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The monocarboxylate carrier from bovine heart mitochondria: partial purification and its substrate-transporting properties in a reconstituted system

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The monocarboxylate (pyruvate) carrier from bovine heart mitochondria was extracted from submitochondrial particles with Triton X-114 in the presence of cardiolipin. By a single hydroxylapatite chromatography step a 125-fold purification of the carrier protein could be achieved. High pyruvate/pyruvate-exchange activity was recovered, when the protein was reconstituted into phospholipid vesicles. No transport activity was observed, when the isolation occurred in the absence of phospholipids. The 2-cyano-4-hydroxycinnamate sensitive pyruvate exchange reaction was strongly temperature sensitive and dependent on the amount of protein reconstituted. Other 2-ketoacids caused competitive inhibition of the pyruvate uptake. Inhibitors of other mitochondrial carries, however, had very low or no effect on the monocarboxylate exchange. The influence of different –SH group reagents on the measured pyruvate/pyruvate-exchange in the reconstituted system was similar to the one observed with intact mitochondria. It is concluded that the described procedures for extraction, purification and reconstitution of the mitochondrial monocarboxylate carrier conserved the functional properties of the protein.

Introduction

The inner mitochondrial membrane contains several specialized systems, which allow the net transport and/or the exchange of metabolic substrates between the matrix and the cytosol. As a result of experiments performed on the isolated intact mitochondria, these systems have been identified and classified according to their substrate specificity and inhibitor sensitivity (for reviews, see Refs.1 and 2).

Recent studies on some of the transport systems of the mitochondrial inner membrane were focussed on the molecular characterization of the proteins involved. Several reports have appeared, describing the partial purification of different mitochondrial carriers. The technique of hydroxylapatite chromatography was used initially to purify the adenine nucleotide translocator [3] but subsequently it was generally applied for the purification of other carriers as well [4]. Depending on the type of detergent used for solubilization and on the phospholipid content of the medium, the protein pattern of the hydroxylapatite eluate was found to be different, when analysed by polyacrylamide gel electrophoresis [5–7]. Apart from the ADP/ATP translocator, porin and the phosphate-transporting protein [7–10] the other bands

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; *p*-CMB, *p*-chloromercuribenzoic acid; *p*-CMBS, *p*-chloromercuriphenylsulphonate; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene; TEMED, *N,N,N′,N′*-tetramethylenediamine.

visible on the gels have not been assigned yet, although the activities of the tricarboxylate [11,12], dicarboxylate [13] and aspartate/glutamate [14] carriers have been found after hydroxylapatite chromatography and reconstitution into phospholipids. As it was recently reported elsewhere [15], also the transport activities of 2-oxoglutarate and moocarboxylate (pyruvate) carriers have been detected in the hydroxylapatite eluate.

The present paper describes in more detail the partial purification of the monocarboxylate carrier from bovine mitochondria. Studies of the transport in the reconstituted systems using different substrates and inhibitors provides more direct evidence for the existence of the monocarboxylate carrier and gives an insight into the role of some of its functional groups.

Materials and Methods

Materials

Pyruvate (sodium salt) was from Boehringer Mannheim GmbH, phenylpyruvate, 2-oxo*iso*caproate and 2-cyano-4-hydroxycinnamate were purchased by Aldrich, cardiolipin (solution 5 mg/ml in ethanol), bovine serum albumin, 1,2,3-benzenetricarboxylic acid, butylmalonate, carboxyatractyloside, antimycin A, rotenon, *p*-CMB, *p*-CMBS were delivered by Sigma, *N*-ethylmaleimide and mersalyl by Serva. Mops, Folin reagent, PPO and POPOP were from Merck, Triton X-114 from Fluka. Asolectin (extract of phospholipids from soy beans) was from Associated Concentrates. Sephadex G-50 was delivered by Pharmacia, hydroxylapatite (HTP-biogel, dry powder) and Dowex AG1-X8, Cl^- form, 100–200 mesh by BioRad. The radioactive substrate [1-^{14}C]pyruvate was from Amersham International. All other chemicals were of analytical grade.

