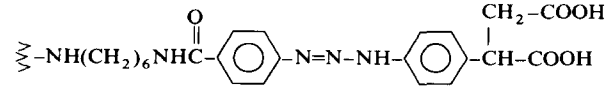
Affinity chromatography

About 2 ml of the sedimented affinity resin was used to pack a small (0.5 × 6 cm) chromatography column. The column was equilibrated with the solution containing 2% Triton X-114, 10 mM Mops buffer (pH 7.2), 1 mM EDTA (pH 7.2) and asolectin (2 mg/ml). In some experiments the medium contained also 20 mM NaCl and 0.7% sodium deoxycholate (see section Results). The total volume of the column was measured with a ferricyanide-containing medium and was found to be about 4 ml. For the affinity chromatography purification of the mitochondrial dicarboxylate carrier, 1 ml of the hydroxylapatite eluate (approx. 150 μg of protein) was loaded onto the column. In the experiments performed in the presence of NaCl and deoxycholate, both chemicals were also added to the eluate at the final concentration of 20 mM and 0.7%, respectively. Loading of the column was followed by slow (30 ml/h) washing with approx. 15 ml of the equilibrating medium. Elution of the specifically bound protein material was performed in one step by applying 5 ml of the medium containing 50 mM malonate and no NaCl. At the end of the experiment, the column was washed with the medium supplemented with 1 M NaCl in order to elute all unspecifically bound proteins. The fractions collected at every chromatography step (0.4 ml each) were analysed for the total protein content, the polypeptide composition and the activity of the dicarboxylate translocator.

Other procedures

For protein determination, the Lowry procedure modified by the addition of SDS was used (for details see Refs. 11–13).

Gel electrophoresis was performed according to Laemmli [15] using a 17.5% polyacrylamide separation gel as described by DePinto et al. [16]. The protein was precipitated by aceton, redissolved in sample buffer and applied for electrophoresis as described previously [12]. The polypeptides were



Scheme I. Affinity chromatography resin synthesized as described under Experimental procedures. The diazo linkage between the spacer and the ligand has been chosen for two main reasons: (1) the double bond makes the resin structure more rigid and prevents folding of the spacer; (2) there is a possibility of cleaving the ligand (± bound protein) from the resin by using a reducing agent (e.g., dithionite).

fixed in 6% formaldehyde and stained with silver nitrate according to the procedure described by Bio-Rad.

Materials

Malonate, succinate, phenylsuccinate, glutamate, malate, diaminohexane, EDTA, Triton X-114 and Dowex AG1-X8, Cl^- form, 200–400 mesh, were supplied by Fluka AG. Before use, Dowex was exchanged into the formate form according to the instructions provided by Bio-Rad. Cardiolipin (solution 5 mg/ml in ethanol), bovine serum albumin, butylmalonate, 2,4,6-trinitrobenzenesulfonate and *p*-nitro-benzoylazide were from Sigma. Folin reagent, 2,5-diphenyloxazol (PPO), 1,4-bis(2-(5-phenyloxazolyl)benzene (POPOP), silver nitrate, deoxycholate and Mops were from Merck. Asolectin (extract of phospholipids from soy beans) was from Associated Concentrates, hydroxylapatite (hydroxylapatite biogel, dry powder) from Bio-Rad and Sepharose-4B from Pharmacia. The radioactive substrates, [2-^{14}C]malonic acid, sodium salt and L-[U-^{14}C]malic acid were from Amersham International. Mersalyl, acrylamide and *N,N,N',N'*-tetramethylenediamine (TEMED) were from Serva. All other chemicals were of analytical grade.

Results and Discussion

Purification of the dicarboxylate carrier by hydroxylapatite chromatography, effect of lipids

The technique of hydroxylapatite chromatography was used initially to purify the adenine nucleotide translocator [17] but subsequently it was applied for the purification of other carriers as well [8–13,18–20]. It is now clear that the activity of at least seven different mitochondrial carriers (ATP/ADP, phosphate, tricarboxylate, dicarboxylate, monocarboxylate, glutamate/aspartate and 2-oxoglutarate) can be identified in the hydroxylapatite eluate prepared under standard conditions, i.e., using media containing 2–4% Triton X-100 or X-114 plus cardiolipin (2 mg/ml) and dry hydroxylapatite powder for the chromatography.

