Purification and Characterization of the Reconstitutively Active Tricarboxylate Transporter from Rat Liver Mitochondria*

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The tricarboxylate transporter has been purified in a reconstitutively active form from rat liver mitochondria. The transporter was extracted from mitoplasts with Triton X-114 in the presence of cardiolipin and citrate and was then purified by sequential chromatography on hydroxylapatite, Matrex Gel Orange A, Matrex Gel Blue B, and Afri-Gel 501. Analysis of the purified material via sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated the presence of one main protein band with an apparent molecular mass of 32.5 kDa.

Upon incorporation into phospholipid vesicles, the purified transporter catalyzed a 1,2,3-benzenetricarboxylate-sensitive citrate/citrate exchange with a specific transport activity of 3240 nmol/4 min/mg of protein. This value was enhanced 831-fold with respect to the starting material. Finally, we characterize the reconstituted transport could be substantially inhibited by isocitrate, malate, and phosphoenolpyruvate, but not by α-ketoglutarate, succinate, malonate, pyruvate, or inorganic phosphate.

In conclusion, these studies describe the first procedure to yield a highly purified tricarboxylate transport protein that both displays a high specific transport activity and can be obtained in quantities that readily enable further structural as well as functional studies. Based on its substrate specificity and inhibitor sensitivity, the purified 32.5-kDa protein appears to represent the complete tricarboxylate transport system found in rat liver mitochondria. Finally, new information is presented concerning the effect of covalent modifying reagents on the function of this transporter.

Citrate moves across the mitochondrial inner membrane via a specific transport system known as the tricarboxylate transporter (for reviews see Refs. 1 and 2). Extensive investigation of this transport system with isolated mitochondria (3–9) has revealed its substrate specificity, kinetic character-

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1 The abbreviations used are: BTC, 1,2,3-benzenetricarboxylate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, N-ethyldmaleimide; pCMB, p-chloromercuribenzoic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis.
Purification of Mitochondrial Tricarboxylate Transporter

TABLE I

Purification of the tricarboxylate transport protein from rat liver mitoplasts

Frozen rat liver mitoplasts (126.9–146.5 mg of protein) were employed as the starting material. Protein yield and total activity values were normalized to 126.9 mg of starting mitoplast protein. Protein fractions were incorporated into asolectin vesicles, and BTC-sensitive citrate transport was then determined as described under “Experimental Procedures.” Data represent means of five separate protein isolations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein yield</th>
<th>BTC-sensitive citrate/citrate exchange</th>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol/4 min % yield nmol/4 min/mg fold enhancement</td>
</tr>
<tr>
<td>Triton X-114 extract</td>
<td>99.16</td>
<td>386.7 100 3.9</td>
</tr>
<tr>
<td>Hydroxylapatite eluate</td>
<td>2.71</td>
<td>608.7 157 224.6 58</td>
</tr>
<tr>
<td>Matrex Gel Orange A flow-</td>
<td>2.35</td>
<td>523.8 135 222.9 57</td>
</tr>
<tr>
<td>through protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrex Gel Blue B eluate</td>
<td>0.04</td>
<td>103.7 27 2591.7 665</td>
</tr>
<tr>
<td>Affi-Gel 501 flow-through</td>
<td>0.02</td>
<td>64.8 17 3240.0 831</td>
</tr>
<tr>
<td>(mercaptoethanol-activated)</td>
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</table>

FIG. 1. Coomassie-stained SDS-polyacrylamide gradient gel electrophoretic pattern of the different stages of purification of the functional tricarboxylate transport protein from rat liver mitoplasts. Proteins were run in a 4.5% polyacrylamide stacking gel followed by a highly resolving 14–20% linear gradient gel. Lane 1, 1.8 μg of each Bio-Rad SDS-PAGE low molecular weight standard protein: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400); lane 2, 15.8 μg (1.3-μl sample) of the Triton X-114 extracted mitoplast protein; lane 3, 9.6 μg (0.26-ml sample prior to precipitation) of the hydroxylapatite eluate protein; lane 4, 7.8 μg (0.26-ml sample prior to precipitation) of the Matrex Gel Orange A flow-through protein; lane 5, 4.4 μg (1.05-ml sample prior to precipitation) of the Matrex Gel Blue B eluate protein; lane 6, 5.1 μg (1.05-ml sample prior to precipitation) of the mercaptoethanol-activated Affi-Gel 501 flow-through protein; lane 7, 1.1 μg (1.05-ml sample prior to precipitation) of the lipid-containing mercaptoethanol-activated Affi-Gel 501 buffer (i.e. Buffer B + 10 μg of asolectin/ml + 28 mM mercaptoethanol) protein. Other conditions were as described under “Experimental Procedures.”

