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Kinetics of the reconstituted dicarboxylate carrier from rat liver mitochondria

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The dicarboxylate carrier from rat liver mitochondria was purified by the Amberlite/hydroxyapatite procedure and reconstituted in egg yolk phospholipid vesicles by removing the detergent with Amberlite. The efficiency of reconstitution was optimized with respect to the ratio of detergent/phospholipid, the concentration of phospholipid and the number of Amberlite column passages. In the reconstituted system the incorporated dicarboxylate carrier catalyzed a first-order reaction of malate/phosphate exchange. *V* of the reconstituted malate/phosphate exchange was determined to be 6000 μmol/min per g protein at 25 °C. This value was independent of the type of substrate present at the external or internal space of the liposomes (malate, phosphate or malonate). The half-saturation constant was 0.49 mM for malate, 0.54 mM for malonate and 1.41 mM for phosphate. The activation energy of the exchange reaction was determined to be 95.8 kJ/mol. The transport was independent of the external pH in the range between pH 6 and 8.

Introduction

Since 1967, when its existence was proposed [1], the properties of the dicarboxylate carrier have been extensively investigated in intact mitochondria. This carrier catalyzes an electroneutral exchange of certain dicarboxylates (e.g., malate, malonate and succinate), inorganic phosphate and inorganic sulphur-containing compounds (e.g., sulphite, sulphate and thiosulphate) [1–8]. It is inhibited by some impermeable dicarboxylate analogues like butylmalonate [9,10] as well as by sulphydryl reagents (but not by *N*-ethylmaleimide) [11–13]. The kinetic properties of the dicarboxylate carrier have also been characterized in intact mitochondria [5,14]. Interestingly, the activity of the dicarboxylate carrier is high in liver and low in heart [15,16], which is related to the absence of gluconeogenesis in the latter tissue.

In various laboratories the mitochondrial dicarboxylate carrier has been partially purified from rat liver [17,18] and bovine heart [19]. Recently, we have isolated the dicarboxylate carrier from rat liver mitochondria by chromatography on Amberlite and hydroxyapatite in the presence of cardiolipin [20]. In SDS-containing gels, the purified fraction consists mainly of one protein band with an apparent M_r of 28000. After incorporation into liposomes the purified protein has been functionally identified as the dicarboxylate carrier [20] by its requirement for a counteranion as well as its substrate specificity and inhibitor sensitivity, which are both very similar to those described for the dicarboxylate transport system in mitochondria.

In this paper, the conditions for optimal reconstitution of the mitochondrial dicarboxylate carrier are described. The values of the transport rate and related kinetic parameters of the exchange between external malate or phosphate and intraliposomal malate or phosphate are reported.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) and Dowex AGl-X8 were purchased from Bio-Rad, Amberlite XAD-2 from Fluka, L-[U-^{14}C]malate, [^{32}P]phosphate and [2-^{14}C]malonate from Amersham International, U.K., and cardiolipin from Avanti-Polar Lipids. Egg-yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), L-3-dioleylphosphatidylcholine, DL-α-dipalmitoylphosphatidylcholine, L-α-phosphatidylinositol and L-lysophosphatidylcholine were purchased

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

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from Sigma, phosphatidylethanolamine from Serdary. Other reagents were obtained as reported [5]. All other chemicals were of the highest purity commercially available.

Isolation of the dicarboxylate carrier. The dicarboxylate carrier was purified from rat liver mitochondria by the Amberlite/hydroxyapatite procedure as described previously [20]. The purity of the 28 kDa band protein previously identified as the dicarboxylate carrier [20] was always higher than 95% as checked by SDS-polyacrylamide gel electrophoresis. Furthermore, the purified dicarboxylate transport protein was not contaminated by other mitochondrial anion-transporting systems including the phosphate carrier, the 2-oxoglutarate carrier and the tricarboxylate carrier.

