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Kinetics of the reconstituted 2-oxoglutarate carrier from bovine heart mitochondria

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The 2-oxoglutarate carrier from the inner membrane of bovine heart mitochondria was purified by chromatography on hydroxyapatite/celite and reconstituted with egg yolk phospholipid vesicles by the freeze-thaw-sonication technique. In the reconstituted system the incorporated 2-oxoglutarate carrier catalyzed a first-order reaction of 2-oxoglutarate/2-oxoglutarate exchange. The substrate affinity for 2-oxoglutarate was determined to be 65 ± 18 μM (15 determinations) and the maximum exchange rate at 25°C reaches 4000–22000 μmol/min per g protein, in dependence of the particular reconstitution conditions. The activation energy of the exchange reaction is 54.3 kJ/mol. The transport is independent of pH in the range between 6 and 8. When the first fraction of the hydroxyapatite/celite column eluate was used for reconstitution, besides the 2-oxoglutarate/2-oxoglutarate exchange, a significant activity of unidirectional uptake was observed. This activity may be due to a population of the carrier protein which is in a different state.

Introduction

2-Oxoglutarate is transported through the inner mitochondrial membrane by an obligatory 1 : 1 exchange with malate or some other dicarboxylic acids. This exchange is catalyzed by a specific transport system, known as the 2-oxoglutarate carrier, which has been extensively investigated in intact mitochondria [1–5]. This transport system plays an important role in several metabolic processes like the malate-aspartate shuttle, the isocitrate-2-oxoglutarate shuttle, gluconeogenesis from lactate and nitrogen metabolism [6,7].

We have recently isolated the 2-oxoglutarate transport protein from pig-heart mitochondria by chromatography on hydroxyapatite and celite in the presence of cardiolipin [8]. In SDS-containing gels, the purified fraction consists of a single polypeptide with an apparent M_r of 31500. This protein corresponds to band 4 of the five protein bands previously identified in the hydroxyapatite pass-through of Triton-X-114-solubilized heart mitochondria [9,10]. After incorporation into liposomes the purified protein has been functionally identified as the 2-oxoglutarate carrier [8], since it retained the same high substrate specificity and susceptibility to inhibitors of the 2-oxoglutarate transport system in mitochondria.

In this paper the conditions for optimal reconstitution of the mitochondrial 2-oxoglutarate carrier are described. Quantitative values of the transport rate and related kinetic parameters of

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

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the [^{14}C]2-oxoglutarate/2-oxoglutarate exchange are reported.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) and Dowex AG 1-X8 were purchased from Bio-Rad, Celite 535 from Roth, [1-^{14}C]-2-oxoglutarate from the Radiochemical Centre (Amersham, U.K.), egg yolk phospholipids (Lecithin from eggs) from Fluka, cardiolipin from Avanti-Polar Lipids and Triton X-114 from Serva. L-3-dioleyl phosphatidylcholine, DL-α-dipalmitoyl phosphatidylcholine, L-α-phosphatidylinositol, and L-lysophosphatidylcholine were purchased from Sigma, phosphatidylethanolamine from Serdary. Mitochondrial phospholipids were isolated by the procedure described in Ref. 11. Phthalonic acid was a gift of Drs. G. Randazzo and A. Evidente. Other reagents were obtained as reported [4].

Isolation of the 2-oxoglutarate transport protein. Bovine heart mitochondria were prepared as described in Ref. 12. The 2-oxoglutarate carrier was purified by the procedure described previously [8] for pig heart mitochondria with some modifications. 5 ml of extract, obtained by solubilization of the mitochondria with 3% Triton X-114 and subsequent centrifugation, were supplemented with cardiolipid (2 mg/ml) and applied on a dry hydroxyapaptite/celite (6 g, ratio 5 : 1) column. Elution was performed with the solubilization buffer (3% Triton X-114 (w/v)/50 mM NaCl/10 mM Pipes (pH 7.0)). Four fractions of 1 ml each were collected. Unless specified otherwise, the second and the third fractions, containing pure 2-oxoglutarate transport protein, were combined and used.