should be noted that we recovered higher transport activity values when a relatively rapid flow rate (4–6 ml/h) was employed. The third purification step consisted of chromatography of the hydroxylapatite eluate on Matrex Gel Orange A. This gel is composed of a synthetic dye (possessing both aromatic and anionic (sulfonate) character) that is coupled to a cross-linked 5% agarose support matrix via an ether linkage to the triazine ring (38). Following application of the hydroxylapatite eluate to the Orange A gel, the functional tricarboxylate carrier appeared in the flow-through. It is important to note that, although treatment with Orange A caused a slight reduction in the reconstituted citrate transport activity values (Table I), analysis of the protein composition of the Orange A flow-through via SDS-PAGE (Fig. 1, lane 4) indicated the substantial removal of three protein bands (with molecular masses of 52, 29, and 28 kDa) by this step. Additionally, analysis of the material that adsorbed to the Orange A confirmed the presence of several protein bands which upon elution were inactive in reconstitution assays (data not shown).

 experimental procedures

RESULTS

Purification of the Reconstitutively Active Form of the Tricarboxylate Transporter from Rat Liver Mitoplasts—The procedure we used to obtain highly purified reconstitutively active tricarboxylate transporter from rat liver mitoplasts is described in detail under “Experimental Procedures.” Therefore, we comment here only on those aspects of the procedure that are critical in obtaining purified functional transporter as well as on the results we obtained with this procedure. Our prior experiments indicated that, in the presence of 3 mg of exogenous cardiolipin/ml and 5 mM citrate, the non-ionic detergent Triton X-114 extracted the functional tricarboxylate transporter. As previously reported (18), maximal total and specific transport activity values were obtained following reconstitution of transporter function in phospholipid vesicles when the extraction was carried out with 3–4% Triton X-114. Moreover, reconstituted transporter function increased with the amount of protein added to liposomes and required intraliposomal citrate. Utilizing these previously optimized extraction/reconstitution conditions in the present investigation, Table I shows that we obtained a reconstituted specific transport activity of 3.9 nmol/4 min/mg of protein with the initial Triton X-114 extract.

The second step in our purification procedure consisted of hydroxylapatite chromatography. It has been demonstrated in several laboratories (20–22, 24, 25, 30–32, 35–37), including our own (18), that hydroxylapatite chromatography causes a large single-step purification of mitochondrial anion transporters. As depicted in Table I, this step resulted in a 58-fold increase in the reconstituted specific citrate transport activity. The enhanced purity of this fraction was further verified by SDS-polyacrylamide gradient gel electrophoretic analysis. As indicated in Fig. 1, lanes 2 and 3, considerably fewer protein bands were present in the hydroxylapatite eluate compared with the initial detergent extract. Additionally, as has been observed previously (18, 24), all but one of the intensely staining protein bands in this fraction display molecular masses in approximately the 30–37-kDa range. Finally, it is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

 Portions of this paper (including “Experimental Procedures” and Fig. 2) are presented in miniprint at the end of this paper. Miniprint
Purification of Mitochondrial Tricarboxylate Transporter

The fourth purification step consisted of chromatography on Matrex Gel Blue B. In addition to possessing hydrophobic and anionic character, this gel also contains a large copper phthalocyanine chromophore (38). Upon application of the Orange A flow-through to the Blue B gel, the functional tricarboxylate transporter was adsorbed. Resin-bound transporter was then eluted with 10 mg of aselectin/ml. As shown in Table I, this step resulted in an additional 12-fold increase in the reconstituted specific transport activity. The enhanced purity of this fraction is verified by the SDS-polyacrylamide gel electrophoretic protein banding pattern depicted in Fig. 1, lane 5, which indicates that the Blue B eluate consisted primarily of two protein bands with apparent molecular masses of 60 and 32.5 kDa. Thus, this step permitted the separation of the tricarboxylate transporter from other proteins in the 30–37-kDa molecular mass region.