The dicarboxylate carrier was reported to be present in the hydroxylapatite eluate prepared from liver, heart and kidney mitochondria [8,9] when using Triton X-114 and cardiolipin in the media for solubilization of the membranes and subsequent hydroxylapatite chromatography. We have found that cardiolipin may actually be replaced by a higher concentration of asolectin, without a substantial loss of the total activity of the carrier recovered in the hydroxylapatite eluate. Instead, much higher specific activity of the malonate/malonate exchange was observed in the asolectin system (27.1 nmol malonate transported/min per mg protein vs. 15.4 nmol/min per mg observed in the presence of cardiolipin), due to a lower total protein content of the eluate (0.18 mg vs. 0.33 mg). The use of the asolectin-containing medium was also found convenient for another reason: no activity of the mitochondrial 2-oxoglutarate translocator [11] was detected in the HTP-eluate prepared in this way (see Table I), thus making the correction for 2-oxoglutarate carrier catalysed malonate/malonate exchange reaction unnecessary. The absence of 2-oxoglutarate transport activity in the hydroxylapatite eluate prepared in the presence of 10 mg/ml asolectin was further confirmed by inhibitor sensitivity of the malonate/malonate exchange measured in proteoliposomes reconstituted with this eluate (Table I). It was found identical to that described for the dicarboxylate translocator in mitochondria (see Refs. 2–5 for comparison).

The polypeptide composition of the Triton X-114 extract of submitochondrial particles as well as of the hydroxylapatite eluate prepared in the presence of 10 mg/ml asolectin is shown in Fig. 1, lanes a and b, respectively. As shown previously [12], some protein bands were missing in the asolectin sample and some were present in a lower amount than in the parallel sample prepared in the presence of cardiolipin.

The actual mechanism of the lipid influence on the hydroxylapatite chromatography of mitochondrial carriers is still a matter of debate. It has been proposed [21] that carriers, being relatively small and hydrophobic, can be embedded in Triton micelles in such a way that their charged groups face the inside of a micelle and thus are not available for an interaction with hydroxylapatite. This would explain an exclusive requirement of Triton X-100 or Triton X-114 for specific elution of a mixture of carriers in the void volume of the

TABLE I

SUMMARY OF THE RESULTS FOR RADIOACTIVE MALONATE ACCUMULATION IN PROTEOLIPOSOMES RECONSTITUTED WITH THE HYDROXYLAPATITE ELUATE PREPARED IN THE PRESENCE OF ASOLECTIN

Solubilization of submitochondrial particles, hydroxylapatite chromatography, reconstitution and malonate-exchange measurements are described in Experimental procedures. The total internal malonate accumulation in the presence (or absence) of different externally added substances is presented.

Catalysed exchange reaction outside 0.2 mM/inside 10 mM	Total malonate accumulation (nmol malonate/min per mg protein)	(%)
Malonate/malonate + no external additions	29.2	100
+ 5 mM phenylsuccinate	7.0	24
+ 5 mM *n*-butylmalonate	9.9	34
+ 5 mM phenylmalonate	11.2	38
+ 0.1 mM mersalyl	5.8	20
+ 0.1 mM pCMB	2.9	10
+ 10 mM phosphate	19.4	67
+ 10 mM succinate	17.3	59
+ 10 mM sulphate	15.6	53
+ 10 mM oxaloacetate	28.4	97
+ 10 mM glutamate	30.1	103
Malonate/malate	34.5	118
Malonate/2-oxoglutarate	3.1	11
Malonate/sucrose	2.2	8
Malonate/malonate (reconstituted with boiled protein)	5.3	18