Incorporation of the 2-oxoglutarate carrier into liposomes. The 2-oxoglutarate carrier was incorporated into liposomes by the freeze-thaw-sonication procedure [8,13]. With the exception of the experiments reported in Table I, egg yolk phospholipids were used for the reconstitution. Liposomes, prepared as described previously [8] in the presence and absence of 2-oxoglutarate, were mixed with the indicated fractions of the hydroxyapatite/celite eluate with a lipid/protein ratio of about 10^4 (w/w). After 5 min at 0°C the mixture was frozen in liquid N_2, thawed in a water bath at 10°C and then pulse-sonicated (0.3 s sonication, 0.7 s intermission) for 6 s (unless specified otherwise) at 0°C. It was found that the detergent/lipid ratio should not exceed a value of 0.24 (w/w), otherwise the activity of the reconstituted 2-oxoglutarate carrier decreases. In the following experiments this ratio was kept at about 0.08.

Assay of 2-oxoglutarate exchange in proteoliposomes. The proteoliposomes were incubated at 25°C for 4 min. When necessary, the external 2-oxoglutarate was removed by passing the proteoliposomes through an anion exchange column (Dowex AG1-X8, 50–100 mesh) in acetate form (0.5 × 8 cm) preequilibrated with 1 ml 10 mM Pipes/20 mM NaCl (pH 7.0) and eluted with the same solution. The eluted proteoliposomes were used for transport measurements by the inhibitor stop method [14]. Transport was initiated by adding carrier-free [^{14}C]oxoglutarate to proteoliposomes prepared in the presence of 2-oxoglutarate. In the case of proteoliposomes, which were prepared in the absence of substrate or contained the substrate only inside, appropriate concentrations of [^{14}C]oxoglutarate were added. After 30 s (unless specified otherwise) the reaction was stopped by the addition of 10–20 mM phthalonate. In control samples phthalonate was added together with the labeled substrate at time zero. In order to remove the external radioactivity, each sample was applied immediately on a column of Dowex AG1-X8, 50–100 mesh, acetate form (0.5 × 5 cm). The liposomes eluted with 1 ml of 20 mM NaCl were collected, vortexed with 4 ml of scintillation mixture (Maxifluor Baker, The Netherlands) and counted. The activity was calculated by subtracting the control values from the experimental samples.

Other methods. Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [15]. The separation gel contained 17.5% acrylamide and an acrylamide-to-bisacrylamide ratio of 150 to give a high resolution of polypeptides of an M_r value close to 30000 [9]. Staining was performed with the silver nitrate method [16]. The molecular weights were determined with the help of Pharmacia low-molecular weight markers. Protein was determined by the Lowry method modified for the presence of Triton [17].

Results

In the previous paper [8], we have shown that the 2-oxoglutarate carrier protein reconstituted in liposomes, which were not loaded with the substrate, catalyzed some uptake of oxoglutarate which proved to be sensitive to phthalonate, the specific inhibitor of the oxoglutarate carrier in mitochondria [18]. At equilibrium this uptake accounted for about 10% of the total amount of substrate transported in reconstituted liposomes loaded with 2-oxoglutarate. These results were obtained by incorporating the complete pass-through, collected after the hydroxyapatite and the celite chromatographic steps, into liposomes.

In order to investigate further the unexpected 2-oxoglutarate uptake into unloaded liposomes, we have fractionated the pass-through obtained by the one step hydroxyapaptite/celite chromatographic procedure described in this paper. The single fractions were analyzed on SDS-gels and tested for 2-oxoglutarate uptake activity in both loaded and unloaded liposomes. The results are reported in Fig. 1. In all the fractions the activity of the 2-oxoglutarate uptake is higher in loaded than in unloaded liposomes (Fig. 1A). The uptake of oxoglutarate in loaded liposomes shows a maximum in the second fraction, whereas that in unloaded vesicles is significant only in the first fraction. Consequently, the difference of activities in the presence and absence of internal substrate, i.e., the oxoglutarate taken up by an exchange mechanism, is lower in the first fraction than in the following ones. The analysis by SDS gel electrophoresis (Fig. 1B) reveals that the first three fractions contain a single protein band with an apparet M_r of 31 500. Therefore this protein extracted from bovine heart mitochondria exhibits the same electrophoretic mobility as the 2-oxoglutarate carrier protein purified from pig heart as described in the previous paper [8]. In the fourth fraction the preparation is contaminated by a protein with an apparent M_r of 35 500, which corresponds to porin [10] from the outer mitochondrial membrane.