Several additional points concerning this chromatographic step merit comment. First, the fact that the transporter could be eluted with lipid suggests that its adsorption to the Blue B gel may have been due partially to hydrophobic interactions. Additionally, based on the observation that the substrate binding site of the tricarboxylate transporter appears to be capable of binding metal ions (6), we speculate that the copper within the Blue B gel may have also participated in transporter adsorption. Finally, we found (a) the volume of the Orange A flow-through that was added to a given volume of Blue B gel and (b) the extent to which the Blue B gel was washed following transporter adsorption to be important parameters in determining the recovery of functional transporter at the aselectin elution step. In the procedure that we have developed, a relatively high ratio of Orange A flow-through to Blue B gel as well as minimal column wash volumes have been utilized to maximize recovery of functional transporter.

The final purification step consisted of chromatography on Affi-Gel 501. This gel is composed of an organomercurial group added to agarose and thus separates proteins based on the presence of accessible sulfhydryl groups. Following the application of the Blue B eluate to Affi-Gel 501, the functional citrate transporter (i.e. the 32.5-kDa protein) appeared in the flow-through, whereas the 60-kDa contaminant protein adsorbed to the gel. Upon incorporation into liposomes, the mercaptoethanol-activated (see below) Affi-Gel flow-through catalyzed a specific citrate transport activity of 3240 nmol/4 min/mg of protein. This value represented a 25% increase in specific activity over that for the preceding Blue B eluate and an 831-fold enhancement relative to the initial detergent extract (Table I). The highly purified nature of the Affi-Gel 501 flow-through is further indicated by the SDS-polyacrylamide gel electrophoretic pattern depicted in Fig. 1, lane 6, which shows the presence of one main mitochondrial protein band (apparent molecular mass of 32.5 kDa). For comparison, lane 7 depicts the electrophoretic pattern of the mercaptoethanol-activated lipid-containing buffer (in the absence of mitochondrial protein) present in the Affi-Gel flow-through.

Two additional points concerning the Affi-Gel 501 step should be noted. First, it was necessary to incubate the Affi-Gel 501 flow-through with mercaptoethanol in order to subsequently recover transporter activity in the reconstituted liposomal system. This finding was especially intriguing because of the observation that incubation of the Blue B eluate (i.e. the transporter fraction immediately preceding the Affi-Gel 501 step) with mercaptoethanol did not cause an increase in the subsequently reconstituted transport activity. Thus, chromatography on Affi-Gel resulted in a mercaptoethanol dependence of transporter function. Possible explanations for this finding are presented under “Discussion.” Second, the 60-kDa protein that adsorbed to the Affi-Gel could be eluted with 30 mM mercaptoethanol. This fraction was inactive in reconstitution assays (data not shown).

In summary, this isolation procedure yields a highly purified preparation of a 32.5-kDa protein which has been identified as the catalytically competent tricarboxylate transporter. Based on liposomal reconstitution assays, the purified protein catalyzes a high magnitude BTC-sensitive citrate/citrate exchange with a specific activity that is enhanced 831-fold relative to the initial detergent extract. Furthermore, the entire purification procedure can be performed by a single individual in 9 h. Finally, we obtain 22 μg (0.68 nmol) of purified tricarboxylate transporter from 127 mg of starting mitoplast protein. This value translates to a yield of 73 μg (2.34 nmol) of purified transporter per rat (we obtain 421 mg of mitoplast protein/rat). Thus, a sufficient quantity of transport protein is obtained by this procedure for most types of functional and structural studies.

**Time Course and Substrate Specificity of the Reconstituted BTC-sensitive [14C]Citrate/[Citrate] Exchange**—The time course of the BTC-sensitive [14C]citrate uptake in exchange for intraliposomal citrate is depicted in Fig. 2. These studies were carried out with proteoliposomes that were formed utilizing the Affi-Gel 501 purified tricarboxylate transporter. The results are depicted in both direct form and after the data have been analyzed assuming an exponential approach to isotopic equilibrium. Fig. 2A indicates that citrate uptake proceeds in a curvilinear (i.e. hyperbolic) manner. Fig. 2B demonstrates that the reconstituted citrate transport reaction displays apparent first order kinetics with respect to the intravesicular unlabeled substrate concentration, an observation that is in agreement with the properties of this carrier in isolated mitochondria (3). A first order rate constant of 0.09 min⁻¹ and a t₀ of 7.76 min have been calculated.

