Mitochondrial and Plasma Membrane Citrate Transporters: Discovery of Selective Inhibitors and Application to Structure/Function Analysis

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Abstract

Cytoplasmic citrate is the prime carbon source for fatty acid, triacylglycerol, and cholesterol biosyntheses, and also regulates glucose metabolism via its allosteric inhibition of phosphofructokinase. It originates either via the efflux of citrate from the mitochondrial matrix on the inner membrane citrate transport protein (CTP) or via the influx of extracellular citrate on the plasma membrane citrate transporter (PMCT). Despite their common substrate, the two transport proteins share little sequence similarity and they transport citrate via fundamentally different mechanisms. We tested the ability of a set of previously identified CTP inhibitors, to inhibit the PMCT. We found that of the top 10 CTP inhibitors only one substantially inhibited the PMCT. Conversely, we identified two other inhibitors that inhibited the PMCT but had little effect on the CTP. All three identified PMCT inhibitors displayed a noncompetitive mechanism. Furthermore, models to explain inhibitor interactions with the CTP are proposed. As part of the present studies a PMCT homology model has been developed based on the crystal structure of the leucine transporter, and a possible citrate binding site has been identified and its composition compared with the two known citrate binding sites present within the CTP. The ability to selectively inhibit the PMCT may prove key to the pharmacologic amelioration of metabolic disorders resulting from the synthesis of excess lipid, cholesterol, and glucose, including human obesity, hyperlipidemia, hypercholesterolemia, and Type 2 diabetes.

Keywords: Citrate; Transporter; Mitochondria; Plasma membrane; Inhibitor discovery; Bioenergetics

Introduction

Citrate is a key intermediate in both catabolism and anabolism and thus occupies a prominent position in eukaryotic energy metabolism (see Figure 1). Two sources of intracellular citrate exist. One source consists of the generation of citrate within the mitochondrial matrix. Thus, when the cell needs to extract energy via oxidation of carbohydrate, lipid, or protein, the resulting intermediates will enter the citric acid cycle at various steps, resulting in the formation of citrate within one turn of the cycle. Intramitochondrial citrate can then be oxidized by the cycle to produce NADH and FADH2 and ultimately yield ATP via oxidative phosphorylation. When the cell has excess energy, citrate is transported out of the mitochondrial matrix across the inner membrane via the mitochondrial citrate transport protein (CTP) (for review see ref. (1)). Citrate can then passively diffuse through an anion selective channel across the outer mitochondrial membrane into the cytoplasm. Once in the cytoplasm, citrate is broken down to acetyl CoA and oxaloacetate, the former providing the immediate carbon source to fuel fatty acid, triacylglycerol, and cholesterol biosyntheses (2-5). The second source of intracellular citrate originates in the blood (6, 7). Thus, extracellular citrate can be transported inwardly across the plasma membrane by a group of proteins which we refer to as the plasma membrane citrate transporters (PMCTs) (6-13). This article will compare and contrast the properties of the CTP and the PMCTs.

The mitochondrial CTP catalyzes an obligatory exchange of tricarboxylates (i.e., citrate, isocitrate) either for each other or for the dicarboxylate malate or for phosphoenolpyruvate (14-18). In higher eukaryotes the transporter catalyzes citrate/malate
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Figure 1. Schematic depiction of the roles of the PMCT and the CTP in supplying citrate to fuel hepatic fatty acid, triacylglycerol, and sterol biosyntheses. Citrate can be transported from the blood across the hepatocyte plasma membrane into the cytoplasm on the PMCT, or it can be effluxed from the mitochondrial matrix across the mitochondrial inner membrane on the CTP. Cytoplasmic citrate derived from either source is then broken down to acetyl CoA by citrate lyase, and the resulting acetyl CoA provides all of the carbon precursor to fuel the fatty acid, triacylglycerol, and sterol biosynthetic pathways.

echange with citrate moving outwardly across the inner membrane (14-16). In yeast, the CTP is thought to catalyze a citrate/isocitrate exchange (17, 18). In both cases, the CTP catalyzes a facilitated-diffusion with dianions being the transported species (16). The CTP is a member of the mitochondrial transporter family. Most members of this family display several common characteristics (for review see ref. 19) including: a size of approximately 300 amino acids and a basic isoelectric point, the presence of 3 homologous sequence domains, and a signature sequence motif of Px(D,E)x(V,I,A,M)(K,R)x(R,K,Q,A)(L,M,F,I) which repeats two-three times. An area of current controversy centers around whether these carriers function as monomers or homodimers (20-22).