In Fig. 2 the time-courses of the 2-oxoglutarate uptake in loaded and unloaded liposomes are reported for the first and the second fraction of the pass-through obtained by hydroxyapatite/celite.

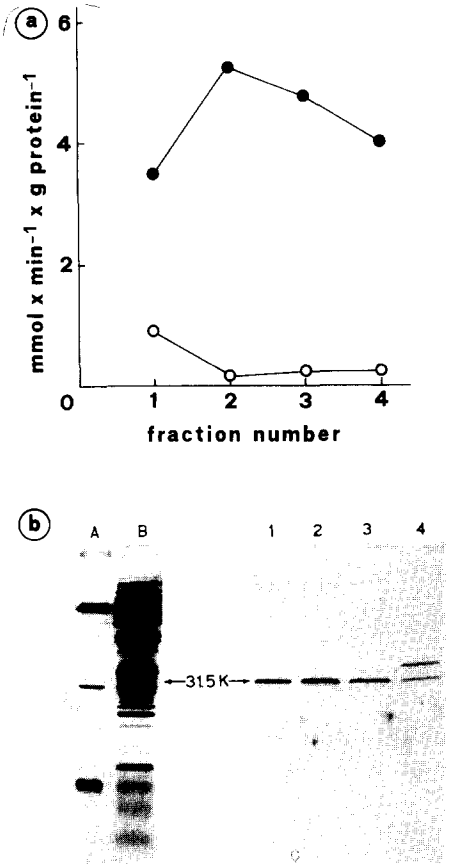

Fig. 1. (a) Uptake of 2-oxoglutarate in loaded and unloaded proteoliposomes reconstituted by incorporating the first four fractions of the hydroxyapatite/celite eluate. Loaded proteoliposomes were prepared in the presence of 1 mM 2-oxoglutarate. [^{14}C]oxoglutarate was added either carrier-free or in 1 mM concentration to loaded (●) or unloaded (○) proteoliposomes, respectively. (b) SDS gel electrophoresis of fractions 1–4 of Fig. 1a: A, marker proteins (bovine serum albumin, carbonic anhydrase and cytochrome *c*); B, mitochondrial extract; 1–4, fractions 1–4 of Fig. 1a.

It can be seen that the uptake of 2-oxoglutarate in the absence of internal substrate is significant only in proteoliposomes prepared by incorporating the first fraction (Fig. 2A), but it is virtually absent when the second fraction has been incorporated (Fig. 2B). Using the third and the fourth fraction time-courses similar to that in Fig. 2B are obtained (not shown). Fig. 2 also shows the effect of the addition of 10 mM cold 2-oxoglutarate on the efflux of labeled 2-oxoglutarate from loaded and unloaded liposomes. The cold substrate was added

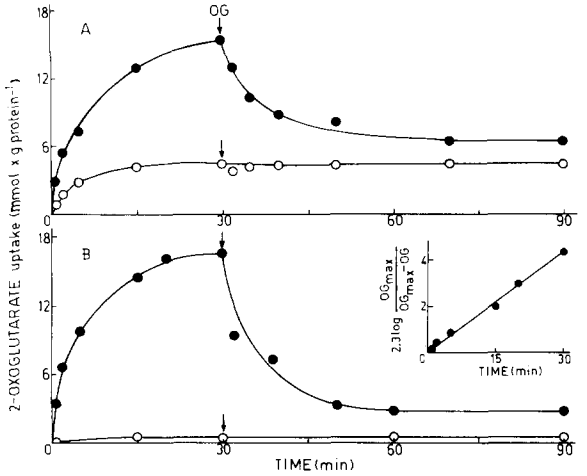

Fig. 2. Time-course of [^{14}C]2-oxoglutarate uptake in reconstituted liposomes and its efflux after the addition of an excess of cold 2-oxoglutarate. Proteoliposomes prepared in the presence (●) and absence (○) of 1 mM 2-oxoglutarate were reconstituted by incorporating the first (A) or the second (B) fraction of the hydroxyapatite/celite eluate. Carrier-free (●) or 1 mM (○) [^{14}C]2-oxoglutarate was added at time zero and the reaction was stopped by adding 20 mM phthalonate after the desired time interval. Where indicated by the arrow, 10 mM cold 2-oxoglutarate was added. The inset in (B) is the logarithmic plot of 2-oxoglutarate exchange. The values used for the inset were obtained by subtracting the values of 2-oxoglutarate uptake in unloaded liposomes from those in loaded vesicles.