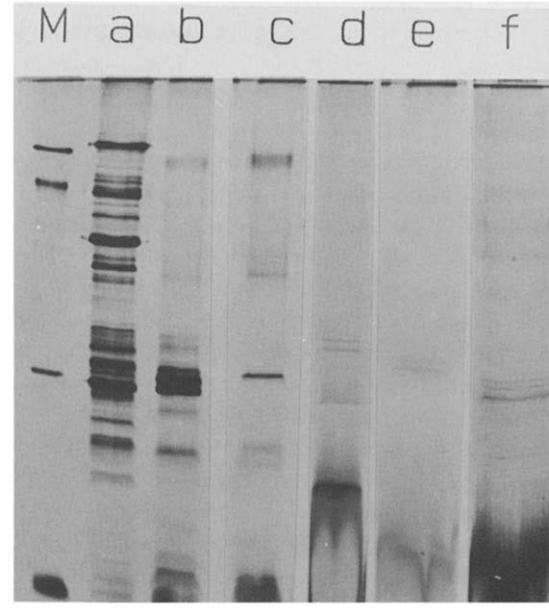


Fig. 1. SDS gel electrophoresis of fractions obtained during the purification of the dicarboxylate transport protein. The following molecular-weight markers (M) were used: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (31 kDa) and cytochrome c (12.5 kDa), (a) Triton extract prepared in the presence of asolectin (10 mg/ml); (b) hydroxylapatite pass-through prepared in the presence of asolectin (10 mg/ml); (c)–(f) fractions collected at different stages of the affinity chromatography performed in the presence of asolectin (2 mg/ml), Triton X-114 (2%) and deoxycholate (0.7%); (c) not bound material collected in the void volume of the affinity chromatography column after loading of the hydroxylapatite eluate; (d) material recovered in the buffer used to wash the column after loading; (e) material eluted with 50 mM malonate; (f) material eluted with 1 M NaCl. For experimental details, see text.

hydroxylapatite column.

Although asolectin was shown to improve hydroxylapatite purification of the dicarboxylate carrier, on the basis of the present data it cannot be excluded that the presence of cardiolipin is specifically required for retaining full activity of the carrier, as originally proposed [8]. Since asolectin contains some cardiolipin (up to 10 mol% according to our estimates), it may still provide a sufficient amount of this lipid for specific interaction with the carrier protein.

Further purification by affinity chromatography

The fractions eluted in the void volume of hydroxylapatite column were applied to affinity chromatography.

In the first set of experiments, the column was equilibrated with the medium containing 2% Triton X-114, 1 mM EDTA and asolectin (2 mg/ml) and run in the same medium, supplied with 50 mM malonate or 1 M NaCl for subsequent elution of bound proteins. It has been observed that about 50% of the loaded carrier activity was retained on the column, the rest being eluted in the void volume (not shown). No apparent specificity of elution was observed: some activity of the carrier was detected in the buffer used to wash the column after loading and the rest was distributed approximately equally between the fractions eluted with 50 mM malonate and, subsequently, 1 M NaCl. Since the fraction eluted with malonate,

however, contained much less protein than the one eluted with NaCl, its calculated specific activity amounted to about 90 nmol malonate transported per min per mg protein. This gave a purification factor of about 250-fold in comparison to the specific activity of the Triton extract of submitochondrial particles (0.36 nmol/min per mg protein). Although such a result is already an improvement in purification the little amount of protein recovered in the malonate-eluted samples made the procedure relatively inefficient. Moreover, the SDS-polyacrylamide gel electrophoresis analysis of the samples revealed the presence of at least five different polypeptide bands in the malonate-eluted material (not shown), pointing to the low specificity of the affinity purification under these conditions. Approximately the same amount of protein was found in the NaCl-eluted samples and they contained the same polypeptides although in different proportions (not shown).

As discussed in Ref. 22, the use of non-ionic detergents in affinity chromatography sometimes leads to unsatisfactory results due to the masking of the ligand by noncharged detergent micelles, thus preventing, at least partially, the formation of a specific ligand-protein complex. It has been suggested [22] that the addition of an anionic detergent, e.g., SDS or deoxycholate, might resolve this difficulty. The presence of salts in the affinity chromatography eluent has also been considered useful in abolishing non specific interactions between proteins and a charged resin (see, e.g., Ref. 23).

Indeed, when 20 mM NaCl and 0.7% sodium deoxycholate were included in the medium employed in the phenylsuccinate-Sepharose affinity chromatography and 50 mM malonate (without NaCl) was used for elution, a much better purification of the dicarboxylate carrier was obtained. Fig. 2 presents the detailed analysis of this experiment, where the total protein content and the dicarboxylate carrier activity were measured in fractions collected throughout the affinity chromatography. As it is shown (filled circles, solid line), the majority of the loaded protein was recovered in the not-bound fractions collected in the void volume of the column as well as in those obtained from washing of the column with equilibrating buffer, prior to the elution with malonate.

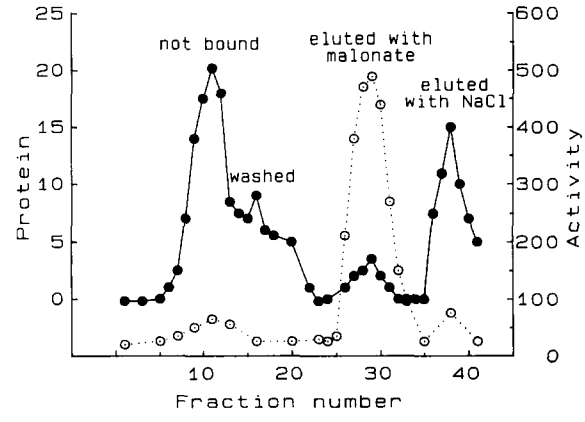


Fig. 2. Elution pattern of the affinity chromatography of hydroxylapatite eluate on the phenylsuccinate-Sepharose column. Chromatography was performed in the presence of Triton X-114, deoxycholate and NaCl. The fractions were analysed for the total protein content (filled circles, solid line) and for the activity of the dicarboxylate carrier after reconstitution (open circles, dotted line). The protein content is expressed in μg of protein/ml of eluate and the carrier activity is presented in nmol malonate/min per mg protein. Experimental details are described under Experimental procedures.

