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Citrate Uniport by the Mitochondrial Tricarboxylate Carrier: A Basis for a New Hypothesis for the Transport Mechanism

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The tricarboxylate transport system located in the inner mitochondrial membrane was studied as an isolated protein reconstituted in proteoliposomes. The effects on the transport of citrate by various reagents, specific for different aminoacid residues, were analyzed. In the group of SH reagents, it was found that *N*-ethylmaleimide is an irreversible inhibitor of the citrate–citrate exchange, while $HgCl_2$ and the mercurial mersalyl cause a rapid unidirectional efflux of citrate from liposomes. It was demonstrated that NEM and mercurials act on different SH groups. Dithioerythritol is not able to reverse the effect of mersalyl unless another reagent, pyridoxalphosphate, is present. Pyridoxalphosphate itself, a reagent specific for NH_2 residues, is an effective inhibitor of citrate exchange transport, as measured in both influx and efflux, but it has no effect on the mercurial-induced efflux. The same behavior was observed with diethylpyrocarbonate, a reagent specific for histidine and tyrosine residues. Interestingly, a slow basic efflux of internal citrate, in the absence of countersubstrate, was observed in proteoliposomes. Because it is inhibited by the same reagents acting on the exchange process, it is deduced that it is catalyzed by the tricarboxylate carrier. The ability of the carrier to perform a uniport of the substrate suggests the presence of a single substrate binding site on the carrier protein. A preliminary kinetic approach indicates that such a transport model is compatible with this theory.

KEY WORDS: Mitochondria; proteoliposomes; tricarboxylate carrier; citrate transport; inhibitors.

INTRODUCTION

In the internal membrane of rat liver mitochondria there is a protein which catalyzes the efflux of citrate, together with a proton, from the matrix in an electroneutral exchange for another tricarboxylate-H^+, a dicarboxylate, or phosphoenolpyruvate (Bisaccia *et al.*, 1993; Palmieri *et al.*, 1972). This transport protein plays an important role in fatty acid synthesis, gluconeogenesis, and the transfer of reducing equivalents across the membrane. The tricarboxylate carrier has been isolated and reconstituted into liposomes in a functionally active state (Bisaccia *et al.*, 1989, 1990; Kaplan *et al.*, 1990). The amino acid sequence has been determined by cDNA sequencing (Kaplan *et al.*, 1993) and is characterized by a tripartite structure typical of the carrier protein family of the mitochondrial inner membrane (Palmieri, 1994; Walker and Runswick, 1993). As in the case of the majority of the mitochondrial metabolite carriers (for review, see Kaplan, 2001), the citrate carrier has been proposed to function according to a sequential reaction mechanism (Bisaccia *et al.*, 1993), which implies that one internal and one external substrate molecule form a ternary complex with the carrier protein. The carrier is inhibited by several covalent labeling agents: pyridoxal 5-phosphate (PLP), for lysine as well as for amino terminals (Gremse *et al.*, 1995), diethyl pyrocarbonate selective for histidine (Kaplan *et al.*, 1990), and sulfhydryl reagents mersalyl, *p*-hydroxymercuribenzoate (*p*-HMB) and *N*-ethylmaleimide (NEM) (Bisaccia *et al.*, 1989; Kaplan *et al.*, 1990). Among the latter reagents, it has also been observed that mercurials strongly inhibit citrate transport activity in intact mitochondria, whereas NEM causes only a slight inhibition.

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The purpose of this study was to contribute to explain this different reactivity of sulfhydryl reagents by using reconstituted proteoliposomes, a system which allows the experimental conditions on both sides of the membrane to be easily controlled in the absence of possible interference from other membrane proteins. We also observed that the effect of mercurials on the citrate carrier is to convert the antiport (citrate/citrate exchange) to a uniport (citrate efflux from proteoliposomes), in a similar way to that reported for other mitochondrial transport systems, such as the aspartate/glutamate carrier (Dierks *et al.*, 1990a,b), the ADP/ATP carrier (Dierks *et al.*, 1990a), the carnitine carrier (Indiveri *et al.*, 1992), and the phosphate carrier (Stappen and Krämer, 1993). This observation led us to better analyze the behavior of the carrier protein in catalyzing the efflux of citrate from proteoliposomes loaded with labelled citrate, in different conditions.