after 30 min incubation of the proteoliposomes with labeled oxoglutarate, i.e., when the radioactivity taken up by the proteoliposomes had approached the equilibrium. The addition of 10 mM cold substrate does not cause any efflux of labeled 2-oxoglutarate when unloaded proteoliposomes have been used, suggesting that these vesicles catalyze only an unidirectional transport. In contrast, in the case of substrate-loaded liposomes the addition of cold 2-oxoglutarate does cause an extensive efflux of radioactivity. This efflux represents 83% of the intraliposomal radioactivity when the second fraction has been used for reconstitution. The efflux of radioactive oxoglutarate amounts to only 58% when the first fraction has been incorporated. In this fraction also the uptake into unloaded liposomes was remarkable. However, the latter value increases to 84% if it is calculated on the basis of the amount of 2-oxoglutarate taken up only by exchange. This amount is obtained by subtracting the substrate taken up unidirectionally, measured in unloaded liposomes, from the total uptake in loaded vesicles. The more or less complete efflux of radioactivity caused by addition of cold 2-oxoglutarate is in agreement with the exchange mechanism of transport one would expect to be catalyzed by the 2-oxoglutarate carrier.

In the light of the results shown in Figs. 1 and 2, in all subsequent experiments only the protein of the second and the third fraction of the hydroxyapatite/celite pass-through has been used to study the activity of the 2-oxoglutarate carrier, since in these fractions the unidirectional uptake is almost negligible. Nevertheless, it was concomitantly measured in the experiments and subtracted in order to obtain the true values of the oxoglutarate/oxoglutarate exchange.

The reaction order of the exchange was investigated by plotting the natural logarithm of the fraction of equilibrium 2-oxoglutarate$_{max}$/(2-oxoglutarate$_{max}$-2-oxoglutarate$_t$) against time. A straight line is obtained (inset of Fig. 2B), demonstrating that the reconstituted 2-oxoglutarate/-oxoglutarate exchange follows a first-order kinetics similar to what has been found in intact mitochondria [4]. The first-order rate constant, k, is 0.15 min^{-1}.

In the previous paper [8] reconstitution was used for monitoring the purification procedure and for characterizing the 2-oxoglutarate transport at near equilibrium. For obtaining reliable kinetic data the conditions which lead to optimal reconstitution should be thoroughly investigated.

An obligatory step for the reconstituion of the 2-oxoglutarate carrier in liposomes is the freeze-thaw procedure. Repeated freeze-thaw steps, however, do not increase the phthalonate-sensitive 2-oxoglutarate exchange (not shown). The effect of sonication following freezing and thawing on the 2-oxoglutarate exchange activity is illustrated in Fig. 3. In the same figure, the control values, i.e., those obtained when the inhibitor was added together with the labeled substrate, were also reported in dependence of the sonication time. These control values can give us information about possible unspecific permeability of the proteoliposomes. First the rate of exchange increases with sonication time, reaches a maximum after 2–3 s sonication and is then diminished progressively.

