Inhibitors of the Mitochondrial Citrate Transport Protein: Validation of the Role of Substrate Binding Residues and Discovery of the First Purely Competitive Inhibitor

Sreevidya Aluvila, Jiakang Sun, David H.T. Harrison, D. Eric Walters, and Ronald S. Kaplan

Department of Biochemistry and Molecular Biology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064
Running title: Inhibitors of the Mitochondrial Citrate Transport Protein

Corresponding author: Ronald S. Kaplan, Department of Biochemistry and Molecular Biology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064, telephone 847-578-8840, fax 847-578-3240, email ronald.kaplan@rosalindfranklin.edu

Number of text pages: 31

Number of tables: 1

Number of figures: 7

Number of references: 27

Number of words in Abstract: 229

Number of words in Introduction: 551

Number of words in Discussion: 1497

List of nonstandard abbreviations: BTC, 1,2,3-benzenetricarboxylate; ZINC compound 792949, 4-chloro-3-[(3-nitrophenyl)sulfamoyl]benzoic acid; CTP, citrate transport protein; $K_{iC}$, competitive inhibition constant; and $K_{iU}$, uncompetitive inhibition constant.

Supplemental Data: 3 Figures + appropriate legends; Total of 4 pages.
Abstract

The mitochondrial citrate transport protein (CTP) is critical to energy metabolism in eukaryotic cells. We demonstrate that 1,2,3-benzenetricarboxylate (BTC), the classical and defining inhibitor of the mitochondrial CTP, is a mixed inhibitor of the reconstituted Cys-less CTP, with a strong competitive component (i.e., a competitive inhibition constant, $K_{ic}$ of 0.12 ± 0.02 mM and an uncompetitive inhibition constant, $K_{iu}$ of 3.04 ± 0.74 mM). Based on docking calculations, a model for BTC binding has been developed. We then determined the $K_{ic}$ values for each of the eight substrate binding site cysteine substitution mutants and observed increases of 62-261-fold relative to the Cys-less control, thereby substantiating the importance of each of these residues in BTC binding. Importantly, we observed parallel increases in the $K_m$ for citrate transport with each of these binding site mutants, thereby confirming that with these CTP variants $K_m$ approximates the $K_d$ (for citrate) and therefore is a measure of substrate affinity. To further substantiate the importance of these binding site residues, in silico screening of a database of commercially available compounds has led to discovery of the first purely competitive inhibitor of the CTP. Docking calculations indicate that this inhibitor spans and binds to both substrate sites simultaneously. Finally, we propose a kinetic model for citrate transport where the citrate molecule sequentially binds to the external and internal binding sites (per CTP monomer) prior to transport.
Introduction

Citrate is a key intermediate at the intersection of carbohydrate and lipid metabolism. Within the mitochondrion, it is part of the TCA (Krebs) cycle; in the cytoplasm, it can be broken down to acetyl CoA in order to support fatty acid, lipid, and cholesterol biosyntheses (Spencer and Lowenstein, 1962; Srere and Bhaduri, 1962; Greville, 1969; Watson and Lowenstein, 1970). The mitochondrial citrate transport protein (CTP) is an obligate antiporter that mediates the exchange of the dibasic form of tricarboxylic acids (e.g., citrate or isocitrate) either for each other in yeast (Kaplan et al., 1995; Xu et al., 2000), or for dicarboxylic acids (e.g., malate, succinate) or phosphoenolpyruvate in higher eukaryotes (Robinson et al., 1971a,b; Palmieri et al., 1972).

Studies of the structure and function of membrane transporters are greatly facilitated by the availability of potent and specific inhibitors. In the case of the mitochondrial ADP/ATP carrier, which is a member of the same family of transport proteins as the CTP, inclusion of an inhibitor facilitated crystallization and subsequent X-ray crystallographic structure determination (Pebay-Peyroula et al., 2003). However, for the CTP, there are only a few such inhibitors known, and their potency is quite low. The best inhibitor available, 1,2,3-benzenetricarboxylate (BTC), was reported to be a competitive inhibitor, with Ki values ranging from 0.07 - 0.16 mM, and IC50 values of 3 - 8 mM (Robinson, 1971; Robinson et al., 1971a,b; Palmieri et al., 1972; Bisaccia et al., 1990). It should be noted that: i) none of these studies were conducted with the yeast mitochondrial CTP (i.e., the subject of the present investigations); and ii) often the data that led investigators to conclude a competitive model for BTC inhibition were not shown (Palmieri et al., 1972; Bisaccia et al., 1990).