MATERIALS AND METHODS

Materials

Hydroxyapatite (Bio-Gel HTP) and Bio-Beads SM-2 were purchased from Bio Rad, Celite 535 was from Roth, Sephadex G-75 from Pharmacia, [1,5-^{14}C]citrate from Amersham International (Amersham, UK), egg-yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), 1,4-piperazinediethanesulphonic acid (Pipes), Triton X-114 and Triton X-100 were from Sigma. All other reagents were of analytical grade.

Purification and Reconstitution of the Tricarboxylate Carrier

The tricarboxylate carrier was purified from rat liver mitochondria as previously described (Bisaccia *et al.*, 1989). The purified protein was reconstituted into liposomes by cyclical removal of the detergent with a hydrophobic column (Palmieri *et al.*, 1995). The composition of the initial mixture used for reconstitution was 200 μL of purified protein in 0.5% Triton X-100 (about 0.1 μg protein), 90 μL of 10% Triton X-114, 100 μL of 10% egg-yolk phospholipids in the form of sonicated liposomes prepared as described earlier (Dulley *et al.*, 1975), citrate at the concentrations indicated in the legends to figures, and 20 mM Pipes pH 7.0 in a volume of 700 μL. After vortexing, this mixture was passed 24 times through the same Bio-Beads SM-2 column (0.5 $\times$ 3.6 cm) pre-equilibrated with the same buffer and the substrate at a concentration identical to the starting mixture. All the operations were performed at 4°C, except the passages through Bio-Beads SM-2 column that were performed at room temperature.

Transport Measurements

The external substrate was removed from reconstituted proteoliposomes by chromatography at 4°C on a Sephadex G-75 column (0.7 $\times$ 15 cm) pre-equilibrated with 50 mM NaCl, 10 mM Pipes at pH 7.0. The transport activity was determined by measuring the flux of labelled citrate from outside to inside (uptake experiments) or from inside to outside (efflux experiments). When measuring efflux, the proteoliposomes containing 10 mM internal citrate were prelabelled by carrier-mediated exchange equilibration. This was achieved by incubating the proteoliposomes with 0.1 mM [^{14}C]citrate at high specific radioactivity for 30 min at 25°C. After this incubation time, the external radioactivity was removed by passing the liposomes through a Sephadex G-75 column as described above. Transport was started, in the case of the efflux experiments, by adding either 1 mM unlabelled citrate in buffer 50 mM NaCl, 10 mM Pipes (pH 7.0) (backward exchange) or buffer alone (uniport) or SH-reagent to the prelabelled proteoliposomes, as indicated in the context of each experiment. The efflux was stopped at the desired time by fast transfer and elution on the Sephadex G-75 column. In the case of uptake experiments (forward exchange), transport was started by adding the labelled substrate to proteoliposomes and stopped by adding 10 mM 1,2,3-benzenetricarboxylate. Proteoliposomes were then transferred on the Sephadex G-75 column and eluted. In control samples, the inhibitor was added at time zero according to the inhibitor stop method (Palmieri *et al.*, 1995).

Each sample of proteoliposomes (100 μL) was eluted by a Sephadex G-75 column (0.6 $\times$ 8 cm) in order to separate the external from the internal radioactivity with 1.3 mL of 50 mM NaCl and collected in 4 mL of scintillation mixture, vortexed and counted (Palmieri *et al.*, 1995). The assay temperature was 25°C. The activity was expressed as mmol citrate/g protein (Fig. 8) or as intraliposomal cpm (Figs. 1–7).

Other Methods

Protein was determined by the Lowry method modified for the presence of nonionic detergents (Bisaccia *et al.*, 1985).

RESULTS

In previous investigations, it was found that the uptake of [^{14}C]citrate by liposomes reconstituted with the purified citrate carrier was inhibited by the addition