Table II depicts information on the substrate specificity of the reconstituted tricarboxylate transport protein. The data indicate that tricarboxylates such as citrate (unlabeled) and isocitrate, as well as the metabolite phosphoenolpyruvate, were effective inhibitors of the [14C]citrate/citrate exchange reaction. Among the dicarboxylates, malate effectively inhibited the reconstituted exchange whereas other dicarboxylates were either considerably less effective (i.e. succinate) or totally ineffective (i.e. malonate). Finally, substrates for other mitochondria.

**Table II**

<table>
<thead>
<tr>
<th>Competing anion</th>
<th>Activity remaining %</th>
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<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Citrate</td>
<td>14</td>
</tr>
<tr>
<td>threo-D,-Isocitrate</td>
<td>19</td>
</tr>
<tr>
<td>Malate</td>
<td>19</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>10</td>
</tr>
<tr>
<td>Succinate</td>
<td>77</td>
</tr>
<tr>
<td>Malonate</td>
<td>108</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>115</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>124</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>141</td>
</tr>
</tbody>
</table>
chondrial anion transporters (e.g. α-ketoglutarate, pyruvate, inorganic phosphate) did not inhibit the reconstituted exchange and in fact may have caused a slight stimulation. Thus, in agreement with the observations from intact mitochondria (1-3, 7), the purified transporter discriminates between its physiological substrates (e.g. citrate, isocitrate, phosphoenolpyruvate, malate) and other anions.

The Effect of Mitochondrial Anion Transporter Inhibitors and Protein Labeling Agents on the Reconstituted Citrate/Citrate Exchange—The sensitivity of the purified tricarboxylate transporter to inhibitors of other mitochondrial anion transporters has been tested. As depicted in Table III, the reconstituted citrate/citrate exchange was substantially inhibited by BTC, a specific inhibitor of the tricarboxylate transporter in intact mitochondria (9). In contrast, the reconstituted transport reaction was, as expected, relatively insensitive to phenylsuccinate, α-cyano-4-hydroxycinnamate, and carboxyatractyside, agents which are considered classical inhibitors of the α-ketoglutarate and dicarboxylate (6, 39), respectively. Finally, α-butyramalonate, an inhibitor of the dicarboxylate transporter (42-44) and to a lesser extent of the tricarboxylate transporter (3) in intact mitochondria, caused a somewhat larger than expected inhibition of the reconstituted transporter. As would be predicted, however, this inhibition was not as great as that observed with BTC. Taken together, the above results indicate that the purified transporter discriminates an inhibitor specificity quite similar to that of the tricarboxylate transport system in intact mitochondria.

Several covalent labeling agents (45) were tested for their effect on the activity of the reconstituted transporter. For example, at a concentration of 2 mM, the sulfhydryl reagents mersalyl, p-chloromercuribenzoic acid, and NEM inhibited the reconstituted citrate/citrate exchange by 82, 85, and 79%, respectively. The inhibitory effects of mersalyl and p-chloromercuribenzoic acid on the purified transporter were consistent with observations obtained with isolated mitochondria (6, 8). Interestingly, NEM caused a considerably greater inhibition of the purified carrier in comparison with its effect on tricarboxylate transport in intact mitochondria (8). This finding suggests that extraction of the transporter from its native membrane may have exposed a sulfhydryl group(s) which is essential for function and is normally inaccessible to NEM and may prove useful in future investigations into the topography of the tricarboxylate transporter in the mitochondrial inner membrane.

Table III depicts results obtained with several other protein labeling agents. N-Acetyl-diazomethane, a tyrosine-selective agent, caused only a slight inhibition of the reconstituted citrate transporter. In contrast, reagents that are selective for histidine (diethyl pyrocarbonate), arginine (2,3-butanedione and phenylglyoxal), and lysine as well as amino terminal (pyridoxal 5-phosphate) residues resulted in considerable inhibition of the reconstituted transport activity.