We have previously used homology modeling to produce a model of the mitochondrial CTP (Walters and Kaplan, 2004). Using a combination of docking calculations and analysis of the
transport kinetics of single point mutations, we have identified two substrate binding sites in this transporter in its cytosolic-facing conformation (Ma et al., 2007) as depicted in Figure 1. Here we describe a number of studies of CTP inhibition. First, we examined the kinetic mechanism of the BTC-mediated inhibition of the yeast mitochondrial Cys-less CTP and found that BTC is a mixed inhibitor with a strong competitive component that affects not only the apparent Michaelis constant ($K_m$) for citrate, but also the apparent maximal transport velocity ($V_{max}$), albeit to a substantially lesser degree. Second, we have measured the competitive binding constant of BTC with previously described (Ma et al., 2007) single point variants of the substrate binding site residues. The results from these studies verify our previous contention that $K_m$ is for the most part related to the $K_d$ for citrate in these variants. Finally, we have utilized our homology model in a high throughput in silico screen of the ZINC database of commercially available compounds (Irwin and Shoichet, 2005), and we have identified a competitive inhibitor that displays a slightly higher affinity for the CTP than BTC. Importantly, in contrast to BTC or citrate, the size, shape, and hydrogen bonding potential of the newly identified inhibitor suggests that it simultaneously spans both of the previously identified citrate binding sites. These results are combined to present a kinetic model for the CTP transport cycle.
Materials and Methods

Construction, overexpression, isolation, and incorporation of Cys-less and single-Cys CTP variants into liposomal vesicles. Single-Cys CTP mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) as previously described (Ma et al., 2004). The Cys-less yeast mitochondrial CTP gene in pET-21a(+) was utilized as the starting template (Xu et al., 2000). Mutations were confirmed by sequencing both strands of the entire CTP open reading frame. BL21(DE3) growth and induction of CTP expression with IPTG were conducted as previously detailed (Kaplan et al., 1995; Xu et al., 1995). Two hours following induction, cells were harvested, lysed, and the inclusion body fraction was purified by centrifugation in a sucrose gradient (Kaplan et al., 1995; Xu et al., 1995). CTP mutants were then solubilized from the inclusion body pellet with 6 ml of ice-cold 1.2% (w/v) sarkosyl. Following centrifugation at 314,000×g for 45 min, the supernatant contained a given solubilized CTP mutant. Each mutant was then incorporated into liposomal vesicles, in the presence of 48–75 mM citrate, via the freeze-thaw sonication procedure as previously described (Kaplan et al., 1990, 1995). Except for the Cys-less CTP, all other single-Cys mutants were reconstituted with 75mM citrate inside the liposome. Immediately prior to the transport assay, a given sample was thawed, sonicated on ice, and the extraliposomal citrate was removed via chromatography on a Dowex column (Ma et al., 2007). The sample was immediately assayed for transport. Protein was quantified via the method of Kaplan and Pedersen (1985).

Characterization of the Type of Inhibition of the Cys-less CTP Mediated by BTC and the ZINC compound 792949 and Determination of Inhibition Constants. We routinely work with a Cys-less form of the yeast mitochondrial CTP which, importantly, displays native-like functional properties (Xu et al., 2000). The type of inhibition of the Cys-less CTP mediated by BTC and ZINC compound, 792949 (i.e., 4-chloro-3-[(3-nitrophenyl)sulfamoyl]benzoic acid) and
the inhibition constants were determined as follows. Proteoliposomes (585 µl) were incubated with 45 µl of either buffer (experimental incubation) or BTC (Sigma Aldrich) (control incubation) for 10 min at 21°C. Transport reactions (13-50 sec) were then triggered by adding 49 µl of the above reaction mix to 25 µl of [1,5-14C] citrate (GE Biosciences; 0.05-14 mM; specific radioactivity = 43-430 ×10^2 cpm/nmol) plus varying concentrations of BTC (0–1.5 mM; Sigma Aldrich) or varying concentrations of compound 792949 (0-2mM; Chembridge). Experimental incubations were quenched by the addition of 3.5 µl of 200 mM BTC, whereas control incubations received an equal volume of buffer. All transport incubations contained a final concentration of 9 mM BTC. Following transport, intraliposomal radiolabeled citrate was separated from the external radiolabel via chromatography on 2.6 ml of Dowex resin in Biorad support columns. The eluted intraliposomal radiolabel was quantified via liquid scintillation counting. The BTC-sensitive transport rate was calculated by subtracting the control value from the experimental value.

The data were then analyzed via Global fit analysis and Lineweaver-Burk (1/v versus 1/S) plots, in which v corresponds to the rate of a given transport reaction. In the case of BTC inhibition, both the $K_{ic}$ (the competitive inhibition constant) and $K_{iu}$ (the uncompetitive inhibition constant) values were calculated using the Global Fit method in the GraphPad Prism program (GraphPad Software, version 4, La Jolla, CA). In this method the observed rate vs [S] data (in the presence and absence of inhibitors) can be fitted simultaneously according to the full non-linear expression for mixed inhibition $v = V_{max} [S]/(K_m(1+[I]/K_{ic}) + [S] (1+ [I]/K_{iu}))$ (Cortes et al., 2001). But in the case of ZINC compound, 792949 inhibition, the $K_{ic}$ value was calculated using the global fit method using the non-linear expression for competitive inhibition $v=V_{max} [S]/(K_m(1+[I]/K_{ic}) + [S])$.