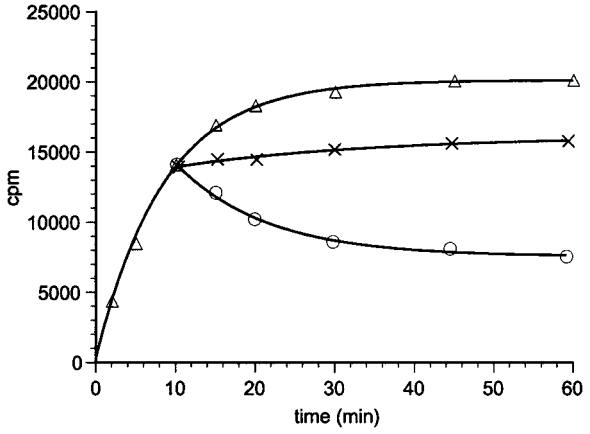

Fig. 1. *Effect of NEM and mersalyl on the time-course of citrate uptake in reconstituted liposomes.* 0.1 mM [^{14}C]citrate was added at time zero to reconstituted liposomes with 10 mM citrate as internal substrate (△). After 10 min, 2 mM NEM (×) or 10 μM mersalyl (○), respectively, were added to two aliquots of vesicles.

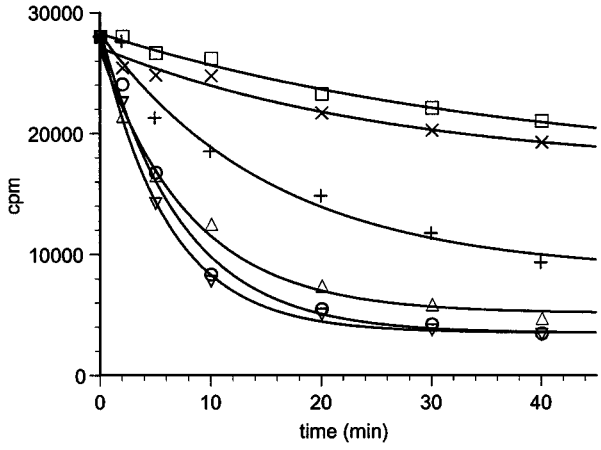

Fig. 2. *Time-course of [^{14}C]citrate efflux from proteoliposomes.* The internal substrate pool of proteoliposomes (10 mM) was prelabelled by carrier-mediated equilibration with external [^{14}C]citrate. After removal of external substrate, the decrease in internal radioactivity was monitored following different additions: buffer (basic efflux □), 2 mM NEM (×), 1 μM $HgCl_2$ (+), 0.3 mM mersalyl (○), 1 mM citrate (△), and 1 mM citrate plus 0.3 mM mersalyl (▽).

of various cysteine-modifying reagents (Bisaccia *et al.*, 1989; Kaplan *et al.*, 1990). In particular, the tricarboxylate carrier was strongly inhibited by mersalyl, *p*-HMB and other mercurials but only partially by NEM. To investigate the different reactivity of the carrier protein to SH-reagents, it was performed a time-course of 0.1 mM [^{14}C]citrate uptake in reconstituted proteoliposomes as exchange in the presence of internal 10 mM citrate. After 10 min of citrate exchange, an aliquot of proteoliposomes was incubated with 10 μM mersalyl and another one with 2 mM NEM. It can be observed (Fig. 1) that the addition of NEM stops the uptake of citrate at the level reached at the time of the addition, while mersalyl promotes a rapid efflux of labelled substrate. Such an effect could be due to the binding of mersalyl to a sulfhydryl group which would lead to a modification of the protein structure, resulting in the activation of a unidirectional transport down the concentration gradient of the substrate.

To verify this hypothesis, efflux experiments were performed using proteoliposomes previously loaded with 10 mM [^{14}C]citrate (Fig. 2). Loaded proteoliposomes were incubated in media containing the buffer alone (control), or added with, respectively, NEM, mersalyl, $HgCl_2$, citrate, or citrate plus mersalyl. It can be observed that externally added citrate causes a rapid efflux of [^{14}C]citrate, by exchange transport (as expected). Mercurial reagents (mersalyl, $HgCl_2$) also cause a similarly rapid efflux, that must necessarily be a uniport. It can also be observed that the efflux induced by the presence of both mersalyl and citrate is quite similar to that induced by mersalyl. As regards NEM, its presence does not cause any efflux.