Bovine heart mitochondria were prepared according to a standard procedure, as described by Yu et al. [16] and stored at −80°C in 250 mM sucrose/0.2 mM EDTA/10 mM Tris-HCl (pH 7.4). Submitochondrial particles were prepared from freshly thawed mitochondria by diluting with 150 mM KCl/10 mM Tris-HCl (pH 7.4) to a final protein concentration of 10 mg/ml and next, in small portions, subjected to sonication, using the large tip of a Branson sonicator (50% duty, sonic power 40). The sonicated material was centrifuged on a Sorvall RC-5B centrifuge at 15000 × *g* for 30 min and the supernatant for 2 h at 50000 × *g*. The transparent brown-red pellet was homogenized and suspended in 50 mM NaCl, 20 mM Mops (pH 7.2) to the final protein concentration of 20–30 mg/ml. Samples of submitochondrial particles were frozen in liquid nitrogen and stored at −80°C.

Solubilization of the submitochondrial particles

A portion of submitochondrial particles, containing 10 mg protein, was solubilized for 20 min at 0°C in the medium containing 3% Triton X-114, 50 mM NaCl, 1 mM EDTA, 20 mM Mops (pH 7.2) and, if not stated otherwise, 10 mg/ml of asolectin (medium A(10)). Solubilization was followed by a centrifugation for 1 h at 140000 × *g* and the supernatant was taken for further purification.

Hydroxylapatite chromatography

0.6 ml of the supernatant, was loaded onto a 0.8 × 6 cm column filled with dry hydroxylapatite and eluted at 4°C with 1.8 ml of the medium containing 4% Triton X-114, 50 mM NaCl, 1 mM EDTA, 20 mM Mops (pH 7.2) and, if not stated otherwise, 2 mg/ml of asolectin (medium A(2)).

Reconstitution

500 mg of asolectin was swollen overnight at 4°C in 4 ml of a medium containing 50 mM NaCl, 10 mM pyruvate and 10 mM Mops, (pH 7.2). Liposomes were formed by sonication with the large tip of the Branson sonifier (50% pulses, 30 W power), with cooling and under nitrogen. 0.9 ml of liposomes were mixed with 50 µl of either solubilized mitochondrial membrane proteins or hydroxylapatite eluate. The samples were kept in ice for 2 min and then quickly frozen in liquid nitrogen, followed by slow thawing at room temperature and sonication for 30 s using a pulse sequence of 20% at 30 W with the micro-tip. In order to remove the external pyruvate portions of 300 µl were passed through small (0.5 × 12 cm) columns packed with Dowex-Cl resin. Equilibration of the ion exchanger and elution was performed with 170 mM sucrose. The volume of the collected liposome opalescent fraction was 600 to

700 μl per column. The resulted dilution of the proteoliposomes was considered in the calculation of the rate of pyruvate exchange.

Measurements of transport

Mitochondria. Bovine heart mitochondria were thawed and diluted to a final protein concentration of 5 mg per ml with 150 mM sucrose/50 mM KCl/10 mM Mops (pH 7.2) and next sedimented (20 min, 15000 rpm). The pellet was taken up in the same buffer, homogenized and sedimented again. The washing procedure was repeated twice. The final suspension of mitochondria was incubated for 15 min with 10 mM pyruvate in the presence of 9 μM rotenone and 12 μM antimycin A [17,18]. In order to separate the external pyruvate, mitochondria were centrifuged in an Eppendorf centrifuge (2.5 min) and resuspended in 250 mM sucrose, 2 mM Mops (pH 7.2). The samples, containing 2 mg protein per ml, were preincubated for 3 min in the presence or absence of 1 mM 2-cyano-4-hydroxycinnamate and the reaction was started by the addition of [1-^{14}C]pyruvate to a final substrate concentration of 0.5 mM. Both preincubation and exchange reaction were performed at 15°C. The transport reaction was terminated by dilution with an ice-cold solution of 210 mM sucrose/10 mM KCl/10 mM pyruvate/2 mM Mops (pH 7.2) and immediately filtered through Milipore filters (0.45 μm). Only the 2-cyano-4-hydroxycinnamate sensitive reaction was taken to calculate the exchange rate.

The pyruvate/pyruvate-exchange activity of freshly isolated mitochondria was measured as described by Nałęcz et al. [18].