**Modeling and in silico screening of potential inhibitors.** High throughput in silico screening was carried out using version 5 of the DOCK software package (Shoichet et al., 1992).
following residues of the mitochondrial citrate transporter were previously identified as key components of substrate binding sites (Ma et al., 2007) and were used to define the target site for the DOCK calculations: lysine 83 and arginines 87 and 189 from site 1, and lysines 37 and 239 and arginines 181, 276, and 279 from site 2.

The ZINC database is comprised of millions of commercially available compounds. We pre-screened the database, as follows. First, we excluded compounds with a calculated partition coefficient above 5.0, since these tend to have poor water solubility. We also excluded compounds with molecular weight over 500, since larger molecules are less likely to fit into citrate binding sites. Finally, we considered only compounds that have net charge of 0, -1, -2, or -3, since the transport pathway has an excess of positively charged side chains.

The output of the initial DOCK screening is a set of the 500 best-scoring compounds, docked into the modeled protein. From the initial set of 500 hits, we purchased and tested 30 compounds. These were selected as follows. First, we visually inspected each hit as it was placed in the transporter model by the DOCK program. We discarded compounds that did not make at least three favorable interactions with the protein that were well separated spatially. Next, we observed that many of the hits could be grouped into clusters having common core structures; we chose the best fitting 1 or 2 analogs for each cluster, in order to have as much diversity as possible in our test set.

Testing of these 30 compounds identified 4 compounds with inhibitory activity (i.e. $\geq 50\%$ inhibition at a concentration of 1 mM). These 4 compounds were used as the basis for a similarity search of the ZINC database, to identify a second set of 13 compounds for testing. We identified 2 actives in this group which were then used for another round of similarity searching, and an additional 16 compounds were identified and tested and resulted in 4 actives. Thus, in total 10 active compounds were identified. Similarity searching was carried out using the Cactvs
algorithm (Ihlenfeldt et al., 2002) to calculate a Tanimoto similarity score; compounds with
greater than 80% Tanimoto similarity were considered for testing.

Both BTC and the most potent inhibitor discovered by screening (i.e., compound 792949)
were docked into the modeled transporter, and positions were optimized by side chain rotation
and energy minimization, using the Molecular Operating Environment software, version 2008.10
(Chemical Computing Group, Montreal).

**Inhibition of the Cys-less CTP by compounds identified from the ZINC database.** The
inhibition of the Cysless CTP by BTC and potential ZINC inhibitors were characterized as
follows. Proteoliposomes (48 µl) were pre-incubated with 3.5 µl of either buffer (experimental
sample) or 200 mM BTC (control sample) for 12 min at 21ºC and were then further incubated
with 0.5 µl of DMSO (dimethyl sulfoxide) or buffer or varying concentrations of BTC (0.05-
5.5 mM) or potential ZINC inhibitors (0.1 µM-1 mM) for 12 min. The ZINC inhibitors were
prepared freshly each day in DMSO (mostly) or buffer. Transport was initiated by the addition of
21.5 µl of 3.6 mM of [1,5-14C] Citrate (GE Biosciences; Specific radioactivity=24 x 10^3
cpm/nmol) and the uptake time was 25 s. Experimental samples were quenched by the addition
of 3.5 µl of 200 mM BTC, whereas the control samples received an equal volume of buffer.
Following transport, intraliposomal radiolabeled citrate was separated from the external
radiolabel via chromatography on short (i.e. 4 cm) Dowex columns in Pasteur pipettes. The
eluted intraliposomal radiolabel was quantified via liquid scintillation counting. The BTC-
sensitive transport rate was calculated by subtracting the control value from the experimental
value. The effect of BTC or a given inhibitor compound on citrate transport is expressed as the
percentage inhibition of the initial BTC-sensitive citrate uptake (measured in the presence of
buffer or DMSO). This was calculated by: i) determining the BTC-sensitive transport rate via
subtraction of the control value from the experimental value, in the absence of the inhibitor; ii)
subtracting the control value from the experimental value obtained in the presence of varying
concentrations of the inhibitor; iii) determining the ratio of this difference to the uninhibited BTC-sensitive citrate transport rate; and iv) application of the formula \((1 - \text{ratio}) \times 100\).