The polypeptide composition of the fractions containing unbound protein eluted within the void volume of the column as well as of those collected during washing of the column with buffer is shown in Fig. 1, lanes c and d, respectively. It is apparent that most of the polypeptides coincide with those of the hydroxylapatite eluate used to load the affinity column (Fig. 1, lane b), with some enrichment in the bands of a relatively high apparent molecular weight. Furthermore, it is shown in Fig. 2 that the rest of the protein, approx. 30% of the amount loaded, was recovered in two peaks eluted subsequently with 50 mM malonate and 1 M NaCl. The activity of the dicarboxylate carrier, instead, was found almost exclusively in the material eluted with 50 mM malonate (open circles, dotted line), suggesting a specific interaction of the carrier with the resin. The flow sheet for the purification of the dicarboxylate carrier recovered in the malonate-eluted material (see Table II) shows that the specific activity of the carrier was increased by about 1300-fold in comparison to the specific activity of the Triton X-114 extract of submitochondrial particles. However, as also shown in Table II, this has been accompanied by a relatively large loss of the total carrier activity:

TABLE II

PURIFICATION OF THE DICARBOXYLATE CARRIER BY AFFINITY CHROMATOGRAPHY IN THE PRESENCE OF 2% TRITON X-114, 0.7% DEOXYCHOLATE AND ASOLECTIN (2 mg/ml)

Affinity chromatography was performed as described under Experimental procedures. Values of protein content represent the total amount of protein recovered in separate samples. 100% protein is referred to the starting material (56.7 mg of mitochondrial protein taken for the experiment). Only the phenylsuccinate-sensitive malonate/malonate exchange is presented (net uptake).

Sample	Protein		Activity		Purification
	(mg)	(%)	total (nmol/min)	specific (nmol/min per mg)	
Submitochondrial particles extracted with asolectin	15.1	26.6	5.29	0.35	1-fold
Hydroxylapatite eluate after solubilization with asolectin, eluted with asolectin	0.16	0.3	4.38	27.4	78-fold
Affinity chromatography eluate	0.004	0.05	1.90	474.6	1356-fold

only about 30% of the activity originally extracted from the membranes was recovered in the final preparation. In general, the lack of total recovery of the transport activities may be due to two different reasons: partial denaturation of the transporter during the purification procedure; and incompetence of the liposome system employed to express fully the activities of the protein. This could be caused by defective reconstitution or by the limiting size of the liposome. One has to remember that the material reconstituted at different steps of the purification procedure differs not only in its protein content, but also detergent and salt concentration, amount of membrane-extracted lipids and other parameters likely to influence the final structure of proteoliposomes formed. Direct comparison of the transport activities measured in these vesicles may therefore lead to somewhat inexact conclusions.

One of the major questions to be answered was whether the malonate/malonate exchange activity measured in the fractions eluted from the affinity chromatography column represents indeed the activity of the dicarboxylate translocator. Table III presents the substrate specificity of the measured reaction. As shown, not only malonate/malonate exchange was catalysed but also malonate/phosphate, malate/malate, malate/malonate and malate/phosphate reactions, all typical for the mitochondrial dicarboxylate carrier. Somewhat unexpected was the observation that malate/malonate and malate/phosphate exchange activities appeared lower than the others, the situation at variance with intact mitochondria [2–5]. It cannot be excluded, however, that some reconstitution problems (see above) were responsible for this observation. On the other hand, no exchange reaction involving either 2-oxoglutarate or citrate was detected. A slight accumulation of radioactivity observed in these samples as well as in the sample loaded with glutamate (malate/glutamate exchange is not catalysed in mitochondria) possibly reflects a slow diffusion of radioactive dicarboxylates into proteoliposomes during the incubation. Furthermore, Table IV presents the inhibitor sensitivity of malate/phosphate and malate/malate exchange reactions catalysed by the reconstituted eluate from the affinity chromatography. The lack of inhibition by *N*-ethylmaleimide, 1,2,3-benzenetricarboxylate, cinnamate and phthalonate as well as the strong inhibition expressed by the typical inhibitors of the dicarboxylate carrier (phenylsuccinate, phenylmalonate and mercurials) points to the fact that indeed only the dicarboxylate exchanger activity is present in the final eluate from the affinity column. DTNB, the SH-group reagent, was also found inhibitory, in line with the known SH-group involvement in the catalytic activity of the dicarboxylate carrier. A slight inhibitory effect of carboxyatractyloside and