In addition, this experiment shows that a spontaneous efflux of labelled citrate is present (control), which is slower than that induced by external citrate or mersalyl, but is measurable and reproducible. These different effects on the carrier activity by the two kinds of reagents, taking into account their different reactivity with SH groups, could be related either to a different modification of a single SH group, located in a strategic site of the carrier protein, or to the presence of two different SH groups, each reacting with one of the reagents. The experiment shown in Fig. 3 was performed in order to discriminate between these possibilities. The efflux was followed in proteoliposomes incubated in buffer (control) or in the presence of mersalyl or in the presence of NEM. While mersalyl, as already seen, induces a rapid efflux of [^{14}C]citrate from proteoliposomes, the time course observed in the presence of NEM is not distinguishable from the control (basic efflux). The addition of mersalyl to an aliquot of NEM treated proteoliposomes after 10 min of incubation causes a rapid efflux of the substrate, similar to the mersalyl-induced efflux. This finding clearly indicates that the efflux induced by mersalyl is independent of the presence of NEM, hence that the mercurial most probably interacts with an SH group different from the one which binds NEM.

Further experiments were performed in order to verify the effect of specific reagents of other aminoacid residues in the efflux process. In this kind of experiment, proteoliposomes preloaded with labelled citrate were

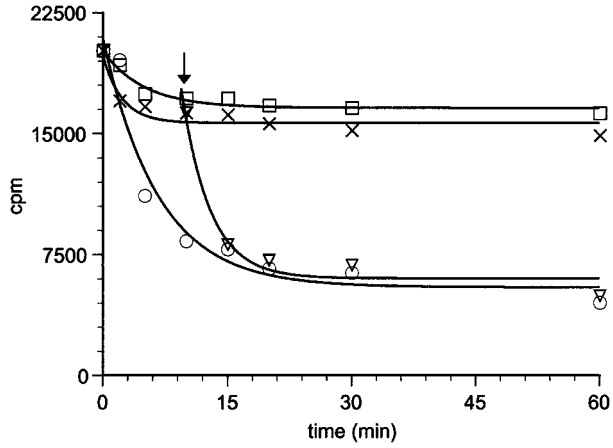

Fig. 3. *Induction of citrate efflux by mersalyl from proteoliposomes in the presence of NEM.* The efflux of 10 mM [^{14}C]citrate from prelabelled proteoliposomes was measured in the presence of buffer alone (50 mM NaCl/10 mM Pipes pH 7.0) (basic efflux □), in the presence of 1 mM NEM (×) or in the presence of 0.3 mM mersalyl (○). As indicated by the arrow, after 10 min from the start of incubation with NEM, 0.3 mM mersalyl was added to proteoliposomes (▽).

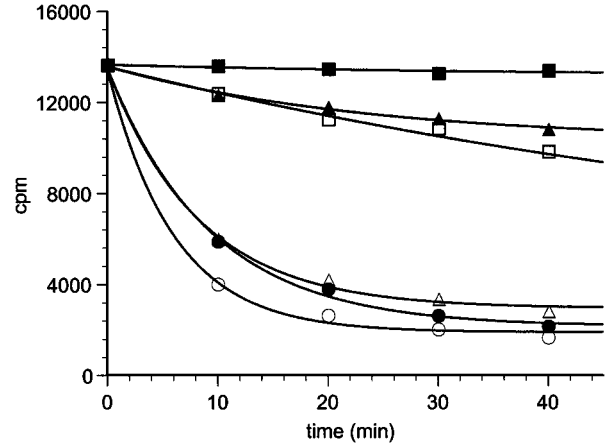

Fig. 5. *Effect of pyridoxal 5-phosphate on the efflux of [^{14}C]citrate from prelabelled proteoliposomes.* The efflux of 10 mM [^{14}C]citrate from prelabelled proteoliposomes was monitored in the presence of buffer alone (50 mM NaCl/10 mM Pipes pH 7.0) (basic efflux □), in the presence of 1 mM unlabelled citrate (exchange △) or in the presence of 0.3 mM mersalyl (○). To aliquots of the same proteoliposomes, 38 mM pyridoxal 5-phosphate was added 1 min before the start of the reaction (■, ▲, ●).

preincubated for 1 min with the inhibitor or with buffer (control) before starting the efflux measurements. Figure 4 shows the effect of diethylpyrocarbonate, which specifically reacts with histidine and tyrosine residues. The fact that diethylpyrocarbonate, in the presence of externally added citrate, completely abolishes the [^{14}C]citrate efflux is a clear indication that this reagent is able to inhibit citrate exchange. It is interesting to observe that the spontaneous efflux (uniport) is almost completely abolished. On the other hand the efflux induced by mersalyl is not influenced by diethylpyrocarbonate.