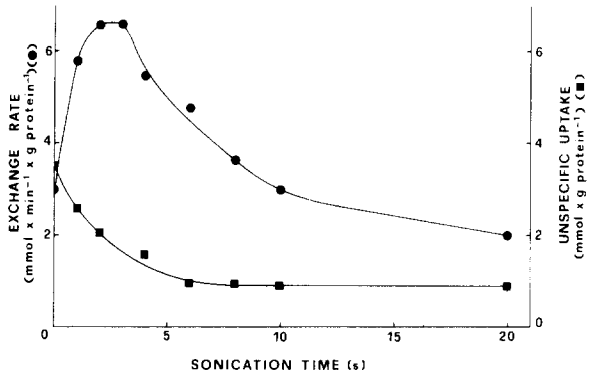

Fig. 3. Effect of sonication of proteoliposomes on the 2-oxoglutarate exchange and on the phthalonate-insensitive uptake of 2-oxoglutarate. Carrier-free [^{14}C]2-oxoglutarate was added to protoeliposomes prepared in the presence of 1 mM 2-oxoglutarate. The exchange rates (●, mmol/min per g protein) represent the difference between the radioactivity in the samples (inhibitor added after 30 s) and the controls (inhibitor added together with the labeled substrate). The phthalonate-insensitive uptake of 2-oxoglutarate (■, mmol per g protein) represents the radioactivity associated to the proteoliposomes in the controls measured after 30 s.

This result is similar to that of other reconstituted systems [19,20], but is in contrast to the effect of sonication time on the activity of the phosphate carrier [21]. The data shown in Fig. 3 suggest the use of a sonication time of 2–3 s in the further experiments. However, the 2-oxoglutarate which is bound to or entrapped in the liposomes in the presence of the inhibitor, i.e., independent of the carrier activity, shows a maximum already without sonication. This amount is drastically decreased during the first few sonication pulses and reaches a constant value after 6 s of sonication. The decrease of the phthalonate-insensitive 2-oxoglutarate uptake suggests that after short times of sonication at least part of the liposomes are unspecifically permeable. Although the carrier protein is more active after 2–3 s of sonication, the results reported in Fig. 3 show that kinetic measurements at low external substrate concentrations require a sonication time of at least 6 s. Thus an unspecific efflux of 2-oxoglutarate from loaded liposomes and a concomitant decrease of the external specific activity is prevented.

The exchange activity of the reconstituted 2-oxoglutarate carrier is influenced by the lipid composition of the liposomes used for reconstitution (Table I). The highest activity was found with liposomes prepared from egg yolk phospholipids. Addition of dipalmitoyl phosphatidylcholine or of mitochondrial phospholipids has no effect on the transport activity. The carrier activity is inhibited by the addition of dioleyl phosphatidylcholine, phosphatidylethanolamine, cardiolipin, phosphatidylinositol, lysophosphatidylcholine and, to a greater extent, by cholesterol. This is in contrast to the effect of various lipids on the activity of other reconstituted anion transport systems. Thus, the phosphate carrier and the tricarboxylate carrier are activated by mitochondrial phospholipids and, specifically, by cardiolipin [20–25]; the ADP/ATP carrier is activated by acidic phospholipids and particularly by cholesterol [26,27].

In order to obtain the basic kinetic data the dependence of the reconstituted 2-oxoglutarate exchange on the substrate concentration was studied by changing the concentration of externally added [^{14}C]2-oxoglutarate, at a constant internal concentration of 10 mM 2-oxoglutarate (Fig. 4). The K_m and V_{max} values for 2-oxoglutarate exchange are 80 μM and 3980 μmol/min per g protein,

TABLE I

INFLUENCE OF DIFFERENT PHOSPHOLIPID COMPOSITION OF LIPOSOMES ON THE RECONSTITUTED 2-OXOGLUTARATE/2-OXOGLUTARATE EXCHANGE ACTIVITY

Reconstitution was performed with liposomes prepared from EYPL or a mixture of 80% EYPL and 20% of the indicated phospholipids. Abbreviations: EYPL, egg yolk phospholipids (Lecithin from eggs, Fluka); DOPC, dioleyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; PE, phosphatidylethanolamine; DPG, cardiolipin; PI, phosphatidylinositol; MPL, mitochondrial phospholipids; LPC, lysophosphatidylcholine. Proteoliposomes were prepared in the presence of 1 mM 2-oxoglutarate. Other conditions as described in Materials and Methods.

