Extraction, partial purification and functional reconstitution of two mitochondrial carriers transporting keto acids: 2-oxoglutarate and pyruvate

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Bovine heart submitochondrial particles were treated with a medium containing Triton X-114 and cardiolipin. The extract was subjected to hydroxyapatite chromatography. Only a few major polypeptides of similar molecular masses were found in the eluate, as shown by electrophoresis in an SDS-polyacrylamide gel stained with silver. The eluate was reconstituted into liposomes and was shown to catalyse two different transport activities: 2-oxoglutarate-2-oxoglutarate exchange sensitive to phthalonate and phenylsuccinate and pyruvate-pyruvate exchange sensitive to 2-cyano-4-hydroxycinnamate. Since both activities were found to have characteristics similar to those described for intact mitochondria, it was concluded that at least two of the polypeptides found in the hydroxyapatite eluate correspond to the two mitochondrial carriers.

Mitochondria *Anion transport* *2-Oxoglutarate carrier* *Monocarboxylate carrier* *Pyruvate transport* *Reconstitution*

1. INTRODUCTION

The transport of substrate anions across the inner mitochondrial membrane is catalysed by specific carrier proteins. Their existence and specificity were first demonstrated by the observation that mitochondria swell when suspended in solutions of certain substrates and subsequently by studying the accumulation of the radioactive substrates (review see [1]). It is now established that the inner mitochondrial membrane contains at least 12 functionally different carriers [1,2]. Recent studies were focused on the molecular mechanism of the transport together with the isolation of the carrier proteins. Some of the mitochondrial carriers have already been isolated in a pure form, e.g. the ATP-ADP translocator [3,4] and the phosphate carrier [5–7]; some others have been extracted and partially purified and their activity has been measured after reconstitution into liposomes, e.g. the tricarboxylate carrier [8,9], dicarboxylate carrier [10,11], glutamate carrier [12] and glutamate-aspartate exchanger [13]. It is interesting to note that most of the purification procedures used hydroxyapatite (HTP) chromatography as an important step, suggesting structural similarities between the different transport proteins. A possible explanation of the efficiency of hydroxyapatite in the purification of hydrophobic proteins has been proposed [14].

Here we report the identification of 2 other mitochondrial carriers present in the HTP eluate, the 2-oxoglutarate and pyruvate transporters.

Abbreviations: HTP, hydroxyapatite; Mops, 3-(*N*-morpholino)propanesulfonic acid; PPO, 1,4-di-(5-phenyloxazolyl)benzene; POPOP, 2,5-diphenyloxazole

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2. MATERIALS AND METHODS

2.1. *Materials*

Beef heart mitochondria were prepared by a standard procedure [15]. Submitochondrial particles were obtained by sonication of mitochondria and subsequent differential centrifugation [16]. Triton X-114, EDTA and phenylsuccinate were from Fluka. Mops, folin reagent, PPO, POPOP and substrates were from Merck. The radioactive substrates (2-oxo[1-^{14}C]glutaric acid, sodium salt and [1-^{14}C]pyruvic acid, sodium salt) were from Amersham; asolectin (extract of phospholipids from soy beans) from Associated Concentrates; cardiolipin (solution 5 mg/ml in ethanol) and 2-cyano-4-hydroxycinnamate from Sigma; hydroxyapatite (HTP-biogel, dry powder) and Dowex AG 1-X8, Cl^- form, 200–400 mesh were from BioRad. Phthalonate was a generous gift from Dr A.J. Meijer, University of Amsterdam. All other chemicals were of analytical grade.

Buffer A for solubilization of the membranes and hydroxyapatite chromatography: 4% Triton X-114, 50 mM NaCl, 1 mM EDTA, 20 mM Mops and cardiolipin (2 mg/ml), final pH 7.2. This solution was always freshly prepared by sonicating (with cooling and under nitrogen) for about 10 min, until clear.

Buffer B enclosed in liposomes: 50 mM NaCl, 10 mM Mops and 10 mM 2-oxoglutarate or pyruvate (final pH 7.2).

Buffer C for transport measurements: 100 mM NaCl, 20 mM Mops (final pH 7.2) and either 2 mM radioactive 2-oxoglutarate (final spec. act. about 0.8 mCi/mol) or 5 mM radioactive pyruvate (final spec. act. about 0.4 mCi/mol). The exact specific radioactivity of buffer C was always measured before the experiment.

Scintillation cocktail: 7 g PPO and 300 mg POPOP were dissolved in 1 l toluene. 700 ml of this solution was mixed with 300 ml of absolute ethanol and used as the scintillation cocktail.