Submitochondrial particles. Submitochondrial particles were diluted with 50 mM NaCl and 10 mM Mops (pH 7.2) to a final protein concentration of 6 mg/ml and next incubated for 2 h at 15°C in the presence of 10 mM pyruvate. In order to separate the external substrate 1 ml samples were passed through columns (1 × 25 cm) of Sephadex G-50 (coarse). Equilibration and elution were performed with 170 mM sucrose. The finally collected submitochondrial particles were diluted with 170 mM sucrose to a protein concentration of 2 mg/ml and transport was immediately measured as described for mitochondria, using the Milipore filtration technique.

Reconstituted system. 0.3 ml proteoliposome samples were used and the reaction was started by the addition of 30 μl of a mixture containing 100 mM NaCl/20 mM Mops (pH 7.2)/5 mM [1-^{14}C]pyruvate. The incubation conditions are given in the legends to the tables and figures. The reaction was terminated by passing the sample through a Dowex column (0.5 × 12 cm), which was equilibrated and eluted with 170 mM sucrose. 200 μl samples were mixed with 5 ml of scintillation cocktail and counted for radioactivity.

The amount of transported pyruvate was calculated from the radioactivity measurements using the following scintillation cocktail: 5 g PPO and 0.2 g POPOP were dissolved in 1 l toluene and then mixed with 0.85 l Triton X-100 and 38 ml acetic acid. The initial specific activity of [1-^{14}C]pyruvate was 0.5 mCi/mol.

Protein determination

The procedure of Lowry et al. [19] was modified by adding 5% SDS to the samples, in order to avoid disturbancies due to the high concentration of Triton. Calibration curves with bovine serum albumin were made in the corresponding media.

Polyacrylamide gel electrophoresis

Gel electrophoresis was performed according to Laemmli [20], using a 17.5% polyacrylamide separation gel as described by De Pinto et al. [10]. The protein was precipitated by adding 5 volumes of cold aceton (stored at −20°C and dissolved in 0.1 M Tris-HCl (pH 6.8)/2% SDS/2% 2-mercaptoethanol/20% glycerol). Electrophoresis was carried out with 25 mA constantly fro 9 h. The polypeptides were fixed in 6% formaldehyde and stained with silver nitrate according to the Bio-Rad procedure.

Results and Discussion

The influence of lipids on the stability of the solubilized carrier

The role of particular lipids in the activity and stability of membrane proteins is of considerable interest. In the inner mitochondrial membrane cardiolipin is considered to be necessary for full cytochrome *c* oxidase activity [21]. Lipids appear also to play a role in the isolation and purification

TABLE I

PURIFICATION OF THE PYRUVATE-TRANSPORTING PROTEIN FROM BOVINE HEART MITOCHONDRIA IN THE PRESENCE OF DIFFERENT PHOSPHOLIPIDS

Solubilization of submitochondrial particles and hydroxylapatite chromatography are described in Materials and Methods. The media containing as the only variable different phospholipids are described as follows: C, cardiolipin (2 mg/ml); A(2), asolectin 2 mg/ml; A(10), asolectin 10 mg/ml; Tx, no phospholipid. The transport activity was measured at 15°C after preincubation with or without 1 mM 2-cyano-4-hydroxycinnamate. Only the inhibitor-sensitive activity is shown. Mitochondria were taken as reference (100%) for the yield of protein. The pyruvate/pyruvate exchange measured in mitochondria and submitochondrial particles were not compared with the activities measured in reconstituted systems (see text). The degree of purification is only given for the hydroxylapatite chromatography step using the corresponding solubilizate as a reference (purification degree = 1).