A second type of citrate transporter has been identified, which is located within the plasma membrane of human liver, human, rat, and mouse brain, and C. elegans (6, 7,10-12). This transporter catalyzes a sodium-coupled citrate import. It is the only known plasma membrane carrier to preferentially transport citrate and has been named NaCT. The NaCTs belong to the SLC13 gene family and in rat, mouse, and C. elegans display a high affinity for citrate (i.e., Km ~ 20-80 μM) (6,11-12), whereas the affinity of the human transporter is somewhat reduced (Km ~ 0.6 - 6 mM, depending on the cell line) (7,10). Nonetheless, even with the human NaCT, the affinity for citrate is greater than for other substrates. The Na+:citrate stoichiometry for each these NaCTs is thought to be 4:1, with the trivalent form of citrate being the transported species, thereby resulting in electrogenic transport. Importantly, the NaCTs represent mammalian orthologs of a plasma membrane citrate transporter originally discovered in Drosophila known as INDY (I'm not dead yet) (8,9,13). Functional differences exist between the NaCTs and INDY. For example, in contrast to the NaCTs, INDY catalyzes a sodium-independent citrate/dicarboxylate exchange across the plasma membrane (9,13). Nonetheless both NaCT and INDY are thought to play key roles in life-span. For example, in Drosophila, reduction in INDY...
activity due to mutation causes a near-doubling in average life-span without a decline in either fertility or physical activity (8). Similarly, functional knockdown of C. elegans NaCT by RNAi leads to a 19% increase in life-span, along with a 40% decrease in body size and a 44% reduction in fat content of the organism (12). Importantly, based upon a combination of expression patterns and transport function, it is thought that disruption of plasma membrane citrate transporters (Note: henceforth, we will use the abbreviation PMCT for “plasma membrane citrate transporter” to refer collectively to the eukaryotic NaCTs and the Drosophila Indy) is likely to alter significantly the energy balance within a wide variety of organisms (8,9,13). Coupled to these insights is the well-documented observation that, with organisms ranging from yeast to non-human primates, caloric restriction (CR) results in significant extension of life-span, a phenomenon due in part to a change in energy utilization (23-25). Recently, Wang et al. (26) reported that CR down regulates PMCT mRNA expression in Drosophila and that optimal life-span extension is observed when expression is decreased to between 25-75% of normal. Additionally, long-lived flies with decreased expression of the PMCT gene are characterized by a decrease in both reactive oxygen species and oxidative protein damage (27), and have numerous phenotypes with flies long-lived due to CR. It is thought that decreased PMCT expression induces a CR-like status by preventing uptake of external citrate, thereby leading to life-span extension (26).

It is of great interest that, despite the fact that the CTP and the PMCTs both transport citrate, there appears to be little similarity in either their sequence (i.e., no significant sequence identity) or their mechanism. Thus, the PMCTs are significantly larger in size (61 kDa versus 32.2 kDa; 12 predicted TMDs versus 6), less polar, and less basic compared to the CTP. With respect to mechanism, most PMCTs (Drosophila Indy being the sole exception (9,13,28)) catalyze a Na:citrate concentrative uptake (6,10,11), whereas the CTP catalyzes an obligatory 1:1 exchange of citrate for either isocitrate, malate, or phosphoenolpyruvate (16).

The discovery of compounds that would selectively inhibit only one of these transporters would serve at least two important purposes. First, it would provide useful tools for further understanding of the structure-function relationships of these transporters. Second, a PMCT-selective inhibitor might mimic caloric restriction, decrease fatty acid and cholesterol biosyntheses, prevent obesity, and extend life-span. We recently reported the discovery of compounds that inhibit CTP (29) via in silico screening of the ZINC database (30). Here, we describe the selectivity of these inhibitors for CTP over PMCT, as well as the discovery of some PMCT-selective inhibitors. Furthermore, we report the development of a homology-modeled structure for the PMCT including identification of a putative citrate binding site. Moreover, we propose molecular explanations for the observed inhibitor selectivity. To our knowledge, these findings represent the first comparative information on inhibitor selectivity, homology-modeled structures, and proposed substrate binding site composition for these different types of citrate transporters.