A similar effect was exerted by PLP, a known protein reagent specific for amino-groups (lysine, amino-terminal), as shown in Fig. 5. In fact, PLP is able to strongly inhibit the exchange efflux and to almost totally block the spontaneous efflux, while the mersalyl-induced efflux was not affected by the presence of PLP.

It is therefore clear that the two reagents tested strongly interfere with the citrate transport mechanism in both forms, exchange and spontaneous efflux, while they have no effect on the mersalyl-induced efflux.

The effects of mercurial reagents, due to the formation of organometallic bonds with protein SH groups, are generally sensitive to dithioerythritol (DTE), which displaces the mercurial reagent leaving the SH groups free. In this case, however, we observed (Fig. 6) that the reagent is ineffective both on spontaneous and citrate-induced effluxes (as expected), and on the mersalyl-induced one. In fact, the addition of DTE (after 5 min) to proteoliposomes treated with mersalyl does not induce any appreciable change in the efflux process, while when the addition is simultaneous with mersalyl the efflux is abolished, obviously due to the rapid reaction of mersalyl molecules with DTE, whose concentration is in large excess over mersalyl.

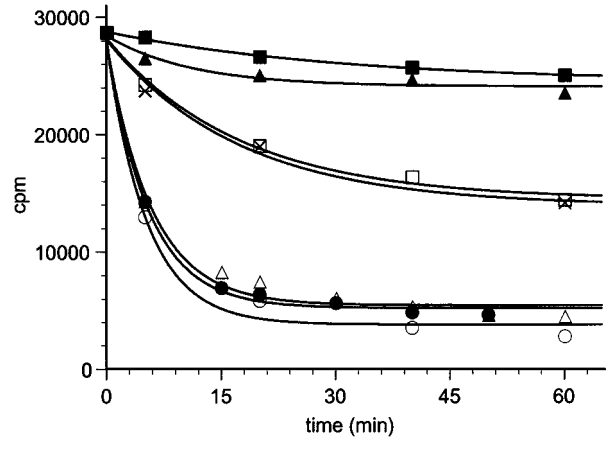

Fig. 4. *Effect of diethylpyrocarbonate on the efflux of [^{14}C]citrate from prelabelled proteoliposomes.* The efflux of 10 mM [^{14}C]citrate from prelabelled proteoliposomes was monitored in the presence of buffer alone (50 mM NaCl/10 mM Pipes pH 7.0) (basic efflux □) and buffer plus DMSO (×), in the presence of 1 mM unlabelled citrate (exchange △) or in the presence of 0.3 mM mersalyl (○). To aliquots of the same proteoliposomes, 10 mM diethylpyrocarbonate was added 1 min before the start of the reaction (■, ▲, ●).

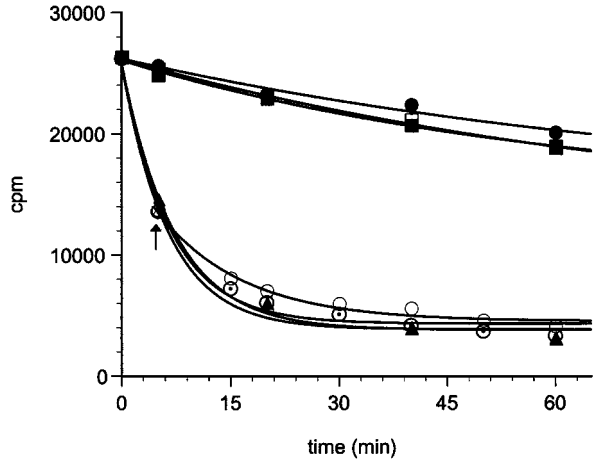

Fig. 6. *Effect of 1,4-dithioerythritol on the efflux of [^{14}C]citrate from prelabelled proteoliposomes.* The efflux of 10 mM [^{14}C]citrate from prelabelled proteoliposomes was monitored in the presence of buffer alone (50 mM NaCl/10 mM Pipes pH 7.0) (basic efflux □); in the presence of 1 mM unlabelled citrate (exchange △) or in the presence of 0.3 mM mersalyl (○). To aliquots of the same proteoliposomes, 10 mM 1,4-dithioerythritol was added 1 min before the start of the reaction (■, ▲, ●). Where indicated by the arrow, 10 mM 1,4-dithioerythritol was added to a parallel sample with mersalyl (⊙).