Reconstitution of the dicarboxylate carrier. The purified dicarboxylate carrier was passed through a Dowex AGl-X8 column, 100-200 mesh, chloride form (0.5 × 8 cm equilibrated with a buffer containing 10 mM Pipes (pH 7.0) and 1 mg/ml egg-yolk phospholipids in the form of sonicated liposomes), in order to remove the interfering anions such as phosphate from the hydroxyapatite and sulphate from the solubilization buffer (see Ref. 20). Liposomes were prepared as described previously [21] by sonication of 100 mg/ml egg-yolk phospholipids in water for 60 min. Reconstitution of the dicarboxylate carrier into liposomes was performed by removing the detergent with Amberlite [22,23] from mixed micelles containing detergent, protein and phospholipids. The composition of the mixture used for reconstitution was: 200 μl of the purified dicarboxylate carrier (7–15 μg protein), 60 μl of 10% Triton X-114, 6.7 mg of phospholipids in the form of sonicated liposomes, 15 mM phosphate or malate, 10 mM Pipes (pH 7) in a final volume of 0.68 ml. After vortexing, this mixture was passed 13 times through the same Amberlite column (0.5 × 3.2 cm) preequilibrated with a buffer containing 10 mM Pipes and 15 mM of the substrate present in the starting mixture. All the operations were performed at 4°C except the passage through Amberlite, which was carried out at room temperature.

Transport measurements. The external substrate was removed by passing the proteoliposomes through a Sephadex G-75 column preequilibrated with 50 mM NaCl/10 mM Pipes (pH 7.0). The eluted proteoliposomes incubated at 25°C for 4 min were used for transport measurements by the inhibitor stop method [24]. Transport was started by adding the labeled substrate ([^{14}C]malate, [^{32}P]phosphate or [^{14}C]malonate) at the concentrations indicated in the legends to tables and figures, and stopped, after the desired time interval, by the addition of 20 mM butylmalonate, a known inhibitor of the dicarboxylate carrier. In control samples, the inhibitor was added together with the labeled substrate. In order to remove the external radioactivity, each sample was passed through a Dowex AGl-X8 column, 100–200 mesh, acetate form (0.5 × 4 cm equilibrated with 40 mM NaCl). The liposomes eluted with 0.9 ml of 40 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. The transport activity was calculated by subtracting the control values from the experimental samples. K_m and V values were determined by a computer-fitting program based on linear regression analysis.

Other methods. Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [25]. The stacking gel contained 5% polyacrylamide and the separation gel contained 17.5% acrylamide and an acrylamide-to-bisacrylamide ratio of 150 [26]. Staining was performed by the silver nitrate method [27]. Protein was determined by the Lowry method modified for the presence of Triton [28]. The internal volume of the proteoliposomes was determined as described in Ref. 22. The activity of other transport systems was assayed by the inhibitor stop method [24] using the following stop inhibitors: *N*-ethylmaleimide (phosphate carrier), 1,2,3-benzenetricarboxylate (citrate carrier), phthalonate (2-oxoglutarate carrier), carboxyatractyloside (ADP/ATP carrier), pyridoxal phosphate (aspartate/glutamate carrier) and α-cyanocinnamate (pyruvate carrier).

Results

Optimal conditions of reconstitution

In a previous paper [20], reconstitution was used for monitoring the purity of the protein during the isolation procedure and for identification of the purified dicarboxylate carrier. For a detailed characterization of the kinetic properties of this carrier protein, the conditions leading to optimal reconstitution should be thoroughly investigated.

The method of reconstitution used in these experiments is based on the removal of the detergent from mixed micelles by repeated passages through columns of Amberlite [22,23]. In order to investigate the efficiency of reconstitution of the purified dicarboxylate carrier, we have measured three parameters, (i) the activity of the malate/phosphate exchange, (ii) the total exchange, i.e., the amount of malate taken up after equilibration, and (iii) the intraliposomal volume, under different experimental conditions.

As shown in Fig. 1, these three parameters increase on lowering the detergent/phospholipid ratio from 2.5 to 1. The specific activity of the malate/phosphate exchange depended mainly on the amount of active carrier molecules incorporated into the liposomal membrane whereas the total exchange (after equilibration) depended on both the amount of carrier protein and the intraliposomal volume. The results of Fig. 1 indicate that both the amount of active carrier incorporated and