**DISCUSSION**

The present investigation has resulted in the development of a procedure for obtaining a highly purified preparation of functional tricarboxylate transport protein from rat liver mitochondria. The purification of this transporter has been accomplished by the extraction of rat liver mitoplasts with the nonionic detergent Triton X-114 in the presence of exogenous cardiolipin and citrate, followed by sequential chromatography of the extract on hydroxylapatite, Matrex Gel Orange A, Matrex Gel Blue B, and Affi-Gel 501. SDS-polyacrylamide gel electrophoretic analysis indicates that the final transporter preparation consists mainly of one mitochondrial protein band with an apparent molecular mass of 32.5 kDa. The high degree of functional competence of the purified transporter (a property that is essential for future mechanistic studies) has been demonstrated by the reconstitution of its function in a liposomal system. We have then proceeded to characterize the purified transporter with respect to its substrate specificity and its sensitivity to both mitochondrial and nonmitochondrial anion transport inhibitors as well as protein covalent labeling agents. To our knowledge, this is the first report of a procedure which yields a highly purified tricarboxylate transport protein that both displays a high specific transport activity and can be obtained in amounts that readily enable further structural as well as functional studies.

The conclusion that the protein which we have purified is in fact the tricarboxylate transporter is based on the following pieces of evidence. First, upon incorporation into phospholipid vesicles, the purified material catalyzes a high magnitude BTC-sensitive citrate/citrate exchange (Table I, Fig. 2). Second, at each stage of purification, the specific activity of the reconstituted transport reaction increases (except between the hydroxylapatite eluate and the Matrex Gel Blue A flow-through where the specific activity values are approximately equal) (Table I). Third, upon reconstitution, the purified protein catalyzes a transport reaction that displays a substrate specificity (Table II) and an inhibitor sensitivity (Table III) which are nearly identical to those observed for the tricarboxylate transport system in intact mitochondria (3, 4, 6-9). In combination, we believe that these results represent conclusive evidence that the procedure which we have developed does indeed result in the purification of the catalytically competent tricarboxylate transport protein.

The highly purified nature of the final tricarboxylate transporter fraction (i.e. the Affi-Gel 501 flow-through) is indicated by both SDS-polyacrylamide gradient gel electrophoretic analysis as well as by specific activity measurements in a reconstituted liposomal system. The electrophoretic analysis indicates the presence of one main mitochondrial protein band with an apparent molecular mass of 32.5 kDa (Fig. 1, lane 6). The reconstitution studies indicate a specific transport activity for this fraction which is increased 831-fold relative to the initial detergent extract. However, the precaution (46) should be noted that the exact extent of the increase in specific activity (and yield of total activity) from one
purification fraction to another may be somewhat inexact due to differences in a variety of factors (e.g. protein composition, efficiency of protein incorporation into the liposomes, detergent and lipid concentrations, etc.) that can potentially influence proteoliposomal structure and thereby affect the observed reconstituted transport function. We suggest that these considerations are the likely cause of the 57% increase (Table 1) in the total transport activity that we observed in the hydroxylapatite eluate relative to the initial detergent extract. Alternatively, it is possible that this increase might reflect a true activation of the transport protein per se if for example hydroxylapatite chromatography resulted in the removal of an inhibitory protein(s). It is noteworthy that similar activity increases have been observed with several other mitochondrial anion transporters (24, 31, 47) in reconstitution assays following hydroxylapatite chromatography.

Another point of interest pertains to our finding that the purified transporter (i.e. the Affi-Gel 501 flow-through) required a treatment with mercaptoethanol in order to subsequently catalyze transport in reconstitution assays, whereas at the immediately preceding stage of purification (i.e. the Blue B eluate) transporter activity was insensitive to mercaptoethanol. Thus, chromatography on Affi-Gel resulted in a mercaptoethanol dependence of transporter function. We speculate that this mercaptoethanol requirement arose due to either: 1) a lipid peroxide-catalyzed oxidation of essential sulfhydryl groups within the transport protein in the presence of the organomercurial resin (consistent with this idea are numerous reports in the literature describing enzyme inactivation due to lipid peroxide-catalyzed oxidation of protein sulfhydryl groups and the enhancement of this process by metals (48, 49)); or 2) the reaction of exposed and essential sulfhydryl groups within the transporter with small amounts of mercury (that may have leached off the Affi-Gel) to form a mercaptide bond(s), thereby preventing the transporter from binding to the gel and inhibiting transporter function. In either scenario, subsequent treatment of the Affi-Gel flow-through with mercaptoethanol would regenerate the original sulfhydryl group(s) within the transporter and thus reactivate transporter function. At present, both explanations appear plausible. Finally, it is important to note that despite uncertainties concerning the mechanism responsible for the observed mercaptoethanol dependence of the Affi-Gel flow-through, this chromatographic step nonetheless yields highly purified reconstitutively active tricarboxylate transporter that is suitable for further investigation.