**Determination of the Kinetic Parameters of CTP Variants.** The kinetic parameters \((K_m\) and \(V_{max}\)) of the Cys-less and the single-Cys CTP variants were determined as follows. Proteoliposomes (45 µl) were preincubated with 5-7 µl of either buffer (experimental sample) or 1M BTC (control sample) for 10-12 min and were then further incubated with or without 2 µl of deionized/distilled water for an additional 10-12 min. The transport reaction was triggered via the addition of 21.5 µl of varying concentrations of [1,5-\(^{14}\)C]citrate (GE Biosciences, 0.05-75 mM, specific radioactivity=28-220 x 10\(^2\) cpm/nmol). In order to enable construction of a stock solution with increased specific radioactivity, the [\(^{14}\)C]citrate source had been concentrated approximately 22-fold by evaporation under vacuum. Following transport incubations that ranged from 13 s to 2.75 h (depending on the intrinsic activity of a given CTP mutant), the experimental sample was quenched by the addition of 5-7 µl of 1M BTC. The control sample received an equal volume of buffer. Transport reactions were conducted at room temperature (21°C). All transport incubations contained a final concentration of 75 mM BTC. Intraliposomal radiolabeled citrate was then separated from the external radiolabel via chromatography on 2.6 ml of Dowex resin in Biorad support columns. The eluted (i.e. intraliposomal) radiolabel was quantified via liquid scintillation counting. Importantly, a much higher level of BTC was utilized in these experiments than previously (i.e., 75 mM versus 9 mM in Ma et al., 2007) in order to more completely quench the transport reaction catalyzed by the single-Cys substitution mutations. Also, an increased quantity of Dowex resin was used to ensure efficient capture of all extraliposomal citrate even at the high substrate concentrations employed. In combination, these changes resulted in a more accurate determination of the \(K_m\) and \(V_{max}\) values of the CTP binding site mutants (See Supplementary Data, Figure 1) than previously reported (Ma et al, 2007). The BTC-sensitive transport rate was then calculated by subtracting the control value from the
experimental value. The rate of uptake versus substrate concentration curves were fitted to the Michaelis-Menten equation \( v = V_{\text{max}} \times S/(K_m + S) \), using a non-linear least squares curve fit in GraphPad Prism. The final \( K_m \) and \( V_{\text{max}} \) values for each single-Cys CTP mutant were calculated by taking the mean of the best fit \( K_m \) and \( V_{\text{max}} \) values derived from each separate \( v \) versus \( S \) profile.

**Determining the \( K_{ic} \) of the cysteine substitution substrate binding site mutants for BTC.**

The competitive inhibition constant (\( K_{ic} \)) of the substrate binding site mutants of CTP was determined as follows. Proteoliposomes (585 µl) were incubated with 91 µl of either buffer (experimental incubation) or BTC (control incubation) for 10 min at 21°C. Transport reactions (5-225 min) were then triggered by adding 52 µl of the above reaction mix to 26 µl of \([1,5-^{14}C]\) citrate (0.1-25 mM; specific radioactivity = 25-2370 ×10^2 cpm/nmol) plus water or varying concentrations of BTC (0–50 mM). It should be noted that prior to the transport experiments, the \([^{14}C]\)citrate source had been concentrated approximately 22-fold by evaporation under vacuum in order to enable construction of a stock solution with increased specific radioactivity. Experimental incubations were quenched by the addition of 7 µl of 780 mM BTC, whereas control incubations received an equal volume of buffer. Transport incubations contained a final concentration of 64 mM BTC. Following transport, intraliposomal radiolabeled citrate was separated from the external radiolabel via chromatography on 2.6 ml of Dowex resin in Biorad support columns. The eluted intraliposomal radiolabel was quantified via liquid scintillation counting. The BTC-sensitive transport rate was calculated by subtracting the control value from the experimental value.

The data were then analyzed as follows. While it was possible to bracket the \( K_m \) for the binding site mutants in the absence of inhibitor, it was not practical to do so in the presence of the BTC inhibitor. At three relatively low concentrations of citrate (below \( K_m \)) for each variant, BTC was varied (i.e., at least 9 concentrations of BTC were tested) and velocity data obtained.
For each mutant at each concentration of inhibitor, the reciprocal slope of a two or three point \( v \) versus \( S \) plot was recorded, fixing the \( x \)- and \( y \)-intercepts to zero. Where significant curvature was found, the high citrate concentration point was excluded from the slope analysis. From this reciprocal slope, \( K_m/V_{\text{max}} \) apparent was estimated and plotted against the BTC concentration (See Supplementary Data, Figure 2). It can be shown that the \( x \)-intercept from this second graph \((K_m/V_{\text{max}})_{\text{app}} \text{ vs. } [\text{BTC}]\)) is equal to the competitive inhibition constant, \( K_{ic} \).
Results

Characterization of the Kinetic Mechanism of the BTC-Mediated Inhibition of the Cys-less CTP.

BTC is considered the classical and defining inhibitor of the mitochondrial inner membrane CTP (Robinson et al., 1971a,b; Palmieri et al., 1972). In the present studies we characterized the kinetic mechanism by which BTC inhibits the yeast mitochondrial Cys-less CTP following its overexpression in *E. coli*, solubilization, and functional reconstitution in a well-characterized liposomal system (Kaplan et al., 1990; Xu et al., 2000). BTC-mediated inhibition of the Cys-less CTP was examined by measuring the [14C]citrate uptake rate in the presence of several different citrate and BTC concentrations. The data were analyzed via both a Lineweaver-Burk Plot and a $v$ vs. $S$ plot (Figure 2). As depicted in Figure 2, Panel A, the Lineweaver-Burk plot indicates that within the range of BTC concentrations tested, the $K_m$ increases by over 1200% and the $V_{max}$ decreases by 24% as the concentration of BTC is increased from 0 to 1.5 mM. The observation that the plotted lines at increasing BTC levels intersect, but do not do so at either the x- or y-axes (see inset in Panel A), indicates that a mixed inhibition pattern is observed. This conclusion is further supported by our observation in Figure 6C that a replot of the y-intercepts (i.e., $1/V_{max}$ values) depicted in Figure 2A versus BTC concentration yields a line with a positive slope, thereby clearly indicating that BTC is not a purely competitive inhibitor. Thus, we conclude that BTC is a mixed inhibitor of the CTP, with a predominantly competitive component. This type of inhibition pattern is further supported by analysis of the data via the Dixon and the Cornish-Bowden plots (data not shown).