2.2. *Solubilization*

A 0.5 ml sample of freshly thawed submitochondrial particles (approx. 15 mg protein) was mixed with 1.2 ml buffer A and gently homogenized. The suspension was kept in ice for 20 min and then centrifuged at 100000 × *g* for 40 min. The brown-red pellet was discarded and the yellowish solubilizate was either reconstituted directly or subjected to hydroxyapatite chromatography.

2.3. *HTP-chromatography*

600 mg dry-HTP gel was placed in a small disposable plastic column and cooled for 15 min at −20°C. The column was loaded with 0.6 ml of the supernatant obtained from the previous step and eluted with an additional 1.8 ml buffer A. The collected eluate (about 0.5 ml) was subsequently used for reconstitution, protein determination and gel electrophoresis.

2.4. *Reconstitution*

Liposomes were made from asolectin by first swelling of the dry lipid in buffer B (125 mg asolectin/ml buffer) for 2 h in the cold and then sonicating the suspension until clear (approx. 10 min, 50% duty), with cooling and under nitrogen. The liposomes obtained were divided into 360 μl samples and kept in ice. Reconstitution was started by addition of 20 μl of either extracted membranes (supernatant after solubilization) or HTP eluate to a sample of liposomes. The samples were mixed, allowed to stand in ice for 2 min, then quickly frozen in liquid nitrogen followed by slow thawing at room temperature. When thawed, each sample was sonicated with a microtip (50% duty, in the cold under nitrogen) until clear and subsequently passed through a small column of Dowex to remove the external substrate. The resin was previously equilibrated with 170 mM sucrose.

2.5. *Transport (2-oxoglutarate-2-oxoglutarate or pyruvate-pyruvate exchange)*

300 μl aliquots of proteoliposomes were used per assay.

2.5.1. For 2-oxoglutarate

The control samples were supplemented with 20 μl of 100 mM phenylsuccinate or 10 μl of 100 mM phthalonate to inhibit the exchange activity and subsequently with 30 μl radioactive substrate solution (buffer C) to give the final external 2-oxoglutarate concentration of 0.2 mM. The assay samples were supplemented first with the substrate (30 μl buffer C) and only after a certain time were inhibited as above. 'Zero-time' samples were supplemented with a mixture of inhibitor and

substrate solutions and were immediately processed further, i.e. quickly passed through a small column of Dowex, to remove external, not exchanged radioactive 2-oxoglutarate. The exchange reaction was measured at 20°C.

2.5.2. For pyruvate

Samples were preincubated for 3 min with or without 1 mM 2-cyano-4-hydroxycinnamate [17]. The exchange reaction was started by addition of substrate (buffer C). The final concentration of pyruvate was 0.5 mM. The incubation was performed at 15°C and the reaction was stopped by passing the mixture through a Dowex column. Aliquots of the eluate were pipetted into scintillation vials, mixed with the scintillation cocktail and counted for radioactivity.

2.6. *Protein determination*

The procedure of Lowry et al. [18] was modified by the addition of SDS (final concentration 5%) to avoid turbidity problems due to the presence of lipid and high concentrations of Triton X-114. Bovine serum albumin was used as a protein standard and the standard curve was prepared in the presence of both Triton, buffer A and SDS.

2.7. *Polyacrylamide-SDS gel electrophoresis*

The electrophoresis was performed according to Laemmli [19] by using a 17.5% polyacrylamide separation gel as described in [20]. Before loading the Triton extract samples were incubated with 4% SDS. The HTP-eluate samples were supplemented with SDS to a concentration of 1% and subsequently extracted with 2 vols chloroform. The aqueous phases were concentrated by ultrafiltration in Minicon B15. The following molecular mass marker proteins were used: phosphorylase *b* (94 kDa), BSA (67 kDa), carbonic anhydrase (30 kDa) and cytochrome *c* (12 kDa). All samples contained 2-mercaptoethanol.

3. RESULTS AND DISCUSSION

3.1. *2-Oxoglutarate carrier*

The procedure used for the extraction of the native 2-oxoglutarate transporter from bovine heart submitochondrial particles consisted of 2 main steps. First, submitochondrial particles were treated with Triton X-114 in the presence of cardiolipin (2 mg/ml). Second, the extracted material was passed through hydroxyapatite. Fig.1 shows the polypeptide patterns obtained after SDS-polyacrylamide electrophoresis of the Triton extract and the HTP eluate. The comparison of the silver-stained polypeptides in both fractions demonstrated a considerable purification by a single HTP-chromatography step. Many bands are visible in the extracted fraction whereas only a few bands can be distinguished in the HTP eluate. They are mainly located in the 30–37 kDa region. Table 1 describes the results of the purification procedure. Only 1% of the total membrane proteins are recovered in the HTP eluate. On the other hand, the specific transport activity is increased more than about 150-fold. The exact purification factor is difficult to estimate due to 2 problems, namely that the measured activity is dependent on the efficiency of reconstitution and that in-