Sample	Protein		Activity		Purification (-fold)
	(mg)	(%)	total (nmol/min)	specific (nmol/min per mg)	
Mitochondria (thawed)	56.7	100	34	0.6	-
Submitochondrial particles	27.0	48	0.4	0.02	-
Membranes extracted with					
cardiolipin 2 mg/ml (C)	18.4	32.4	193	10.5	1
asolectin 10 mg/ml (A(10))	18.4	32.4	129	7.0	1
asolectin 2 mg/ml (A(2))	18.4	32.4	129	7.0	1
no lipid (Tx)	22.2	39.4	115	5.2	1
HTP eluates after solubilization with					
cardiolipin (2 mg/ml)					
eluted with C	0.33	0.6	342	1037	99
A(10)	0.27	0.5	127	473	45
A(2)	0.30	0.5	354	1180	112
Tx	0.36	0.6	13	36	4
asolectin (10 mg/ml)					
eluted with C	0.39	0.7	98	250	36
A(10)	0.09	0.2	60	633	90
A(2)	0.24	0.4	213	887	126
asolectin (2 mg/ml)					
eluted with C	0.63	1.1	63	100	14
A(10)	0.45	0.8	31	68	10
A(2)	0.57	1.0	140	247	35
no lipid					
eluted with C	0.42	0.8	0	0	-
Tx	0.55	1.0	0	0	-

of membrane proteins and cardiolipin, for instance, seems to be required for the isolation of an active phosphate carrier [5]. Partial purification of other carriers from the inner mitochondrial membrane such as those of dicarboxylate [13] and oxoglutarate [15] was also successfully performed only in the presence of cardiolipin.

In order to optimise the lipid reuqirement, monocarboxylate carrier solubilization and purification were performed in media of different phospholipid content. Two sources of lipids were used: pure cardiolipin and asolectin, a mixture of different phospholipids extracted from soy beans. A ^{31}P-NMR analysis of the asolectin employed in this study showed that it contained mainly phosphatidylcholin and phosphatidylethanolamine, but also about 10% of the total phosphorus was ascribed to cardiolipin (Prof. Seelig, personal communication). The influence of the different lipid conditions on the protein recovery and pyruvate/pyruvate-exchange activity are summarized in Table I. The amount of the extracted protein was not dependent on the quality of the added lipid, but was slightly increased when only Triton X-114 was used for solubilization. About 70% of the submitochondrial protein was extracted, a compara-

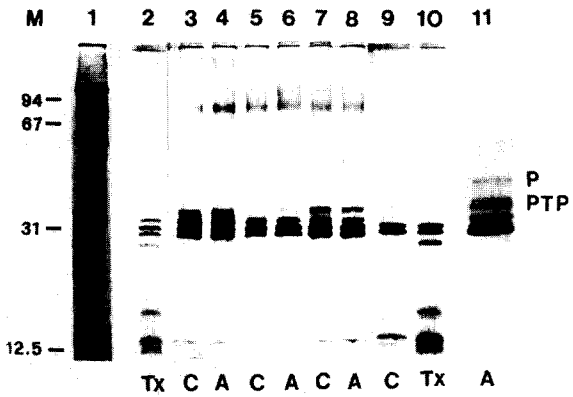

Fig. 1. Polypeptide pattern of the Triton extract and hydroxylapatite eluates obtained in the presence of different amounts of phspholipids. 40 μg protein of Triton extract (lane 1) and 12–15 μg of hydroxylapatite eluate (lanes 2–11) were applied to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The lipid conditions used for the solubilization of submitochondrial particles are given by the lane numbers as follows: 2 mg/ml cardiolipin (lane 2–4), 10 mg/ml asolectin (lanes 5, 6 and 11), 2 mg/ml asolectin (lanes 7 and 8), no lipid added (lanes 9 and 10). The lipid content of the media used for the subsequent hydroxylapatite chromatography is indicated at the bottom of the lanes as follows: 2 mg/ml cardiolipin (C); 2 mg/ml asolectin (A); no lipid added (Tx). Lane 11 shows the molecular weight range between 30 K and 35 K in an expanded scale. P and PTP indicate the putative proteins porin and phosphate carrier, respectively (see text). The following molecular-weight markers (M) were used: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (31 kDa) and cytochrome *c* (12.5 kDa).