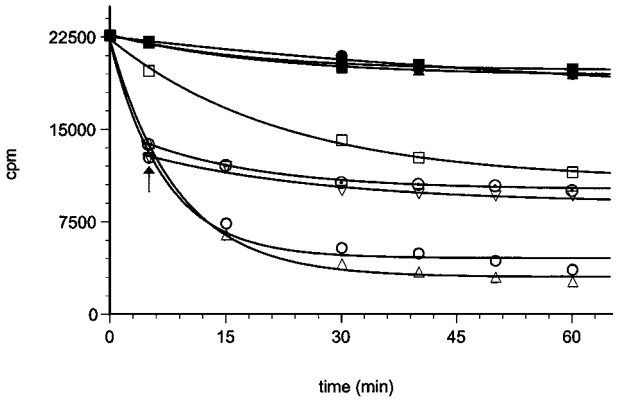

Fig. 7. *Effect of pyridoxal 5-phosphate plus 1,4-dithioerythritol on the efflux of [^{14}C]citrate from prelabelled proteoliposomes.* The efflux of 10 mM [^{14}C]citrate from prelabelled proteoliposomes was monitored in the presence of buffer alone (50 mM NaCl/10 mM Pipes pH 7.0) (basic efflux □), in the presence of 1 mM unlabelled citrate (exchange △) or in the presence of 0.3 mM mersalyl (○). To aliquots of the same proteoliposomes a mixture of 38 mM pyridoxal 5-phosphate and 10 mM 1,4-dithioerythritol was added 1 min before the start of the reaction (■, ▲, ●). Where indicated by the arrow, the same mixture was added to parallel samples with mersalyl (uniport ⊙) or with citrate (exchange ▽).

On the other hand, a different result was observed when we tested the combined effect of PLP and DTE on the efflux processes. In this case, spontaneous, citrate-induced and mersalyl-induced effluxes were all inhibited, as shown in Fig. 7. On the basis of the results in Figs. 5 and 6, the block of uniport and exchange effluxes can only be attributed to PLP. As regards the effect on mersalyl-induced efflux, it is clear that the simulataneous presence of mersalyl and DTE added at the start of the incubation nullifies the effect of mersalyl (as already seen in Fig. 6). On the other hand, when DTE and PLP were added 5 min after the start of mersalyl-induced efflux, we observed that the efflux is blocked at the level reached at that time, in contrast with the effect obtained when DTE alone is added (see Fig. 6).

Throughout the experiments reported above, we noticed that a spontaneous efflux occurs in the absence of external substrate. It is much slower than the exchange efflux, but is measurable and reproducible, and specifically involves the citrate carrier, since it is inhibited by substances that are able to inhibit the exchange, as shown in Fig. 4 for diethylpyrocarbonate and in Fig. 5 for PLP. The possible implications of such an observation in the description of the carrier's transport mechanism led us to perform a kinetic test. In this kind of experiment, the rate of citrate uptake was measured in the "double substrate" mode, i.e., by using varying concentrations of both external and internal citrate. The result is described as a double reciprocal plot in Fig. 8, where it can be seen that straight lines, each obtained by using different external citrate concentrations at a fixed internal citrate, tend to meet at a point on the negative side of the abscissa. The possible meaning of this pattern will be an object of the Discussion section.

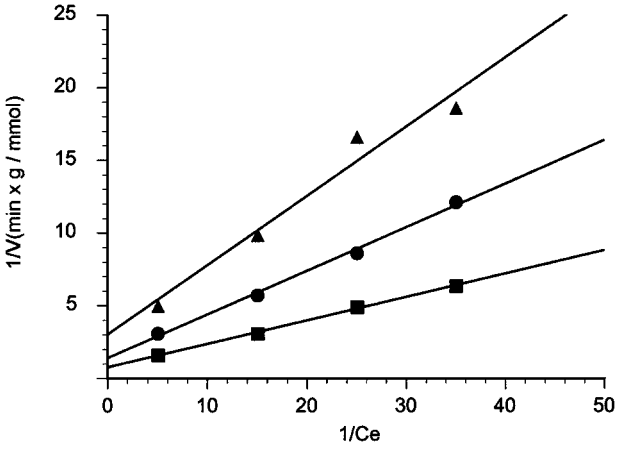

Fig. 8. *Two-substrate analysis, by the forward-exchange method, of the citrate/citrate exchange reaction catalyzed by the reconstituted citrate carrier.* Double reciprocal plot showing the dependence of exchange rate on external citrate concentrations. The rate is measured as [^{14}C]citrate uptake in 2 min. The concentrations of the internal citrate were 2.5 mM (▲), 5 mM (●), and 10 mM (■).