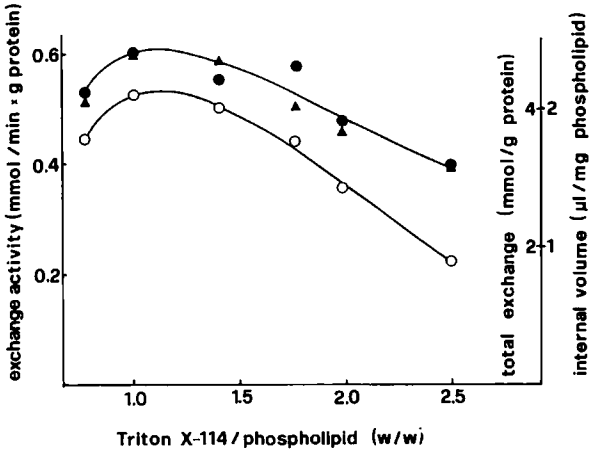

Fig. 1. Reconstitution of the dicarboxylate carrier: variation of the detergent/phospholipid ratio. The proteoliposomes were prepared as described in Materials and Methods except that increasing concentrations of Triton X-114 were used. 0.1 mM [^{14}C]malate was added to proteoliposomes which contained 15 mM phosphate. The exchange activity (○), the total exchange calculated from the exchange equilibrium after 20 min (●) and the internal volume (▲) were determined.

the number (or size) of liposomes increase by increasing the phospholipid concentration with respect to Triton X-114 until a ratio of about 1 is reached. A similar inactivation of the reconstituted ADP/ATP carrier and aspartate/glutamate carrier by Triton X-114 has been observed using the same method of reconstitution [22].

Fig. 2 shows that both the rate of malate/phosphate exchange and the intraliposomal volume increased with the number of passages through Amberlite, reaching a maximum after 13 passages. This behaviour is similar to that reported for the aspartate/glutamate carrier recon-

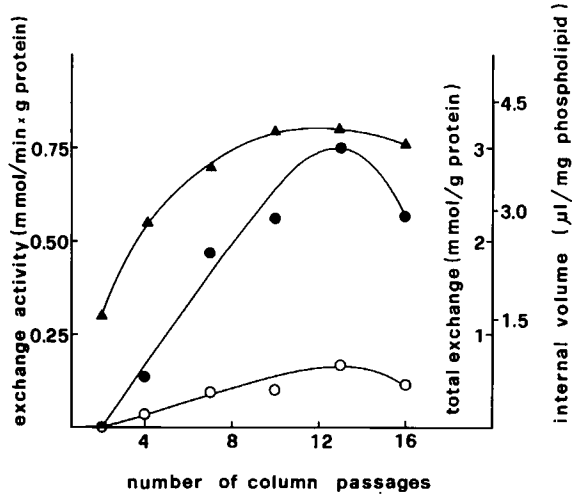

Fig. 2. Reconstitution of the dicarboxylate carrier: variation of the number of the Amberlite column passages. The proteoliposomes were prepared as described in Materials and Methods except that the number of passages through the same Amberlite column was varied as indicated. 0.1 mM [^{14}C]malate was added to proteoliposomes which contained 15 mM phosphate. The exchange activity (○), the total exchange calculated from the exchange equilibrium after 20 min (●) and the internal volume (▲) were determined.

TABLE I

Dependence of the rate of the reconstituted malate/phosphate exchange on the phospholipid composition of liposomes

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 (L-α-phosphatidylcholine from turkey eggs, Sigma); DOPC, dioleyl phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, cardiolipin. 1 mM [^{14}C]malate was added to proteoliposomes loaded with 15 mM phosphate.

Phospholipid composition	Specific activity (µmol/min per g protein)	Total exchange (µmol/30 min per g protein)
EYPL	2980	8870
+ DOPC	2860	8550
+ DPPC	2030	5620
+ PE	2730	9910
+ PS	2950	8060
+ PI	2300	9960
+ DPG	2490	8780

stituted with dodecyl octaoxyethylene ether [22], although in our case a lower number of passages through Amberlite is required for optimal activity. In the light of the results shown in Figs. 1 and 2, in all subsequent experiments a Triton X-114/phospholipid ratio of 1 and 13 passages through a single Amberlite column have been used. In further experiments which were performed for optimizing the reconstituted system we found that the optimum conditions with respect to the absolute concentration of phospholipids were 10 mg/ml, and with respect to the ratio Amberlite/detergent were 50 (w/w).