It is important to compare our purified preparation of tricarboxylate transporter from rat liver mitochondria with two other purified preparations that have been reported recently (21, 22). Claey and Azzi (21), utilizing a substantially different procedure from that reported here, described the purification of highly purified tricarboxylate transporter from bovine liver mitochondria. Several important differences between their preparation and our preparation from rat liver mitochondria are apparent. First, upon functional reconstitution, the specific activity of our rat liver transporter is approximately 5-fold higher than the value they obtained with the bovine liver transporter (i.e. 3240 nmol/4 min/mg of protein versus 600 nmol/4 min/mg of protein (calculated from Ref. 21, Table 1)). Thus, the rat liver preparation has the important advantage of catalyzing a high magnitude specific transport activity, a requirement for future studies into the roles of specific structural domains of the transporter in the transport mechanism. As Claey and Azzi (21) have pointed out, their relatively low specific transport activity may be a consequence of obtaining the starting tissue from a slaughter-house, which thereby prevents the use of very fresh material for the protein isolation. A second important distinction between the two preparations is the different apparent molecular mass values for the purified transporter that are indicated by SDS-PAGE. We obtain a value of 32.5 kDa for the rat liver transporter, whereas Claey and Azzi (21) report a value of 37-38 kDa for the bovine liver preparation. At present, it is unclear whether these values reflect a true species-specific difference in the tricarboxylate transport protein or reflect differences that arise due to the protein purification and/or the SDS-PAGE conditions. It is noteworthy that there is precedence for species-specific differences in the structure of mitochondrial anion transport proteins (e.g. the phosphate and the ADP/ATP transporters) (50). Quite recently, Bisaccia et al. (22), utilizing a procedure which involves the sequential chromatography of a Triton X-100 extract on hydroxylapatite and celite, reported the purification of the functional tricarboxylate transporter from rat liver mitochondria. They found that the tricarboxylate transporter migrated as one protein band on SDS-PAGE with an apparent molecular mass of 30 kDa. Our finding that the tricarboxylate transporter consists of a single 32.5-kDa protein is in basic agreement with their report, especially when one accounts for the different electrophoretic conditions employed in the two studies. Moreover, the reconstituted specific transport activity that we obtained is similar to the value reported by Bisaccia et al. (22) (i.e. 3240 nmol/4 min/mg of protein versus 5400 nmol/4 min/mg of protein (calculated from Ref. 22, Table 1)). The slight difference in activity values may reflect in part the different reconstitution and assay conditions employed in the two studies. Most significantly, although both procedures result in a similar yield of purified tricarboxylate transport protein from the initial detergent-solubilized protein (i.e. 0.02%), our procedure makes use of a considerably greater quantity of starting material and thus yields an 11-fold greater quantity of purified transporter per preparation than the amount reported by Bisaccia et al. (22) (i.e. 22 versus 2 μg). Thus, we believe that an important advantage of our purification procedure is that it provides an amount of purified tricarboxylate transport protein that will readily enable studies into the structure as well as the function of this transporter. It should also be mentioned that, in the present investigation, our results are reproducible with different batches of hydroxylapatite. This finding is critical because batch to batch variation in this resin is known to markedly influence the ability of hydroxylapatite to yield reproducible results in anion transporter purification procedures (24). Finally, upon functional reconstitution, transporter purified by the method described in this paper as well as by the method of Bisaccia et al. (22) displayed quite similar substrate specificities and sensitivities to both sulfhydryl reagents and inhibitors of other anion transporters. Minor exceptions were the increased sensitivity to NEM and the insensitivity to malonate and phenylsuccinate that we observed relative to the findings of Bisaccia et al. (22).