As depicted in Figure 2, Panel B, the $v$ versus $S$ data were then analyzed via Global fit analysis for mixed inhibition enabling calculation of both the $K_{ic}$ (the competitive inhibition constant) and $K_{iu}$ (the uncompetitive inhibition constant) values. The competitive inhibition constant, $K_{ic}$ is $0.12 \pm 0.02$ mM and the uncompetitive inhibition constant, $K_{iu}$ is $3.04 \pm 0.74$.
Importantly, the $K_{iu}$ is 25-fold higher than the $K_{ic}$ value, confirming that BTC is primarily, but not exclusively a competitive inhibitor of the Cys-less CTP.

**Proposed Modes of BTC Binding to the CTP.** Since the CTP transport path has numerous arginine and lysine side-chains, and BTC has three carboxylate groups, it is not surprising that there are numerous possible ways to dock BTC. We have previously identified two substrate binding sites in the CTP (Ma et al., 2007). When we carried out the computational docking of BTC to our model of the CTP structure, we observed a large cluster of docking poses with BTC in the second (deeper) substrate binding site, and a smaller cluster of docking poses with BTC in the first substrate binding site. Representative configurations are shown in Figure 3. Figure 3A shows that ion pair interactions form between the BTC carboxylate groups and the side chains of Lys83, Arg87, and Arg189; all of these were previously identified as part of substrate binding site 1. As illustrated in Figure 3B, the BTC carboxylates can interact with the side chains of Lys37, Lys134, Arg181, Lys239, and Arg276; all of these except Lys134 were previously identified as components of substrate binding site 2. It is noteworthy that we have previously hypothesized that Lys134 resides in close juxtaposition to site 2, such that it is capable of forming an ionic hydrogen bond to the C5-COO$^-$ of citrate (Ma et al., 2007).

Our proposed BTC binding modes garner further support by the experimental results depicted in Table 1. Since BTC is a mixed inhibitor of the Cys-less CTP with a strong competitive component ($K_{ic} = 0.12 \pm 0.02$ mM and $K_{iu} = 3.04 \pm 0.74$ mM) and since both citrate and BTC are structurally very similar (see Supplementary Data Figure 3, Panel B), we assume that the two ligands mainly bind to the CTP at the same sites. Consequently, if each of the substrate binding site residues plays a prominent role in binding BTC, then we would expect to observe an increase in the $K_{ic}$ value for BTC with each of the binding site mutants when compared to the Cys-less CTP control. We tested this notion experimentally by measuring the $[^{14}C]$citrate transport rate in the presence of varying citrate and inhibitor concentrations. For each inhibitor
concentration, v versus S data were used to calculate \( \frac{K_m}{V_{max}} \) apparent. Replots of \( \frac{K_m}{V_{max}} \) apparent vs. [Inhibitor] were constructed in order to determine the \( K_{ic} \) value of each mutant (note: the x-intercepts in these plots correspond to the \( K_{ic} \) value; see Supplementary Data, Figure 2). As depicted in Table 1, we observed a 62-261-fold increase in the \( K_{ic} \) values of the binding site mutants for BTC. Furthermore, since each of the single-Cys binding site mutants displayed a large increase in their apparent \( K_m \) values compared to the Cys-less control (i.e., 4-50 fold increases; see Table 1 and Supplementary Data, Figure 1), and a significant reduction in their \( V_{max} \) values and catalytic efficiencies (See Supplementary Data, Figure 1; and Ma et al., 2007), we infer that with each of the binding site mutants the \( k_2 \) (\( k_{cat} \)) for transport is severely depressed and therefore \( K_m \) approximates \( K_d \). This hypothesis is consistent with the observation that the increases in the binding site single-Cys mutant \( K_{ic} \) values for BTC parallel the observed increases in the \( K_m \) values for these mutants, thereby supporting our contention that with these mutants the \( K_m \) values approximate the \( K_d \) values and therefore primarily reflects the affinity of the transporter for substrate.