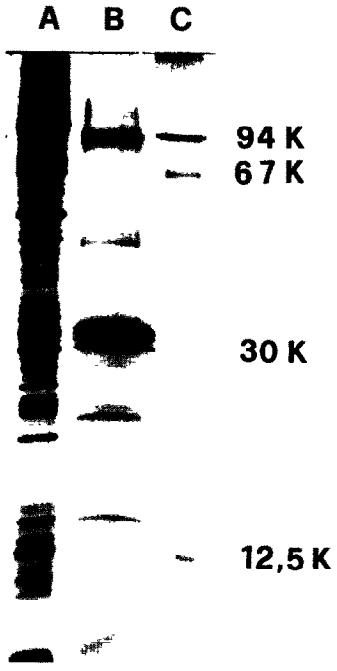

Fig.1. Polypeptide composition of the Triton extract and HTP eluate. SDS gel electrophoresis was performed in a 17.5% polyacrylamide separation gel as described in section 2. The polypeptides were fixed with formaldehyde and stained by the silver nitrate method. About 25 μg of the Triton extract (A), 10 μg of the HTP eluate (B) and 0.3 μg of each marker protein (C) were loaded.

Table 1

Purification of the 2-oxoglutarate carrier from bovine heart submitochondrial particles

Fraction	Protein yield		Phenylsuccinate-sensitive 2-oxoglutarate uptake		Purification (-fold)
	(mg)	(%)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	
Triton X-114 extract of SMP	13.70	39.3	7.2	0.53	1
HTP eluate	0.36	1.0	29.6	82.11	155

Frozen submitochondrial particles (35 mg protein) were used as starting material. Both fractions (Triton extract and HTP eluate) were reconstituted and the exchange reactions were measured for 1 min as described in section 2. Only the phenylsuccinate-sensitive activity is presented. The data represent mean values from 5 separate experiments

complete solubilization of the carrier protein and its possible partial denaturation by the detergent can occur. A similar point was considered recently in the case of the isolation of the dicarboxylate carrier from mitochondria [11].

Fig.2 shows the time course of the phenylsuccinate-sensitive uptake of external 2-oxo[1-^{14}C]glutaric acid in exchange for internal 2-oxoglutarate. Fig.2 indicates that the uptake proceeds linearly for about 1 min and then reaches a plateau at about 82 nmol oxoglutarate/mg protein. The presence of radioactivity in the zero-time sample is due to penetration of substrate into the liposomes during the time necessary to pass the sample through Dowex and/or radioactive oxoglutarate which is binding to the liposomes.

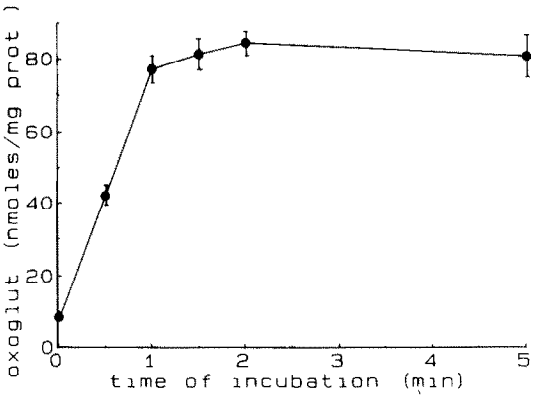

Fig.2. Time dependence of phenylsuccinate-sensitive 2-oxo[1-^{14}C]glutarate uptake during 2-oxoglutarate-2-oxoglutarate exchange in reconstituted proteoliposomes prepared from the HTP eluate. Procedures of reconstitution and transport measurements are described in section 2. All points represent mean values of activity calculated from 3 separate experiments.

Table 2 summarizes the inhibitor sensitivity and substrate specificity of the reconstituted oxoglutarate exchanger. The data indicate that both phthalonate and phenylsuccinate are potent inhibitors of the carrier. Furthermore, dicarboxylates such as malate, malonate and oxaloacetate substantially lower oxoglutarate uptake, whereas glutamate and citrate do not compete. The finding that malate is a more effective inhibitor than malonate and oxaloacetate is in agreement with the observation that the K_m for malate in intact mitochondria is much lower than the values for the other acids [1].