ble value with that obtained by Kaplan and Pedersen [13] in their solubilized mitoplasts fraction. Transport activity was twice as high when solubilization was carried out in the presence of cardiolipin, indicating a stabilization effect of cardiolipin on the extracted pyruvate carrier. The presence of asolectin during solubilization gave an intermediate transport activity not depending on the amount of the added lipid. Low lipid concentrations in the medium (A(2)) used for hydroxylapatite chromatography gave, with any solubilization condition employed, the highest specific transport activity in the eluate. No lipid added to the elution medium caused loss of activity, although the recovery of protein was relatively high. Polypeptide analysis of these fractions by SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes 2 and 10) showed increased amounts of low-molecular-weight contaminants, indicating enhanced proteolysis. The presence of high amounts of lipid (A(10)) during hydroxylapatite chromatography seemed to prevent protein elution in general and also that of the carrier. By comparison of the purification procedures listed in Table I, it can be concluded that a high cardiolipin concentration is necessary during solubilization and that a low-lipid-containing medium for hydroxylapatite chromatography should be used to obtain an optimal degree of purification and high activity of the purified and reconstituted pyruvate transporting protein. However, since in a large scale preparation, it would be easier to utilize asolectin rather than pure cardiolipin, the procedure with 10 mg/ml asolectin for solubilization and 2 mg/ml of phospholipid mixture for further purification was used in the present study.

It should be noticed that with the described purification procedure not only a considerable increase in the specific activity, but also a significant gain in total activity was achieved (Table I). This increase in total transport activity after purification might reflect differences in the reconstitution conditions: 300 μg of the total solubilized

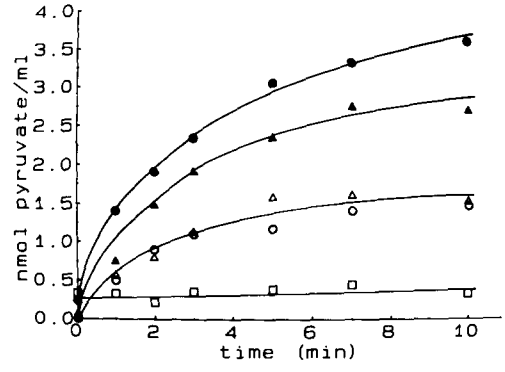

Fig. 2. Pyruvate/pyruvate exchange at different protein concentrations measured as a function of time. Solubilization was performed with the medium A(10) containing 10 mg asolectin per ml and hydroxylapatite elution with medium A(2) (2 mg asolectin per ml), as described in Materials and Methods. Reconstitution was performed with two different amounts of protein and in the absence of protein. Transport was measured at 15°C after 3 min preincubation with or without 1 mM 2-cyano-4-hydroxycinnamate and is given as nmol pyruvate taken up per ml of incubated liposomes. The final protein concentration was: 0 μg/ml (□), 0.4 μg/ml (▲, △) or 0.8 μg/ml (●, ○). The closed symbols represent the values obtained in the absence, the open ones in the presence of 2-cyano-4-hydroxycinnamate.

membrane protein per ml of liposomes but only 7.5 μg in the case of the hydroxylapatite eluate were used for reconstitution. It seems reasonable that a successful reconstitution of a carrier molecule depends on the lipid-to-protein ratio used. Such a phenomenon could also be observed: when a double amount of hydroxylapatite eluate was reconstituted, the pyruvate-exchange activity was increased only by about 80% (Fig. 2).

Rather low transport activities were measured with thawed mitochondria and submitochondrial particles. However, with 70% of the submitochondrial particle protein recovered in the Triton extract a 500-fold increase in specific activity was achieved in the reconstituted system. This indicates that the observed low-transport activities of the mitochondria and submitochondrial particles is not a consequence of loss or inactivation of the carrier, but rather due to the low quality of those membranes compared to the proteoliposomes (e.g., high carrier-independent pyruvate permeability). This suggestion could be confirmed by the much higher transport activity of freshly prepared bovine heart mitochondria (6.7 nmol/min per mg), a value comparable with the specific activity of the Triton extract measured in the reconstituted system.

From titration studies with 2-cyano[^{14}C]cinnamate [22] or 2-cyano-(1-phenylindol-3-yl)acrylate [23] on mitochondria, a stoichiometry of 50–100 pmol of binding sites per mg of total mitochondrial protein was estimated. Assuming a molecular weight of this carrier of approx. 30000 it should correspond to 1.5–3 μg/mg or to 0.15–0.3% of the total protein. After hydroxylapatite chromatography 0.4–0.6% of the original mitochondrial protein was recovered, indicating that other proteins besides the carrier have to be present after purification. This was confirmed by polyacrylamide gel electrophoresis (Fig. 1).