Materials and Methods

Overexpression, Isolation, and Incorporation of Cys-less CTP and PMCT into Liposomal Vesicles

The overexpression, isolation, and incorporation of Cys-less CTP were performed as previously detailed (17,29,31,32). The PMCT was overexpressed in Pichia pastoris, solubilized in n-dodecyl-α-D-maltopyranoside (DDM, Anatrace), and purified via NiNTA (Qiagen) and Talon (Clontech) chromatography. The purified PMCT was incorporated into liposomal vesicles, in the presence of 50mM citrate and 10 mM NaCl, by the freeze-thaw-sonication method as described before (17,33). Immediately prior to transport, a given proteoliposomal sample was thawed, sonicated on ice, and passed through Dowex resin in a Pasteur pipette to remove the external citrate. The collected proteoliposomes were then immediately assayed for transport. Protein was quantified as detailed previously (34).

Determining Transporter Inhibition by Compounds from the ZINC Database

The inhibition by ZINC compounds of BTC-sensitive citrate transport catalyzed by the Cys-less CTP was characterized as previously described (29). The effect of ZINC compounds on the NEM-sensitive citrate transport catalyzed by the PMCT was determined as follows. 45 µl of proteoliposomes were incubated with 3.5 µl of either 300 mM N-ethylmaleimide (NEM, control reaction) or water (experimental reaction) for 10 min at ~20°C. 1.4 µl of

either dimethyl sulfoxide (DMSO) or ZINC inhibitors (Ct = 1 mM) were then added to the reaction mix and incubated for another 10 min. Transport reactions were initiated by the addition of 21.5 µl of [1,5-14C]citrate (Ct = 1.0 mM; specific radioactivity 22×10^3 cpm/nmol; GE Healthcare) and NaCl (Ct = 50 mM). The reaction was quenched after 6 min uptake, with water or 300 mM NEM, for control and experimental reactions, respectively. Immediately following transport, 60 µl of the reaction mix was loaded onto a 2.6 ml Dowex Bio-Rad column, to separate the intraliposomal radiolabeled citrate from the external radiolabel. The eluted intraliposomal radiolabel was quantified via liquid scintillation counting and the NEM-sensitive transport rate was calculated by subtracting the control value from the experimental value. The effect of a given ZINC compound on citrate transport is expressed as the percentage inhibition of the initial BTC (with the CTP)- or NEM (with the PMCT)-sensitive citrate uptake (measured in the presence of buffer or DMSO) and was calculated as previously detailed (29).

Characterization of the Type of PMCT Inhibition Mediated by Compounds from the ZINC Database

Transport reactions were conducted as described above with the following modifications. Varying concentrations of [1,5-14C]citrate were employed ranging from 0.05 to 0.75 mM with specific radioactivity values that ranged from 3.5×10^4 to 25.0×10^4 cpm/nmol. The uptake time was 3-6 min. Within each round of transport, a v versus [S] curve was obtained in the presence of a single concentration of inhibitor. Controls contained DMSO instead of inhibitor.

Construction of a Homology-based Model of the PMCT

We used the Membrane Protein Explorer software package (35), version 3.2, to assess the topology of the PMCT. The translocon analysis module (36) predicts 12 transmembrane helical domains. The most closely related structure in the Protein Data Bank (PDB) is a prokaryotic sodium-coupled leucine transporter, LeuT. It has 12 transmembrane domains. Several structures have been determined for this transporter, with different substrate plus non-competitive inhibitor combinations. While the overall sequence homology is relatively low, we were able to construct a reasonable alignment for the 12 transmembrane domains. Both the substrate and inhibitor sites are formed by the transmembrane domains. We chose the PDB structure 2Q72, which includes a substrate molecule (leucine) plus the non-competitive inhibitor imipramine and has resolution of 1.7 Å (37). Homology modeling was carried out as described previously (38) using Molecular Operating Environment (MOE), version 2009.10 (Chemical Computing Group, Montreal, Canada). While the current model is considered to be only a rough approximation, it serves as a starting point to identify potentially important residues that can be investigated through single point mutation experiments. For example, there are four arginines (R33, R103, R104, and R274) in the transport path that could be important for citrate binding.