All this led us to the conclusion that the oxoglutarate exchanging activity found in the HTP eluate is identical to the mitochondrial 2-oxoglutarate transport activity.

3.2. *Pyruvate (monocarboxylate) carrier*

The Triton extract of mitochondria and its further purification products employed in the study of the 2-oxoglutarate transporter were also checked

Table 2

The effect of inhibitors and the substrate specificity of the partially purified, reconstituted 2-oxoglutarate exchanger

Addition (mM, final)	2-Oxoglutarate accumulation (uncorrected[a])		Net uptake of radioactive 2-oxoglutarate (corrected[a])	
	(nmol/min per mg protein)	(%)	(nmol/min per mg protein)	(% inhibition)
None	114.2	100	84.8	–
2.9 mM phthalonate	29.4	26	0.0	100
5.7 mM phenylsuccinate	30.8	27	1.4	98
28.6 mM malate	41.1	36	11.7	86
28.6 mM malonate	50.3	44	20.9	75
28.6 mM oxaloacetate	44.5	39	15.1	82
28.6 mM glutamate	116.5	102	87.1	none
28.6 mM citrate	111.9	98	82.5	3

[a] The corrected values are obtained by subtracting the phthalonate-insensitive accumulation of 2-oxoglutarate

The HTP eluted carriers were reconstituted and the 2-oxoglutarate-2-oxoglutarate exchange activity was measured as described in section 2. The values of activity represent the total accumulation of radioactive 2-oxoglutarate, not corrected for the inhibitor sensitivity and the net uptake of 2-oxoglutarate, corrected for phthalonate-insensitive accumulation. The data are mean values from 2 separate experiments. The listed anions were adjusted to about pH 7.2 (except for phthalonate and oxaloacetate which were adjusted to about 6.7) and were added externally. The samples were preincubated for 1 min prior to the addition of the radioactive 2-oxoglutarate. The temperature was 20°C and the time of incubation was 2 min. The reaction was stopped by quickly passing the mixture through a Dowex column

for the possible presence of the pyruvate (monocarboxylate) carrier. This protein has been reported to catalyse 2 reactions in mitochondria, the ΔpH-dependent uptake of monocarboxylates and their exchange. Since it is difficult to maintain and control a stable ΔpH across a liposomal membrane, the exchange reaction has been chosen as a standard assay for the monocarboxylate carrier activity. Only the 2-cyano-4-hydroxycinnamate-sensitive reaction was considered specific in the activity calculations. The mechanism of inhibition of the pyruvate transporter by cinnamate derivatives, possibly involving a covalent binding to -SH groups, has been a matter of debate (review see [21]). It was proposed by Halestrap et al. [21] that cinnamate derivatives bind on the inner side of the inner mitochondrial membrane, this being a possible reason for the need of a preincubation time when using these inhibitors.

Here it was found that both the Triton extract of the inner mitochondrial membrane and the HTP eluate, after reconstitution into liposomes, were able to catalyse an active, 2-cyano-4-hydroxycinnamate-sensitive exchange of pyruvate. Table 3 summarizes the results of the purification procedure. The calculated purification factor for the HTP chromatography amounted to about 75-fold, i.e. was considerably lower than in the case of the oxoglutarate carrier (see table 1). Such a result may be a consequence of inaccurate insertion of the carrier into the liposome membrane or of an inactivation during the purification procedure. An even lower purification factor was recently reported for the aspartate/glutamate exchanger using a similar procedure [13]. The purification procedure for the monocarboxylate carrier might be improved by using different phospholipids, detergents and salts.

The reconstituted pyruvate carrier was found to

Table 3

Purification of the monocarboxylate carrier from bovine heart submitochondrial particles

Fraction	Protein yield		Pyruvate exchange		Purification (-fold)
	(mg)	(%)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	
Triton X-114 extract of SMP	13.70	39.3	60.2	4.4	1
HTP eluate	0.36	1.0	119.2	331.0	75

Frozen submitochondrial particles (35 mg protein) were used as starting material. Proteins from both fractions (Triton extract and HTP eluate) were reconstituted and the exchange reactions were measured for 2 min as described in section 2. Only the 2-cyano-4-hydroxycinnamate-sensitive pyruvate exchange is shown. It represents 50–70% of the total pyruvate accumulation. The data represent mean values from 3 separate experiments

be specific for monovalent keto acids and not influenced by dicarboxylates and tricarboxylates and its inhibitor sensitivity (not shown) was similar to what has been described for mitochondria. These 2 findings suggest that, under the conditions described, together with other carriers also the mitochondrial monocarboxylate transporter is co-purified.

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