Polypeptide analysis by polyacrylamide gel electrophoresis

The proteins of the hydroxylapatite eluates were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. In the molecular-weight range between 30000 and 35000, where the mitochondrial carrier proteins so far analyzed were located, at least six bands could be distinguished. In all the samples eluted in the presence of lipids those six polypeptides were visible, although stained with different relative intensities. From their isolation behaviour in the different media, two polypeptides might be attributed to already described proteins. Band P (see Fig. 1, lane 11) was heavily stained only in eluates, where the cardiolipin concentration was very low during solubilization (medium A(2) in Table I, lanes 7 and 8 in Fig. 1). This is a typical behaviour for the mitochondrial porin described by De Pinto et al. [10]. An opposite behaviour was observed for two other bands (indicated in Fig. 1 by PTP), which were only strongly stained in eluates originated from solubilization in the presence of high amounts of cardiolipin (medium C in Table I, lanes 3 and 4 in Fig. 1). Such properties were rather specifically attributed to the phosphate translocator [5,6,10]. Since the amounts of those polypeptides (P and PTP) were found to be lowest in the eluate, where one of the highest pyruvate transporting activity was measured (Table I: extracted with A(10) and eluted with A(2)), the monocarboxylate carrier should be identified with one (or more) of the other noncharacterized polypeptides.

Pyruvate / pyruvate-exchange activity

2-Cyano-4-hydroxycinnamate was shown to inhibit the activity of the monocarboxylate carrier [24], when either the uptake or exchange was measured in mitochondria. Since it was reported that movement of monocarboxylic acids could also occur due to simple diffusion across the phospholipid membrane [25], the exchange reaction in the reconstituted system was measured with proteoliposomes containing different amounts of protein in the presence and absence of 2-cyano-4-hydroxycinnamate (Fig. 2). The control liposomes were subjected to the same treatment as proteoliposomes, except that instead of the hydroxylapatite eluate the same volume of the corresponding buffer (medium A(2)) was added. These liposomes without any protein incorporated revealed no activity. The exchange reaction, shown in Fig. 2 as a function of time, was faster and gave a higher steady-state value for the system with higher protein content. 1 mM 2-cyano-4-hydroxycinnamate inhibited the reaction, although a certain uptake of substrate was still observed. This

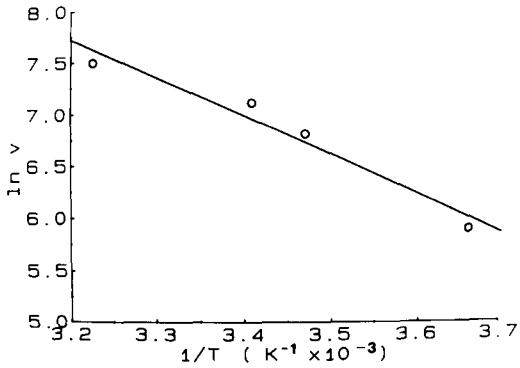

Fig. 3. Temperature dependence of the pyruvate/pyruvate-exchange reaction. Hydroxylapatite eluate was obtained and reconstituted as described in Materials and Methods. Proteoliposomes were preincubated for 3 min with or without 1 mM 2-cyano-4-hydroxycinnamate at the temperatures 0°C, 15°C, 20°C and 37°C. The reaction was started by the addition of substrate and terminated at different times by passing the incubate through a Dowex column. Only the 2-cyano-4-hydroxycinnamate sensitive initial velocity was taken into consideration.

value could not be decreased neither by a 2-fold increase of NaCl concentration nor by longer incubation with the inhibitor. The observed uptake could have been the result of binding or diffusion of substrate occurring in the case of proteoliposomes, but not with liposomes. 5 or 10 times lower concentrations of the inhibitor (0.2 or 0.1 mM) as it was used for the inhibition of the pyruvate transport in intact mitochondria [17,24] did not change significantly the inhibitory effect of 2-cyano-4-hydroxycinnamate. An unspecific effect of the inhibitor on the pyruvate permeability of the liposomal membrane could therefore be excluded, also at the higher concentration (1 mM) used here. In all further calculations only the 2-cyano-4-hydroxycinnamate sensitive reaction was considered.