Docking of Substrate to the PMCT Model

Docking calculations were carried out using the Docking module of MOE. A database was first constructed, containing 220 conformations of citrate. Conformers were then selected randomly and docked in the region of the four arginines R33, R103, R104,
Results and Discussion

Discovery of Selective Inhibitors of the CTP and the PMCT

Previously, we had identified new inhibitors of the CTP via in silico screening of the ZINC database based on a modeled CTP structure followed by experimental testing of potential lead compounds (29). We identified 10 compounds that, at a concentration of 1 mM, yielded at least 50% inhibition of reconstituted CTP function. The structures of these inhibitors are depicted in Figure 2. One of these, compound 792949, was demonstrated to be the first purely competitive CTP inhibitor and is thought to span and simultaneously bind to both CTP substrate binding sites. In the present studies, we examined the effect of these 10 inhibitors on the reconstituted function of the PMCT. As depicted in Table 1, we observed that in contrast to its effect on the CTP, at 1 mM compound 792949 caused little effect on PMCT function (i.e., 85% inhibition of the CTP versus 6% inhibition of the PMCT). We note that of the other 9 CTP inhibitors, only compound 4180643 also significantly inhibited the PMCT (i.e., 73 versus 79% inhibition, PMCT versus CTP, respectively). Thus 9 of the 10 CTP inhibitors were selective for the CTP over the PMCT.

Conversely, when the CTP-inactive candidates were screened against PMCT, two PMCT-selective inhibitors were found. Thus, upon testing 44 additional compounds that were identified via our initial in silico CTP screen, but which did not in fact substantially inhibit reconstituted CTP function, we discovered two compounds, 39396 and 339393, which at 1 mM, caused substantial inhibition of PMCT function (i.e., 60 and 49% inhibition, respectively; see Table 1 and Figure 2 for structures). Thus, our results clearly demonstrate that it is possible to selectively inhibit either of these transporters in the presence of the other and suggests substantive differences between the CTP and PMCT substrate binding sites.

Characterization of the Inhibition Mechanism of Identified PMCT Inhibitors

We next analyzed the inhibition mechanism of the best two PMCT inhibitors (i.e., compounds 39396 and 4180643). Figure 3, Panels B and E depict v
versus [S] data obtained at multiple inhibitor concentrations. Lineweaver-Burk analysis of these data (Panels C and F) reveals a noncompetitive inhibition pattern with both compounds. Global fit analysis of the v versus [S] data indicates Ki values of 0.34 mM and 0.30 mM for compounds 39396 and 4180643, respectively. Interestingly, the inhibitor that is specific for PMCT, compound 39396, is a hydrophobic, uncharged molecule, suggesting interaction primarily with hydrophobic PMCT residues. In contrast, compound 4180643, which inhibits both the PMCT and the CTP, contains two carboxylate groups, suggesting an interaction with basic residues, and closely resembling typical CTP inhibitors.

Relatedly, we note that several highly effective commercial drugs act through a noncompetitive inhibition mechanism (e.g., nevirapine, HIV reverse transcriptase (39); donepezil, acetylcholinesterase (40); ibuprofen, one subunit of cyclooxygenase II (41)).