Lipids have been shown to modulate definitely the activity of reconstituted mitochondrial anion carriers [29–32]. Table I compares the activity of the reconstituted dicarboxylate carrier in liposomes with various lipid compositions. The highest rate of the malate/phosphate exchange was found using liposomes prepared from egg-yolk phospholipids. Addition of dioleylphosphatidylcholine, phosphatidylethanolamine or phosphatidylserine had very little or no effect on the transport activity. The rate of exchange was inhibited by cardiolipin and phosphatidylinositol and, to a greater extent, by dipalmitoylphosphatidylcholine. The fact that the total exchange was not decreased by cardiolipin and phosphatidylinositol indicates that these phospholipids inhibit the carrier activity without affecting the incorporation of the carrier molecules into the liposomes. Vice versa, the decrease in the total exchange by dipalmitoylphosphatidylcholine suggests a negative influence of this phospholipid on the incorporation of the dicarboxylate carrier into the liposomes. It should be noted that the intraliposomal volume did not varied significantly by changing the lipid composition (not

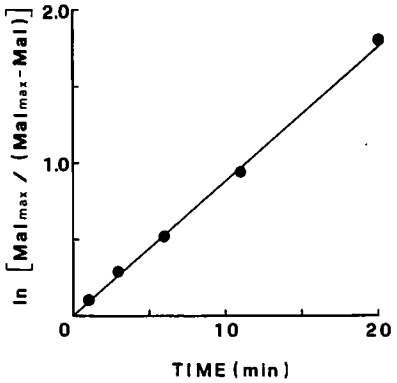

Fig. 3. Logarithmic plot of reconstituted malate/phosphate exchange, demonstrating first-order kinetics: ln $malate_{max}/(malate_{max} - malate_t) = kt$. 0.1 mM [^{14}C]malate was added to proteoliposomes which contained 15 mM phosphate. $Malate_{max}$ means the amount of malate taken up after the equilibrium was reached; when measured after 30 min it was 3.2 mmol/g protein.

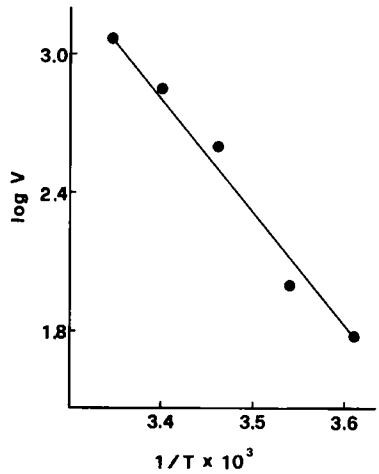

Fig. 4. Arrhenius plot of the temperature dependence of the reconstituted malate/phosphate exchange activity. 0.1 mM [^{14}C]malate was added to proteoliposomes which contained 15 mM phosphate and were incubated at the indicated temperatures. The exchange activity, V, is expressed in mmol/min per g protein.

shown). The inhibition of the dicarboxylate carrier activity by cardiolipin and phosphatidylinositol resembles that observed with the reconstituted 2-oxoglutarate carrier [33] and contrasts with the activation of the phosphate carrier and the tricarboxylate carrier by cardiolipin [31,32] and the activation of the ADP/ATP carrier by acidic phospholipids [29,30].

Reaction order and temperature dependence of [^{14}C]-malate/phosphate exchange

The reaction order of the malate/phosphate exchange was investigated by plotting the natural logarithm of the fraction of equilibrium $malate_{max}/(malate_{max} - malate_t)$ against time [34]. As shown in Fig. 3, a straight line was obtained, demonstrating that the reconstituted malate/phosphate exchange follows first-order kinetics similar to the results found for succinate and sulphate uptake in intact mitochondria [14,35]. The first-order rate constant, k, was determined to be 0.09 min^{-1}.

Fig. 4 shows the temperature dependence of the rate of malate/phosphate exchange. In an Arrhenius plot a straight line was obtained in the range from 4°C to 26°C. The activation energy as derived from the slope was 95.8 kJ/mol.