Phospholipid composition	Specific activity (μmol/g per min)	Total exchange (μmol/g per 30 min)
EYPL	6320	17000
EYPL + DOPC	4900	11200
EYPL + DPPC	6370	17400
EYPL + PE	3790	6700
EYPL + DPG	4180	12620
EYPL + PI	3950	8600
EYPL + MPL	6430	11850
EYPL + cholesterol	1360	2430
EYPL + LPC	3740	10560

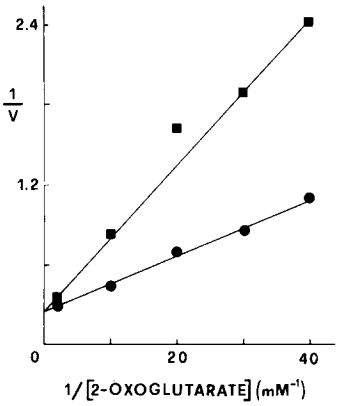

Fig. 4. Dependence of the rate of 2-oxoglutarate exchange on the external 2-oxoglutarate concentration and competitive inhibition by phthalonate. Proteoliposomes were prepared in the presence of 10 mM 2-oxoglutarate and then passed through a Dowex column in order to remove the external substrate. [^{14}C]2-oxoglutarate was added at the indicated concentrations. When present, 0.1 mM phthalonate was added simultaneously with [^{14}C]2-oxoglutarate. (●) Control; (■) with phthalonate. The exchange activity V is expressed in mmol/min per g protein.

respectively, at 25°C. In 15 experiments an average value of 65 ± 18 μM for the K_m and 3780 ± 460 μmol/min per g protein for the V_{max} was determined. In accordance with what has been found for mitochondria [18], phthalonate proves to be a competitive inhibitor with respect to 2-oxoglutarate with a K_i of 60 μM.

In the experiment shown in Fig. 4, application of different external 2-oxoglutarate concentrations has required passage of the loaded proteoliposomes through a Dowex column in order to remove the external substrate. This procedure could lead to partial inactivation of the 2-oxoglutarate carrier. In fact, when the rate of 2-oxoglutarate exchange is measured in proteoliposomes, prepared in the presence of 5 mM 2-oxoglutarate and sonicated for 2–3 s, by adding carrier-free [^{14}C]2-oxoglutarate, and hence eliminating the passage through the Dowex column, a value of 13600 ± 1200 μmol/min per g protein is obtained (eight determinations). Although the higher V_{max} is partially due to the shorter sonication time there remains a significant difference to the V_{max} calculated on the basis of the data shown in Fig. 4, which can be attributed to the inactivation by the Dowex column passage. Thus, we tried to estimate

TABLE II

K_i VALUES FOR DICARBOXYLATES COMPETING WITH 2-OXOGLUTARATE FOR THE EXCHANGE REACTION

The K_i values were calculated from double reciprocal plots of the rate of 2-oxoglutarate exchange activity vs. substrate concentrations. The experimental conditions were the same as in Fig. 4. The competing anions were added simultaneously with [^{14}C]2-oxoglutarate at the appropriate concentrations.

Dicarboxylate	K_i (mM)
L-Malate	0.27
Malonate	1.0
Succinate	1.2
Maleate	1.3
Oxaloacetate	2.6
Phthalonate	0.06
p-Iodobenzylmalonate	0.07
Phenylsuccinate	0.4
Butylmalonate	0.7

the maximal activity of the reconstituted 2-oxoglutarate exchange by another type of measurement. Proteoliposomes were prepared by 2–3 s of sonication in the presence of substrate concentrations from 1 to 10 mM, and the transport activity was measured by adding carrier-free [^{14}C]2-oxo-

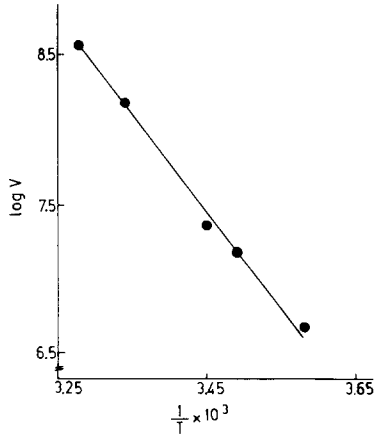

Fig. 5. Arrhenius plot of the temperature dependence of the reconstituted 2-oxoglutarate exchange activity. Carrier-free [^{14}C]2-oxoglutarate was added to proteoliposomes which were prepared in the presence of 1 mM 2-oxoglutarate and incubated at the indicated temperatures. The exchange activity V is expressed in μmol/min per g protein.

glutarate. Thereby a V_{max} of 22200 μmol/min per g protein and a K_m of 2 mM was obtained (three experiments).