DISCUSSION

The data herein reported provide strong evidence for two features of the mitochondrial tricarboxylate carrier protein. The first one is that two distinct SH groups of the carrier protein seem to be involved in the transport process, the second is the ability of the carrier, not only to catalyze the exchange transport, but also to promote a basic efflux of the internal substrate, which necessarily is a uniport.

As regards the involvement of two SH groups, the experimental data clearly indicate that the sulfhydryl group reacting with NEM is certainly involved in the transport mechanism, since the inhibitor is able to block both the influx and the efflux of citrate in the exchange process. This observation comes out from the experiments shown in Fig. 1 (forward exchange) and Fig. 3 (backward exchange). Mercurials behave differently, since they promote a rapid efflux from proteoliposomes both when added during the uptake (Fig. 1) and in efflux experiments where it is present at time zero (Fig. 2). Furthermore, we have observed that mersalyl is able to induce efflux even when added to proteoliposomes treated with NEM (Fig. 3). This behavior can only be explained by the hypothesis that mercurials interact with an SH group different from the one irreversibly modified by NEM, and that the latter SH, even when bound by NEM, does not influence the interaction of the former with mercurials.

The interaction of mercurials with the carrier seems therefore to convert the carrier molecule in a system operating a rapid uniport, in analogy with what has been reported in the literature for other carrier systems (Dierks *et al.*, 1990a,b; Indiveri *et al.*, 1992; Stappen and Krämer, 1993). The rapid uniport thus induced is easily observed in experiments where efflux of labelled substrate takes place, but in our experimental conditions influx experiments cannot be monitored, due to the very small active internal volume of proteoliposomes. In the light of the present finding, the inhibitory effect exerted by mersalyl and other mercurials on the forward exchange process, reported in the literature for a number of isolated carrier proteins (Krämer and Palmieri, 1992), could be explained, at least in some cases, by a rapid depletion of the internal substrate, switched by such "inhibitors."

Another peculiarity of the transport system, compared with what is generally reported in the literature, is the lack of reversibility of the mersalyl-induced efflux by DTE (Fig. 6). Actually, we also find that DTE becomes able to block the mersalyl-induced efflux (Fig. 7), provided that PLP is present as well. This data can be tentatively explained by supposing that the binding of mersalyl to the specific SH group causes a conformational change in the carrier protein such that the organometallic bond is inaccessible to DTE. On the other hand PLP, by binding to one or more amino-groups, would produce a different protein conformation, allowing DTE to interact with the organometallic bond, and thus reversing the mersalyl effect.

The second feature of the citrate transport system, evidenced in the present paper, is the ability of the carrier protein itself to catalyze a basic efflux. The presence of this unidirectional flux is in contrast with the transport mechanism previously stated for the tricarboxylate carrier, described as a sequential random antiport (Bisaccia *et al.*, 1993), since the latter requires the formation of a ternary complex of the carrier with both the external and the internal substrates, leading to an obligatory exchange with a 1:1 stoichiometry. This contrast makes it necessary to formulate an alternative hypothesis for the transport mechanism, which takes into account the coexistence of the exchange process and the unidirectional flux. The simplest explanation is that the carrier protein has a single binding site for the substrate and, both in the bound form and in the free one, can rearrange itself between two conformations, one presenting the binding site on the outer face and the other exposing the site on the inner face of the membrane. The steps involved in the transport process are the following:

$$S_e + C_e \leftrightarrows CS_e \qquad K_S = \frac{[C_e] \cdot [S_e]}{[CS_e]}$$

$$CS_e \leftrightarrows CS_i \qquad k_2$$

$$CS_i \leftrightarrows C_i + S_i \qquad K_S = \frac{[C_i] \cdot [S_i]}{[CS_i]}$$

$$C_i \leftrightarrows C_e \qquad k_3$$

where S stands for substrate (citrate in our case), C for the free carrier molecule, CS for the carrier–substrate complex, subscripts "i" and "e" for the phases inside and outside the proteoliposomes, K_S is the dissociation constant, which is assumed to be the same in both phases, k_2 and k_3 are the kinetic constants of the rearrangement steps from "outside" to "inside" (and vice versa) of the carrier–substrate complex and, respectively, of the free carrier molecule.