K_m and V values of malate and phosphate transport

In order to obtain the basic kinetic data of the dicarboxylate carrier the dependence of the exchange rate on substrate concentration was studied by changing the concentration of externally-added [^{14}C]malate or [^{32}P]phosphate at constant internal concentration of 15 mM malate or 15 mM phosphate, respectively. The data from typical experiments are shown in Fig. 5 as double-reciprocal plots. In both malate-loaded (Fig. 5A) and phosphate-loaded (Fig. 5B) liposomes, straight lines were obtained intersecting at the ordinate. The different

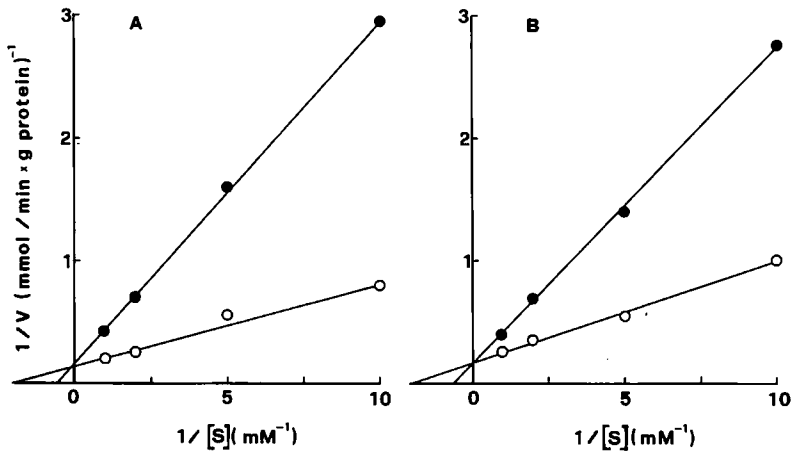

Fig. 5. The dependence of the rate of malate and phosphate uptake in proteoliposomes on substrate concentration. [^{14}C]Malate (○) or [^{32}P]phosphate (●) was added at the concentrations indicated to proteoliposomes loaded with 15 mM malate (A) or with 15 mM phosphate (B). The exchange activity, V, is expressed in mmol/min per g protein.

TABLE II

K_m and V values for the uptake of malate, malonate and phosphate in phosphate-loaded proteoliposomes

Experimental conditions as in Fig. 5B except that [^{14}C]malonate was also used as external substrate. The values given in the table are the means ± S.E. of 5–23 experiments.

Substrate	K_m (mM)	V (mmol/min per g protein)	No. expts.
Malate	0.49 ± 0.05	6.0 ± 1.6	23
Malonate	0.54 ± 0.10	5.9 ± 0.9	5
Phosphate	1.41 ± 0.35	6.0 ± 1.4	15

slopes indicate that the relative half-saturation constants were different. In these experiments, both in malate-loaded and phosphate-loaded liposomes the K_m for phosphate (1.8 mM) was more than 3-fold higher than that for malate (0.5 mM). The V value was approx. 6700 μmol/min per g protein for the malate/malate and phosphate/malate exchanges and approx. 6300 μmol/min per g protein for the malate/phosphate and phosphate/phosphate exchanges. Thus, V of the dicarboxylate carrier was independent of the types of substrate present on the two sides of the membrane. Table II reports mean values and standard errors of K_m and V for the uptake of malate, malonate and phosphate in phosphate-loaded liposomes. It should be noted that the standard error of the V values was rather high when comparing different experiments, presumably due to variations in the amount of active carrier molecules present in each preparation of the purified carrier. Nevertheless, the V values for the three substrates as compared in one experiment were not significantly different.

Influence of pH

The influence of the pH on the rate of malate/phosphate exchange is illustrated in Fig. 6. In the presence of 1 mM external malate, the rate of the exchange increased markedly on increasing the pH from 5.5 to 7 (Fig. 6A). Double-reciprocal plots of the exchange rate versus malate concentration show, however, that raising the pH from 6.2 to 7.0 decreased the K_m of malate from 1.0 mM to 0.6 mM without affecting the V (Fig. 6B). This result demonstrates that the activity of the dicarboxylate carrier is independent of the pH at high substrate concentrations, as found previously in intact mitochondria [36].

Discussion

In a previous paper [20] we have characterized the reconstituted dicarboxylate carrier with respect to its substrate specificity and inhibitor sensitivity. The experiments reported here on optimization of the reconstitution of the dicarboxylate carrier were performed in order to obtain a reliable basis for the determination of the kinetic data of this carrier protein. Among the parameters which may influence the effectivity of reconstitution, particularly the ratio of detergent and phospholipids on the one hand and the number of passages through the Amberlite column on the other hand were found to be important for obtaining high transport activities. It should be mentioned that the method of reconstitution based on detergent removal by chromatography on Amberlite XAD 2 [22,23] applied here not only resulted in several-fold higher transport activities than those observed with the freeze-thaw-sonication procedure (data not shown) but also led to the formation of larger proteoliposomes, which are more suitable for kinetic studies.