The total uptake of radioactivity, as well as the net 2-cyano-4-hydroxycinnamate sensitive reaction were temperature dependent. Fig. 3 shows an Arrhenius plot of the pyruvate/pyruvate-exchange reaction in the reconstituted system at 0, 15, 20 and 37°C. The calculated activation energy of 31 kJ/mol for the pyruvate exchange is lower than the one calculated for the net transport into mitochondria (113 kJ/mol, Ref. 24). This process is, however, rather slow compared to the exchange reaction. Still the activation energy was high enough to suggest a carrier mechanism rather than a transport through a channel.

Substrate specificity and inhibitor sensitivity

It is known that the activity of the pyruvate carrier in mitochondria can be inhibited by different monocarboxylates, as for instance phenylpyruvate [17]; the 2-ketoacids originating from the branched chain amino acids are also transported by the same system [17,18]. Fig. 4 presents the effect of two alternative substrates on the pyruvate-exchange activity. These substrates were added together with pyruvate. Both, phenylpyruvate and 2-keto*iso*caproate, inhibited the reaction to the level obtained in the presence of 2-cyano-4-hydroxycinnamate. Such an inhibition could indicate that competition occurred between pyruvate and those substrates, which is in agreement with the substrate specificity of the monocarboxylate-transporting protein in mitochondria.

The effect of various substances known to inhibit different carriers from the inner mitochondrial membrane is presented in Table II. 2-Cyano-4-hydroxycinnamate strongly inhibited the reaction, whilst 1,2,3-benzenetricarboxylate, an inhibitor of the tricarboxylate carrier from heart

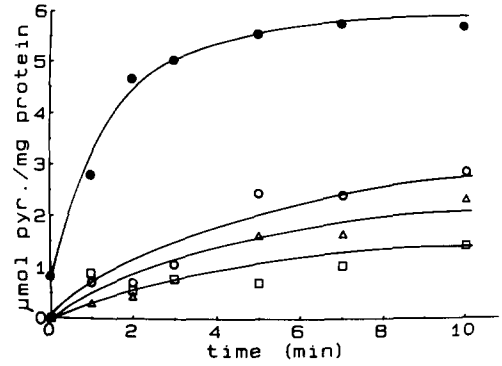

Fig. 4. Effect of other 2-ketoacids on the pyruvate/pyruvate exchange in the reconstituted system. Solubilization, hydroxylapatite-chromatography and reconstitution were performed as described in Materials and Methods. The transport reaction was measured at 15°C after 3 min of preincubation without (●) or with (○) 1 mM 2-cyano-4-hydroxycinnamate. The addition of 5-mM 2-keto*iso*caproate (□) or 5 mM phenylpyruvate (△) occurred simultaneously with the addition of [1-^{14}C]pyruvate.

TABLE II

EFFECT OF INHIBITORS OF DIFFERENT MITOCHONDRIAL CARRIERS ON THE PYRUVATE/PYRUVATE EXCHANGE IN RECONSTITUTED SYSTEMS

Solubilization, purification and reconstitution of the monocarboxylate carrier were performed as described in Materials and Methods. The transport reaction was measured for 3 min at 15°C after 3 min preincubation with or without the indicated inhibitor. 1,2,3-benzenetricarboxylate and butylmalonate were added simultaneously with pyruvate.

Inhibitor	Concentration (mM)	Exchange after 3 min	
		(μmol/mg protein)	(%)
None	–	2.5	100
2-Cyano-4-hydroxy cinnamate	1.0	0.3	12
1,2,3-Benzenetri carboxylate	1.0	2.5	101
Carboxyatractyl oside	0.005	1.9	75
Phenylsuccinate	1.0	1.1	45
Butylmalonate	1.8	2.5	102
N-ethylmaleimide	2.0	2.5	100
N-ethylmaleimide (pretreated) *	0.5		55–70
Mersalyl	0.5	2.0	80

* The HTP-eluate was treated with 2 mM *N*-ethylmaleimide for 3 min at 15°C. After stopping the reaction with 5 mM cysteine reconstitution was performed and the pyruvate/pyruvate exchange was measured.

mitochondria [26] did not affect pyruvate exchange.