The inhibition of the reconstituted 2-oxoglutarate exchange by various dicarboxylates was analyzed in the presence of different substrate concentrations. L-Malate and other dicarboxylates were identified as competitive inhibitors, since they were found to increase the K_m without changing the V_{max} of the 2-oxoglutarate exchange. The inhibition constants, K_i, are summarized in Table II, where substrates and inhibitors of the 2-oxoglutarate carrier [4,18,28] are listed.

The temperature dependence of the rate of 2-oxoglutarate exchange is shown in Fig. 5. The rate of 2-oxoglutarate exchange increases about 7 times with increasing the temperature from 6° to 32°C. A straight line is obtained in an Arrhenius plot over this range. The activation energy as derived from the slope is 54.3 kJ · mol^{-1}.

Fig. 6 illustrates the influence of the pH on the rate of 2-oxoglutarate exchange. In the presence of low substrate concentrations, the rate of 2-oxoglutarate exchange increases on decreasing the pH from 8.0 to 6.0 (Fig. 6A). If, however, the substrate is present at a high concentration, the exchange is more or less independent of the pH. Fig. 6B shows that raising the pH from 7.0 to 8.0 increases the K_m of oxoglutarate from 53 to 104 μM without affecting the V_{max}. This confirms that the activity of the 2-oxoglutarate carrier is independent of the pH at high substrate concentrations similar to the observations in mitochondria [4].

Discussion

In the course of our experiments for characterizing the kinetics of the reconstituted 2-oxoglutarate carrier we were confronted with some methodological problems of transport measurement. When optimizing the sonication step, which is necessary for the reconstitution, we realized that sonication has a great influence not only on the exchange activity, but also on the amount of 2-oxoglutarate which is unspecifically associated to the proteoliposomes. This unspecific, i.e., phthalonate insensitive, association decreases with increasing sonication time. Since it is not inhibitor sensitive, i.e., not due to the action of the 2-oxoglutarate carrier, and since it appears already without sonication, this phenomenon may be caused by temporary opening and closing of vesicles which thus may include labeled substrate. This effect, therefore, is more or less abolished after appropriate sonication time, when the proteoliposomes have become tight because of the ultrasonic pulses. In order to decrease the un-

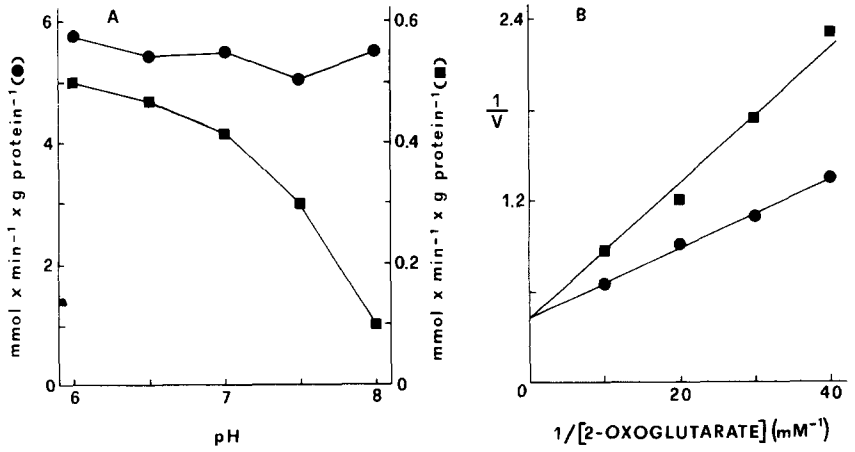

Fig. 6. Effect of pH on 2-oxoglutarate exchange. (A) Carrier-free [^{14}C]2-oxoglutarate was added to proteoliposomes which were prepared in the presence of 0.08 mM (■) or 3 mM (●) 2-oxoglutarate and incubated at the indicated pH values. (B) The experimental conditions were similar to those described in Fig. 4 at pH 7.0 (●) and 8.0 (■). The exchange activity V is expressed in mmol/min per g protein.

specific background, we had to use longer sonication times especially when transport values at low substrate concentrations or at short reaction times had to be measured.