Figure 4 displays the effect of a given binding site mutation on the CTP’s relative binding energy for BTC compared with that of the Cys-less CTP (i.e., \( \Delta \Delta G \)) versus the effect of a given mutation on the \( K_m \) for citrate transport (i.e., \( \log \frac{K_m'}{K_m} \), where \( K_m' \) is the Michaelis constant for a given CTP binding site mutant and \( K_m \) is the constant for the Cys-less control). Since binding energy is related to the log of the dissociation constant by the expression \( \Delta G = -2.303RT\log K \) and since the individual binding energies between each binding site residue and a given ligand are expected to add to the total binding site free energy, one expects a constant relationship between the \( \Delta \Delta G \) for BTC and the logarithm of the relative \( K_m \) for citrate if \( K_m \) in fact approximates \( K_d \). As depicted in Figure 4, we observed that 6 of the 8 binding site mutants reside on the best fit line, thereby indicating a strong correlation between the binding energy of a particular residue for both BTC and citrate. We observe that binding site mutant R276C lies above the best fit line.
indicating a greater than expected contribution of this residue to BTC binding, whereas one binding site mutant (i.e., K239C) lies to the right of the best fit line suggesting a stronger than expected contribution of this residue to the $K_m$ for citrate transport. Interestingly, each of these two residues is located within binding site 2. The observation that the Cys-less CTP resides significantly to the right of the line is readily explained by the contribution of $k_{cat}$ towards the $K_m$ value, in contrast to all of the single Cys binding site mutants that display $k_{cat}$ values that are dramatically reduced.

**Identification of new CTP inhibitors.** We next proceeded to identify new inhibitors of the CTP via *in silico* screening of the ZINC database which, at the time of these studies, consisted of > 3 million commercially available small molecules (see “Materials and Methods”). An initial set of 30 compounds were purchased and tested. This was followed by two additional rounds of searching the database for compounds similar to the initial sets of active inhibitors. In total, 59 compounds were experimentally tested for inhibition of citrate transport. Ten of these produced at least 50% inhibition when tested at 1 mM. For comparison, in this assay, BTC produced 79% inhibition at this concentration. The active inhibitors identified are shown in Figure 5, along with their ZINC database identifier codes and their % inhibition at 1 mM. The most potent inhibitor, compound 792949, produced 85% inhibition of citrate transport.

**Inhibition Mechanism of Compound 792949.** We characterized the kinetic mechanism by which compound 792949 inhibits the yeast mitochondrial Cys-less CTP by measuring the transport rate at a variety of different citrate and inhibitor concentrations. As depicted in Figure 6 the data were analyzed via both a Lineweaver-Burk Plot and a $v$ vs. $S$ plot. The Lineweaver-Burk plot (Panel A) indicates that with the range of inhibitor concentrations tested, the apparent $K_m$ increases (38-fold) whereas no significant change in the apparent $V_{max}$ was observed (see inset in Figure 6A and y-intercept replot in Panel C). The observation that for 792949 the best-fit lines in Panel A display essentially the same y-intercept is evidence of a purely competitive inhibitor.
Based on a Global Fit analysis (Figure 6, Panel B), the competitive inhibition constant, $K_{ic}$ is 0.048 ± 0.007 mM. Importantly, as depicted in Figure 6, Panel C a re-plot of the y-intercepts obtained in Panel A as a function of the 792949 inhibitor concentration yield a line whose slope ($2.1 ± 3.2 \times 10^{-5}$) does not significantly deviate from 0 (shown by the red dashed line), thus providing compelling graphical evidence that this inhibitor is purely competitive. For comparison, we also depict a re-plot of the data obtained with BTC (from Figure 2, Panel A). In this case it is evident that the slope of the re-plotted line ($14.2 ± 1.8 \times 10^{-5}$) is non-zero, thus clearly indicating a mixed inhibition pattern.

Docking of compound 792949 into the CTP homology model (Figure 7) produced a configuration in which the nitro-group forms ionic hydrogen bonds with Lys83 and Arg87 of binding site 1; the sulfonamide group forms two ionic hydrogen bonds to Arg181 of binding site 2; and the carboxylate group forms ionic interactions with Lys37, Arg276, and Arg279 of binding site 2. In addition, the nitro-substituted ring has hydrophobic interaction with Leu120, and the chloro-substituted ring has hydrophobic interaction with Phe76 (not shown in Figure 7). Thus, compound 792949 is capable of spanning portions of substrate binding sites 1 and 2, an observation in line with the fact that this inhibitor is about double the length of either citrate or BTC (Supplementary Date Figure 3, Panel A).
Discussions