TABLE III

SUBSTRATE SPECIFICITY OF THE DICARBOXYLATE CARRIER PURIFIED BY AFFINITY CHROMATOGRAPHY

Affinity chromatography, reconstitution of the carrier and activity measurements were performed as described under Experimental procedures. Incubation with externally added radioactive substrate was performed for 10 min at room temperature. Only malonate-eluted fractions (see Fig. 2) were studied. Total accumulation of radioactivity, not corrected for inhibitor sensitivity, is presented.

Catalysed exchange reaction outside 0.2 mM/inside 10 mM	Total substrate accumulation (nmol/min per mg protein)
Malonate/malonate	483
Malonate/phosphate	592
Malonate/2-oxoglutarate	12
Malate/malate	554
Malate/malonate	284
Malate/phosphate	291
Malate/2-oxoglutarate	39
Malate/citrate	21
Malate/glutamate	18

TABLE IV

INHIBITOR SENSITIVITY OF THE DICARBOXYLATE CARRIER PURIFIED BY AFFINITY CHROMATOGRAPHY

Conditions of the experiment as in Table III. Inhibitors were added to proteoliposomes prior to the radioactive substrate and the mixture was preincubated for 2 min. The actual incubation was performed for 15 min at room temperature. Control activities measured in the absence of any inhibitor (and taken as 100% activity, i.e., 0% inhibition) were following: for malate/phosphate exchange – 280.7 nmol malate/min per mg protein; for malate/malate exchange – 536 nmol malate/min per mg protein.

Inhibitor added (final concentration)		% Inhibition of the exchange reaction	
		malate/phosphate	malate/malate
10 mM	phenylsuccinate	93	96
1 mM	*N*-ethylmaleimide	7	0
5 mM	1,2,3-benzenetricarboxylate	0	6
0.1 mM	pCMBs	63	49
0.1 mM	pCMB	80	not measured
1 mM	cinnamate	0	not measured
0.1 mM	carboxyatractyloside	27	not measured
1 mM	phthalonate	1	6
7 mM	phenylpyruvate	not measured	20
1 mM	mersalyl	92	95
10 mM	phenylmalonate	76	89
1 mM	DTNB	80	not measured

phenylpyruvate, instead, possibly expresses some unspecific inhibitory action of these compounds, e.g., a detergent-like effect of 0.1 mM carboxyatractyloside and a surface charge change effect introduced by 7 mM phenylpyruvate. In both cases a small inhibition of the dicarboxylate carrier activity should be predicted.

The polypeptide composition of the material eluted from the affinity column with 50 mM malonate is shown in Fig. 1, lane e. Only two protein bands were found in these fractions, having the apparent molecular weight of 36 kDa and 34 kDa, respectively. It seems clear that at least one of them represents the mitochondrial dicarboxylate carrier, since the activity of the translocator was almost exclusively present in the fractions eluted with malonate (Fig. 2) and neither of the two polypeptides in question appeared in other fractions collected from the affinity chromatography (Fig. 1, lanes c–f). Although at present it cannot be stated which of these two protein bands represents the mitochondrial dicarboxylate carrier and what the nature of the second polypeptide co-purified with the carrier is, the following different hypothesis can be made.

(1) The dicarboxylate carrier is a non-symmetrical dimer composed of two monomers having a slightly different molecular weight.

(2) The dicarboxylate carrier co-purifies with an unknown polypeptide which may result from an interaction between the polypeptides in question or may be due to interaction of both of them with the resin. (For instance, the second band may reflect another carrier such as the 2-oxoglutarate, which may bind to phenylsuccinate but is unable to catalyse transport. A similar phenomenon has been observed for the mitochondrial tricarboxylate carrier, see Ref. 24).

(3) It is possible that the smaller polypeptide (34 kDa) represents a degradation product of the carrier (36 kDa) still retaining the ability of bind-

ing phenylsuccinate and thus being specifically purified by the affinity chromatography.

(4) Finally, it cannot be excluded that one of the two polypeptides is the biosynthetic precursor of the other.

In all cases the primary structure analysis should give an answer to the above questions.

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