**Development of a Homology-modeled PMCT Structure and Prediction of the Residues Comprising the Substrate Binding Site**

Figure 4 depicts a homology model of the *C. elegans* PMCT that we developed based on the crystal structure of the sodium-driven leucine transporter (see Materials and Methods). Like the leucine transporter, the PMCT is predicted to consist of 12 transmembrane domains. It is open on the extracellular face and closed on the cytosolic face. The model places four arginines in the transport path: R33 of TMD-1, R103 and R104 of TMD-3, and R274 of TMD-6. Docking calculations, which indicate that all four of these can simultaneously bind to a citrate molecule, has led to our proposal that these residues form the citrate binding site within the PMCT, as depicted in Figure 5, Panel A. In addition, the hydroxyl group of citrate is in position to hydrogen bond to the -SH of C412 which may also comprise part of this site. Comparison of this PMCT citrate binding site with the two substrate binding sites previously identified in the CTP (reference (32) and Figure 5, Panels B and C) shows substantial differences in the number and...
arrangement of positively charged sidechains and in the overall shape of the binding pocket. In PMCT, citrate interacts with 4 arginines; in CTP site 1, it binds to 2 arginines and 1 lysine; in CTP site 2 it binds to 3 arginines and 2 lysines.

**Correlation of Inhibitor Binding with Transporter Structure**

It is notable that compound 339393, which inhibits only PMCT, differs by only the removal of a single methylene group from 4180643, which inhibits both transporters. We have modeled 4180643 in the active site of CTP (Figure 6, Panel A) and find that the carboxylate attached to the pyrrole nitrogen can interact favorably with both K83 and R87. The corresponding (shortened) substituent in 339393 (Panel B) could still bind to K83 but would be unable to reach R87, which would account for its decreased inhibitory activity with the CTP.

The PMCT inhibitor 39396 is a hydrophobic molecule having only one hydrogen bond donor and two acceptors. It is a non-competitive inhibitor (Figure 3), indicating it does not directly compete for the substrate binding site. It is noteworthy that the LeuT crystal structure, which we used as a template, includes both a bound substrate molecule (deep in the transport pathway) and a non-competitive inhibitor, imipramine (nearer the extracellular surface of the pathway) (37). Our PMCT model has a number of hydrophobic residues in the area corresponding to the imipramine site of LeuT. Future modeling efforts will investigate this outer region as a possible site for non-competitive inhibitor binding.

**Concluding Remarks**

An important application for transport inhibitors is in efforts to grow crystals of these transporters for X-ray diffraction studies. The structure determination of the mitochondrial ADP-ATP carrier (upon which our CTP model is based) (43) was facilitated by use of the inhibitor carboxyatractyloside, which locks the transporter into a single conformation. Thus, a goal of our future studies will involve the use of identified inhibitors to enhance the crystallizability of PMCT. A second important application has to do with the likely
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Figure 5. Citrate interactions with PMCT and CTP binding sites. Protein backbones are shown as green ribbons. Nitrogen atoms are blue, oxygen atoms red, sulfur atoms yellow; transporter carbon atoms are green, and citrate carbon atoms are magenta. Panel A, In PMCT, citrate interacts with R33 of TMD1, R103 and R104 of TMD3, R274 of TMD6, and C412 of TMD10. Panel B, In site 1 of CTP, citrate interacts with K83 and R87 of TMD2 and with R189 of TMD4. Panel C, In site 2 of CTP, citrate interacts with K37 of TMD1, R181 of TMD4, K239 of TMD5, and R276 and R279 of TMD6.

Figure 6. Inhibitors modeled in the CTP transport path. Panel A, Compound 4180643, a significant CTP inhibitor, interacts with K83 and R87 of TMD2, K134 of TMD3, and K239 of TMD5. Panel B, Compound 339393, a much less effective inhibitor of CTP, has a shorter substituent on the pyrrole-nitrogen; its carboxylate can still interact with K83, but is unable to reach R87 on TMD2.

significance of the PMCTs in human physiology and pathology. Thus, eventual development of PMCT inhibitors that display high specificity and potency may enable a novel approach to the pharmacologic amelioration of metabolic disorders resulting from the synthesis of excess lipid, cholesterol, and glucose, including human obesity, hyperlipidemia, hypercholesterolemia, and Type 2 diabetes. Along these lines, the discovery of initial PMCT inhibitors and the development of initial testable models for PMCT structure including the composition of the translocation pathway and the substrate binding site represent the first steps toward our long-term goal of designing small molecules that can effectively inhibit PMCT function.

Acknowledgements
This work was supported by National Institutes of Health Grant GM-054642 to R.S.K.

Conflicts of Interest
No potential conflicts of interest to disclose.

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