Our amino acid modification studies indicate that the purified and reconstituted tricarboxylate transport protein can be substantially inhibited by sulfhydryl reagents, thus suggesting that a cysteine residue(s) may be essential to the transport mechanism. These findings are in agreement with observations concerning the sensitivity of this transporter to sulfhydryl reagents with intact mitochondria (6, 8) as well as with purified transporter preparations (21, 22). Moreover, the fact that other mitochondrial anion transporters are also sensitive to sulfhydryl reagents (for review see Refs. 1 and 2) suggests a mechanism for anion transport across the mitochondrial inner membrane that may involve similar structural
elements among the different anion transporters, an idea that has recently been proposed (51, 52) based on the amino acid sequence homology between the ADP/ATP carrier (53), the phosphate carrier (51, 52), and the uncoupling protein (55). Additionally, our observation (Table III) that the purified tricarboxylate transporter is highly sensitive to reagents which are selective for histidyl (diethyl pyrocarbonate), guanidyl (2,3-butanedione, phenylglyoxal), and lysyl (pyridoxal 5-phosphate) residues suggests that these types of amino acid residues are also important for the transport mechanism. While based on analogy to other anion binding (56-59) and proton transporting (60) systems one can readily envision how each of these types of residues could be mechanistically important in the transport of a negatively charged substrate (i.e. citrate) and/or a proton, our data should be interpreted cautiously since none of the above reagents are absolutely selective for only a single type of residue. Nonetheless, these results should prove useful in future studies in which we will characterize the inhibitor-transport protein interactions more extensively.

In conclusion, the purification and characterization of the reconstitutively active tricarboxylate transporter from rat liver mitochondria represent important first steps toward an eventual understanding of the molecular mechanism of citrate transport across the mitochondrial inner membrane. Investigations are currently under way in this laboratory which seek to elucidate the structure, mechanism of action, and regulation of this transporter in normal and diseased states.

Acknowledgments—We would like to thank Amanda L. Bastian, Steven M. Blackwell, and Nancy R. Blackwell for their expert technical assistance during certain of these experiments. Additionally, we are grateful to Dr. Thomas M. Lincoln for critically reading this manuscript.

REFERENCES


**EXPERIMENTAL PROCEDURES**

**Materials** - Sperms phosphodiesterase type IV (i.e., phosphodiesterase) bovine heart cardiolipin (Sigma Chemical Co., St. Louis, MO), Tris (hydroxymethyl) aminomethane (Tris), triphosphate, ATP, and other reagents were purchased from Sigma Chemical Co., St. Louis, MO. Heparin was purchased from Chas S. Phipps & Sons Co., Inc., Philadelphia, PA. 

Purification of the Tricarboxylate Transport Protein - The mitochondrial tricarboxylate transporter was isolated and characterized. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. 

**Preparation of Citrate-Loaded Proteoliposomes** - Citrate vesicles were prepared by batch co-sonication as previously described. 

**Preparation of BT Solutions and their Control Buffers** - Four stock solutions of BT (100 µM) were prepared. The final concentration of BT in each solution was 100 µM. 

**Determination of the Substrate Specificity of the Purified Transport Protein** - The net flux of the ATP-dependent transport of citrate into proteoliposomes was measured. 

**RESULTS** - The purification of the mitochondrial tricarboxylate transporter was successful. The transporter was isolated and characterized. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. 

**DISCUSSION** - The purification of the mitochondrial tricarboxylate transporter was successful. The transporter was isolated and characterized. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. To improve the solubility of the transporter, the matrix was dialyzed against a solution containing 1 M KCl and 0.1 M Tris-HCl (pH 8.0). To increase the yield of the transporter, a buffer system consisting of 1 M KCl and 0.1 M Tris-HCl (pH 8.0) was prepared. 

**Figure 1** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 2** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 3** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 4** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 5** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 6** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 7** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 8** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 9** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 10** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 11** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined. 

**Figure 12** - Time dependence of the ATP-sensitive citrate uptake. Panel A, direct plot of citrate uptake rate. The purified tricarboxylate transporter was incorporated into liposomes and incubated in a solution containing 100 µM ATP. To determine the time course of the transport reaction, aliquots of the reaction mixture were withdrawn at various time points and the amount of citrate taken up by the proteoliposomes was determined.