Butylmalonate was shown to be a potent inhibitor of the dicarboxylate carrier in heart mitochondria [27] as well as in a reconstituted system of a prepurified fraction of this carrier [13]. Although the same inhibitor affected pyruvate oxidation in rat heart mitochondria, this effect could be demonstrated not to occur at the level of pyruvate transport [27]. This is in agreement with our observation, that butylmalonate did not inhibit the pyruvate/pyruvate exchange in our carrier reconstituted system (Table II).

The 25% inhibition of the pyruvate-exchange reaction by carboxyatractyloside (Table II), an inhibitor of the ADP/ATP carrier [28], could not be finally explained. Carboxyatractyloside might inhibit unspecifically by binding to the proteoliposomes.

N-ethylmaleimide was reported not to affect pyruvate/pyruvate exchange in intact mitochondria [29]. In the reconstituted system *N*-ethylmaleimide was a poor inhibitor (Fig. 5) and the effect could hardly be seen after 3 min of incubation (Table II) even if it was already present during the preincubation period. However, when the *N*-ethylmaleimide treatment occurred before the reconstitution the cinnamate-sensitive pyruvate-exchange rate was only abotu 50–70% of the control. This result would favour the idea, that the monocarboxylate translocator may contain an –SH group which is essential for its activity and is located on the inner side of the inner mitochondrial membrane. On such a basis an inhibition mechanism for 2-cyanocinnamate and its derivatives was proposed by Halestrap et al. [30,31]. Since the hydrophobic part of the cinnamate derivatives is essential for the inhibition, the SH-group might be located in a hydrophobic surrounding. The chemical structure of phenylsuccinate, an inhibitor of dicarboxylate carrier [32], does not include the possibility of a reaction with such an active site what would explain the 50% inhibition observed (Table II). In order to investigate further an involvement of –SH group(s) into the monocarboxylate transport the pyruvate/pyruvate-exchange reaction was performed in the presence of other compounds reacting with –SH groups [33]:

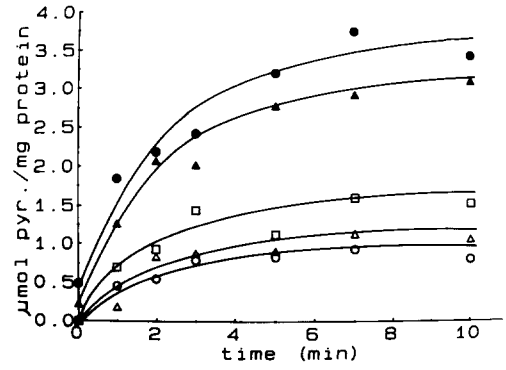

Fig. 5. Effect of –SH group reagents on the pyruvate/pyruvate-exchange activity in the reconstituted system. Solubilization, hydroxylapatite-chromatography and reconstitution were performed as described in Materials and Methods. Transport was measured at 15°C after 3 min preincubation without (●) or with the following additions: 2 mM *N*-ethylmaleimide (▲), 0.2 mM *p*CMB (△), 0.2 mM *p*CMBS (□), 1 mM 2-cyano-4-hydroxycinnamate (○).

p-CMB, *p*-CMBS and mersalyl (Fig. 5, Table II). As it is shown in Fig. 5 the effect of *p*-CMB is similar to that of 2-cyano-4-hydroxycinnamate, whilst *p*-CMBS is less inhibitory, most probably due to its higher polarity and therefore greater difficulty to pass through the phospholipid bilayer. However the differnece between the inhibition of *p*-CMB and *p*-CMBS seems not to be significant enough, especially in comparison with the relative high non-inhibited pyruvate exchange, to conclude for a location of an essential –SH group at the inner side of the membrane. Further evidence is necessary to support this hypothesis.

Since the 2-cyano-4-hydroxycinnamate-sensitive pyruvate-exchange reaction in the reconstituted system described here had substrate and inhibitor characteristics similar to those observed in mitochondria, we can conclude that the monocarboxylate carrier was successfully prepurified by a single hydroxylapatite chromatography step and reconstituted in its active form into phospholipid vesicles. The –SH group reactivity data suggest that the reconstituted carrier molecules were properly oriented.

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