The present investigations characterize CTP inhibitors and their effects on the kinetics of substrate binding site cysteine substitution mutants, and have resulted in several novel findings that are critical to understanding the mechanism of this metabolically important transporter. First, upon reconstitution of the Cys-less CTP variant in liposomal vesicles (which we have previously demonstrated displays native-like functional properties (Xu et al., 2000)), BTC, the defining inhibitor of the CTP, functions as a mixed inhibitor with a strong competitive component (Figure 2). Docking calculations conducted with BTC and our homology-modeled CTP structure (Figure 3) indicate that while BTC can bind to both substrate binding sites 1 and 2, it displays a clear preference for the second binding site. Second, with the eight residues that were previously identified as forming the two substrate binding sites within the CTP, we observed that increases in the $K_i$ of each mutant for BTC, relative to the Cys-less control, generally parallel the observed increases in the $K_m$ values of these mutants for citrate transport. This finding, in combination with our earlier finding that the $k_{cat}$ value for each of these mutants is dramatically reduced, lends strong support to our contention that with these single-Cys CTP variants the $K_m$ for citrate transport approximates the $K_d$ for citrate, and therefore primarily reflects the affinity of the transporter for substrate. Third, our results demonstrate the effectiveness of the application of DOCK-based high throughput in silico screening to a homology-based transporter model in facilitating the discovery of novel competitive inhibitors. The best inhibitor that we identified (i.e., compound 792949) has a slightly higher affinity for the CTP than does BTC and is a purely competitive inhibitor. Most importantly, in contrast to BTC, it is of sufficient length that it is predicted to bind to both of the citrate binding sites simultaneously, which may result in a CTP that is locked in a single conformation.
Since the transporter binding sites contain a number of positively charged side-chains, it is not surprising that the identified inhibitors contain one or two carboxylates. BTC is a relatively small molecule, and its interactions are primarily with positively charged residues in the second of two previously identified substrate binding sites. It is interesting to note that 7 of the 10 newly identified inhibitors from the ZINC database also contain a sulfonamide group which is often acidic (Scior et al., 1997). Furthermore, many of these compounds are of significantly greater length than BTC, thus raising the possibility that they may be able to bind to residues in both citrate binding sites simultaneously.

With the recent identification of two distinct citrate binding sites that reside within the hydrophilic transport pathway of the CTP in the cytoplasmic-facing conformation (Ma et al., 2007), a number of different kinetic mechanisms for transport can be proposed. One such mechanism would posit that two molecules of citrate are required to be bound prior to transport of the citrate bound at binding site 2 (the more interior binding site; See Figure 1). However, such a model gives rise to non-hyperbolic velocity vs. substrate concentration curves and parabolic Lineweaver-Burk curves, which are not seen in the wild-type (Xu et al, 2000), Cys-less, or other mutated forms of the transporter (See Supplementary Data, Figure 1). Alternatively, one might suppose that in the presence of a citrate molecule bound at site 2, the binding of a second citrate molecule to site 1 might actually inhibit the transport process. This would give rise to substrate inhibition and a checkmark appearance to the Lineweaver-Burk curve. There is no evidence of substrate inhibition even when citrate concentrations are greater than six times that of $K_m$. Thus, only mechanisms that bind and transport one citrate molecule at a time through one transport pathway/monomer should be further contemplated. The fact that cysteine substitution for each of the CTP binding site residues all give rise to transporters with highly elevated $K_m$ values can be best explained by a sequential model where citrate first binds to the outer binding site (site 1) with an equilibrium dissociation constant of $K_{d1}$ (for the reaction
Citrate·Site1 ↔ Citrate + Site1) and then to the inner binding site (site 2) with an equilibrium constant of $K_{d2}$ (for the reaction Citrate·Site2 ↔ Citrate·Site1) to give an overall binding constant of $K_d = K_{d1} \times K_{d2}$. This implies that a citrate molecule bound at site 2 is not able to directly dissociate back to the external milieu (i.e., the cytosol). Functionally, this model implies that: i) a single molecule of citrate moves sequentially from site 1 to site 2, but can not physically contact all of the residues in these two sites simultaneously; and ii) all 8 residues that comprise binding sites 1 and 2 contribute in an essential manner to citrate binding prior to the transport event. For these binding site variants, where $V_{max}$ is greatly reduced (See Supplementary Data, Figure 1), $K_m$ should approximate $K_d$. This assumption was shown to be substantially correct by the nearly linear relationship across all the cysteine variants between the change in the binding energy ($\Delta G$) of the inhibitor BTC relative to that of the Cys-less control ($\Delta \Delta G$) and the log of the ratio of the $K_m$ of the variants to the $K_m$ of the Cys-less control transporter (See Figure 4). If $K_m$ had a substantial contribution from $k_{cat}$, we would expect a great deal more scatter in this plot. The observation that six of the variants essentially lie on the best fit line strongly suggests that the $K_m$ for citrate transport closely approximates the $K_d$ for citrate binding. It should be pointed out that the K239C variant not only significantly lies to the right of the best fit line but also is the variant with the highest $V_{max}$, suggesting that for this mutant, $K_m$ has a significant $k_{cat}$ component. The unusually high BTC binding constant for the R276C variant can be understood as this residue having a more significant role in BTC binding than citrate binding. Importantly, both of these residues reside within binding site 2, which modeling studies suggested to be the favored BTC binding site.