The second problem which arose during the kinetic measurements was the observation of unidirectional substrate uptake. It is not easy to separate experimentally the true 1 : 1 exchange and the unidirectional uptake, which is necessary for measuring the kinetics of the 2-oxoglutarate/2-oxoglutarate exchange. However, we have solved this problem by fractionation of solubilized 2-oxoglutarate carrier protein, thus obtaining a pure fraction which catalyzes only the exchange reaction. Apart from this, it is interesting that a fraction of the isolated carrier exists, which consists of a protein band of the same mobility in SDS-gels and which catalyzes substantial unidirectional uptake of 2-oxoglutarate when reconstituted into liposomes. The indications for the uniport reaction are (i) the uptake into unloaded liposomes and (ii) the lack of efflux after addition of excess of cold substrate. Furthermore, the uptake in unloaded liposomes cannot be explained by simple binding because the amount of 2-oxoglutarate taken up by this process exceeds the number of carrier molecules incorporated into the liposomes by about three orders of magnitude. Also the possibility of a half-cycle reaction, i.e., a single inward translocation event without backward conformational change of the protein, is ruled out by the discrepancy between the amount of carrier molecules and transported substrate. Obviously the solubilized and reconstituted 2-oxoglutarate carrier can exist in two states or possibly two conformations which catalyze different mechanisms of transport reactions. It is unlikely that the two different activities can be catalyzed by a single carrier molecule at the same time, since the 2-oxoglutarate, which was taken up into unloaded liposomes due to the unidirectional transport, cannot be equilibrated with externally added 2-oxoglutarate as would occur if carriers catalyzing exchange were also active in these liposomes. The occurrence of a population of carrier molecules catalyzing the unidirectional flux may possibly be explained by some influences during solubilization and purification, e.g., the interaction with the dry hydroxyapatite/celite in the course of the adsorption chromatography, which would predominantly affect the first eluted fraction.

In the reconstituted system, the optimization of which has been discussed above, we have determined some important kinetic data of the 2-oxoglutarate carrier from the inner mitochondrial membrane. The reconstituted carrier catalyzed a 2-oxoglutarate/2-oxoglutarate exchange which can be described as a first-order reaction. The temperature dependence of the transport reaction from 6° to 32°C is linear in an Arrhenius plot. The activation energy was determined to be 54.3 kJ/mol, which is very close to the value obtained for the reconstituted phosphate carrier [21] and the reconstituted ADP/ATP carrier [27]. As is the case for the 2-oxoglutarate transport in mitochondria [4], the reconstituted carrier is practically independent of the applied pH in the range between 6 and 8.

In the previous paper [8] we have characterized the reconstituted 2-oxoglutarate carrier with respect to its substrate specificity and inhibitor sensitivity. Here it is shown that the well-known substrates and inhibitors of the 2-oxoglutarate carrier all compete for the same binding site, showing K_i values between 0.06 mM for the specific inhibitor phthalonate and 2.6 mM for the poor substrate oxaloacetate. The reported K_i values are similar to those found in intact mitochondria [4,18,28]. Under optimal conditions, i.e., short sonication time and no passage through Dowex ion exchanger for the removal of external substrate, a V_{max} value of 22200 μmol/min per g protein is measured at 25°C. The turnover number, which was calculated assuming that the isolated protein is pure and consists of a monomer of 31.5 kDa, corresponds to 700 min^{-1}. This turnover number is lower than that reported for the reconstituted phosphate carrier [22,29,30] and is in the same range as found for the reconstituted ADP/ATP carrier [31] and the reconstituted aspartate/glutamate carrier (unpublished results).

Under conditions suited for the measurement of substrate affinity the external K_m for 2-oxoglutarate was determined to be 65 μM. Since in this case longer sonication times and the Dowex column have to be used, the corresponding V_{max} value reached only about 4000 μmol/min per g protein. Although the experiments for the deter-

mination of the external K_m for 2-oxoglutarate reproducibly lead to the value reported above, a measurement under different experimental conditions, i.e., short sonication without separation of external substrate, led to a much higher K_m value of about 2 mM. It may be that this high value is due to the affinity of 2-oxoglutarate to the inner side of the 2-oxoglutarate carrier. In fact, Sluse et al. [3] have reported a K_m value of 1.04 mM for internal 2-oxoglutarate in intact mitochondria.

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