Under optimal conditions, a V value of 6 mmol/min per g protein was measured at 25 °C. This value was found to be very similar for three different externally added substrates (Table II) and did not depend on the nature of the internal substrate (Fig. 5A and B). Thus, as found in intact mitochondria [5], the translocation step catalyzed by the dicarboxylate carrier seems not to

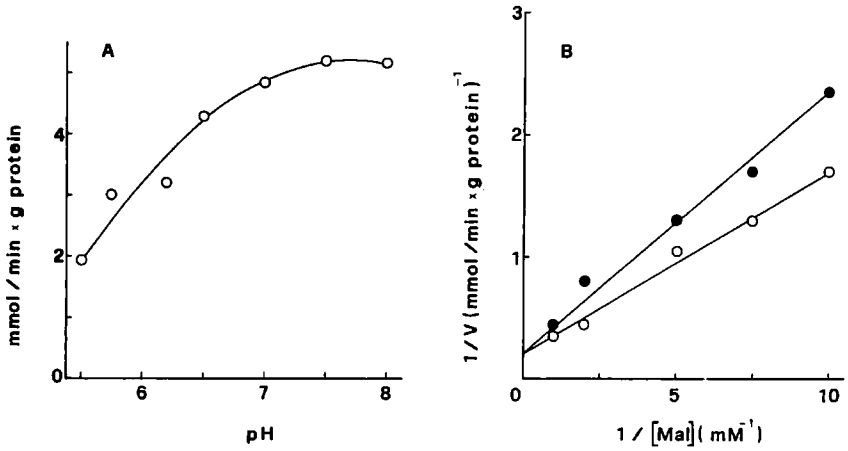

Fig. 6. Effect of pH on the reconstituted malate/phosphate exchange. (A) 1 mM [^{14}C]malate was added to proteoliposomes which contained 15 mM phosphate and were incubated at the indicated pH values. (B) Experimental conditions as in Fig. 5B at pH 6.2 (●) and 7.0 (○). The exchange activity, V, is expressed in mmol/min per g protein.

be influenced very much by the kind of substrate transported. The turnover number, which was calculated assuming a pure isolated protein monomer of 28 kDa, corresponds to 340 min^{-1}. This turnover number is lower than that reported for the reconstituted phosphate carrier [37–39] and is in the same range as found for the reconstituted ADP/ATP carrier [40], the reconstituted 2-oxoglutarate carrier [33] and the reconstituted aspartate/glutamate carrier (unpublished result). The half-saturation constant of phosphate for the reconstituted dicarboxylate carrier is relatively low as compared to malate and malonate. It is important to note that identical K_m values were found for phosphate and malate in both malate-loaded (Fig. 5A) and phosphate-loaded (Fig. 5B) proteoliposomes. This indicates that the half-saturation constant of the carrier for a particular substrate does not depend on the counteranion.

The activity of the reconstituted dicarboxylate carrier is influenced by the lipid composition of the liposomes (Table I). In particular, cardiolipin and phosphatidylinositol were found to inhibit the carrier activity without affecting the incorporation of the carrier molecules into the liposomes. This result is similar to what reported for the 2-oxoglutarate carrier [33] and is in contrast to the activation of other reconstituted mitochondrial carriers by cardiolipin or acidic phospholipids [29–32]. As observed in mitochondria for the exchange between external succinate or sulphate and internal phosphate [14,35], the reconstituted dicarboxylate carrier catalyzes a first-order reaction. The reconstituted exchange activity showed a high temperature dependence with an activation energy of 95.8 kJ/mol. This is very close to the value obtained for the reconstituted ADP/ATP carrier [41], and somewhat higher than the values obtained for the reconstituted phosphate carrier [37] and 2-oxoglutarate carrier [33], respectively. The reconstituted dicarboxylate carrier was practically independent of the applied external pH in the range between 6 and 8, as observed also for the uptake of dicarboxylates in intact mitochondria [36].

Acknowledgements

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