To test this hypothesis, a kinetic approach is useful in order to correlate the initial rate of substrate transport with the substrate concentrations. Taking into account that a stationary state is experimentally verified (the initial rate of citrate transport is constant for some minutes), velocity equations for uptake and efflux can be obtained by adapting the Haldane procedures of enzyme kinetics (Segel, 1975) to the membrane transport process described in the scheme. The reasonable assumption is made of a "rapid

equilibrium" kinetics. This means that the protein rearrangement steps are the rate determining ones for the total transport process, since they are considered to be much slower than the substrate–carrier interactions, that practically reach equilibrium. On the basis of this assumption, the equilibrium constant of the latter, K_S, and the kinetic constants of the former, k_2 and k_3, will be the parameters present in the velocity equations of the transport process, in both influx and efflux. On this basis, because, by using labelled external or internal substrates, we actually measure the rate of the steps to which k_2 refers, velocity equations are obtained by defining the influx (v_{inf}) and the efflux (v_{eff}) rates respectively, as

$$v_{inf} = k_2[CS_e] \quad \text{and} \quad v_{eff} = k_2[CS_i]$$

and considering that v's are constant as long as [CS]'s are stationary.

Finally, considering the influx, the following equation is obtained:

$$v = k_2[CS_e] = \frac{V_M}{1 + \frac{k_2K_S + k_2S_i}{k_3K_S + k_2S_i}} \cdot \frac{S_e}{S_e + \frac{2k_3K_S + (k_2+k_3)S_i}{(k_2+k_3)K_S + 2k_2S_i}K_S} \tag{1}$$

where maximal rate,

$$V_M = k_2C_T \quad \text{and} \quad C_T = [C_e] + [C_i] + [CS_e] + [CS_i]$$

A similar equation can be obtained for efflux. However, focusing on the uptake of citrate, a simple study of Eq. (1) leads to the observation that the rate of uptake is expected to have a hyperbolic saturation dependence with respect to the external citrate concentration (S_e) at any intraliposomal citrate concentration (S_i), and hence double reciprocal plots will be linear. Furthermore, the slope of the straight lines is again a function of S_i, such that they will meet at a point on the negative side of the abscissa. The kinetic data shown in Fig. 8 clearly fit with this model.

We can therefore consider the model of "exchange by uniport" as a reasonable hypothesis for the tricarboxylate carrier mechanism, since it is in agreement with our experimental results, and in particular explains the basic efflux data. According to the model, the carrier can perform exchange with a part of its molecules (back-rearrangement of the complex with the countersubstrate) and uniport with the other (back-rearrangement of the free carrier molecule). Experimentally, the rate of the basic efflux is considerably lower than the exchange rate, which implies that kinetic constant k_3 is correspondingly lower than k_2; this in turn means that, in the presence of both internal and external substrate, the carrier actually operates an exchange with the already established 1:1 stoichiometry (Bisaccia *et al.*, 1993), whereas pure uniport only occurs in the absence of a countersubstrate (efflux in the absence of external citrate). On the other hand, k_3 must not be so small as to be negligible with respect to k_2: it would practically mean $k_3 = 0$, implying that the free carrier molecule would not be able to rearrange inside the membrane. In fact, in that case, Eq. (1) would change so that, in the double reciprocal plot, the slopes of the straight lines would become independent of S_i (i.e., the lines would become parallel). In that case (and only in that case) the model would become an obligatory exchange, corresponding to the classical ping-pong mechanism.

It has also to be noted that the mersalyl-induced uniport cannot simply be explained by a change in the values of some parameters in the rate equation, induced by the binding of the reagent. In fact, the acquired insensitivity of the system to inhibitors such as PLP and diethylpyrocarbonate suggests a more radical change in the transport mechanism and also, possibly, in the substrate specificity.

Clearly, a detailed kinetic study will be necessary to reinforce experimental evidence for this "exchange by uniport" model, as well as to kinetically characterize the tricarboxylate carrier.

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