A number of different models for BTC binding can be proposed to explain how BTC, a molecule very similar in structure to citrate, causes mixed inhibition (Figures 2A&B and 6C) rather than purely competitive inhibition as has been assumed for more than 35 years (Robinson et al., 1971; Robinson et al., 1971a,b; Palmieri et al., 1972; Bisaccia et al., 1990). While the
competitive (and more pronounced) aspect of this inhibition is obviously due to the similarity in structure of BTC to that of citrate and the fact that its two binding sites overlap with the two citrate binding sites in the modeled cytosolic-facing conformation, the uncompetitive aspect of BTC inhibition may provide some clues about the mechanics of citrate transport. To understand the uncompetitive component of BTC inhibition one may posit that BTC binds to site 1 when the transporter is in its alternative matrix-facing conformation (not modeled). BTC binding might stabilize the matrix-facing conformation and decrease the rate at which CTP progresses through its transport cycle. By interfering with the conformational cycling, \( V_{\text{max}} \) would decrease. We further posit that external citrate, perhaps due to its hydrophilicity, is not capable of binding to this matrix-facing conformation of site 1 as evidenced by the lack of substrate inhibition. Importantly, this mechanism would hold true whether CTP functions as a monomer or a homodimer, an issue that is controversial (Kotaria et al., 1999; Palmieri et al. 1992; Bamber et al., 2007). This interference in the transport cycle could manifest itself in the context of either a single monomer or as part of a coordinated dimeric structure where one protomer is in the cytosolic-facing conformation and the other is in the matrix-facing conformation. The presence of BTC in site 1 of the protomer in the matrix-facing conformation might limit the ability of the other protomer to change conformation.

From this analysis, strictly competitive inhibitors such as compound 792949 must only be capable of binding to CTP site 1 and/or site 2 in the cytosolic-facing conformation. Binding to other sites or site 1 in the matrix-facing conformation leads to an uncompetitive component in the inhibition pattern. In the case of 792949 itself, its lack of uncompetitive inhibition can be readily explained by the model whereby the molecule enters sites 1, proceeds to site 2, and in contrast to citrate then binds to residues from both sites simultaneously.
In conclusion, these studies have provided important insight into the mechanism of the mitochondrial CTP and have led to the development of a physical model explaining its modes of interaction with BTC. Additionally, utilizing high throughput in silico screening of the ZINC database with the homology-modeled CTP, we have discovered a novel inhibitor (i.e., compound 792949) that has a slightly higher affinity than BTC, is purely competitive, and likely spans both substrate binding sites. This inhibitor may prove to be an effective conformational-stabilizing tool in our CTP structural studies.
Acknowledgments

We thank Austin Kirschner, John J. Irwin, and Brian K. Shoichet for helpful discussions.
References


Footnotes

This work was supported by the National Institutes of Health [Grant GM-054642].

Address correspondence to: Dr. Ronald S. Kaplan, Department of Biochemistry and Molecular Biology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064. E-mail: ronald.kaplan@rosalindfranklin.edu
FIGURE LEGENDS

Figure 1. **Ribbon diagram of the homology modeled CTP structure in the cytosolic-facing conformation.** Portions of helix I and helix VI have been removed for clarity. Key residues of citrate binding sites 1 and 2 are shown as stick structures in red and blue, respectively. The approximate boundaries of the mitochondrial inner membrane are shown as magenta lines.

Figure 2. **Determination of kinetic mechanism and inhibition constants of BTC-mediated inhibition of the Cys-less CTP.** Panel A: Lineweaver-Burk plot depicting the effect of BTC on the citrate transport activity. The citrate/citrate exchange (0.1-10mM external/48mM internal) was determined in the absence (purple line) and in the presence of BTC (green, 0.157mM; blue, 0.524mM; and red, 1.5mM lines) as described under Materials and Methods. Non-linear regression analysis of $v$ vs. $[S]$ data was used to calculate $V_{\text{max}}$ (apparent) and $K_m$ (apparent) for each inhibitor concentration. These lines were then plotted on a double reciprocal plot. (Inset) – Showing the y-intercept ($1/V_{\text{max}}$) of each line. $V_{\text{max}}$ (apparent) decreases while $K_m$ (apparent) increases with increasing concentration of the inhibitor, BTC. Panel B: Global fit analysis of $v$ vs. $[S]$ data (in the presence and absence of inhibitors) fitted simultaneously according to the full non-linear expression for mixed inhibition.

Figure 3. **Important binding interactions for the docked conformations of BTC with the mitochondrial CTP model.** Top views, CTP backbone shown as green ribbon, with transmembrane domains identified using Roman numerals, with interacting side-chains shown as stick structures colored by atom type, and BTC shown as a stick structure with carbon atoms colored orange. Ion-pair interactions are shown using magenta dotted lines. Bottom, schematic representation, with ion-pair interactions shown as dotted lines. A, Binding of BTC in previously identified (Ma et al., 2007) citrate binding site 1 ; B, Binding of BTC in citrate binding site 2. Portions of helix VI in panel A, and helices I and II in panel B, have been removed for clarity.
Figure 4. Contribution of CTP binding site residues to the binding energy for BTC versus citrate. This figure plots the change in the free energy of BTC binding (ΔΔG) of the single-Cys binding site mutants relative to the Cys-less control versus the log of the ratio of the corresponding Km values for citrate transport. Data were extracted from Table 1.

Figure 5. Newly discovered inhibitors of the yeast mitochondrial citrate transporter. For each compound, the ZINC database ID number and the % inhibition at 1 mM concentration are listed.

Figure 6. Determination of kinetic mechanism and the competitive inhibition constant for ZINC compound 792949-mediated inhibition of the Cys-less CTP. Panel A: Lineweaver-Burk plot depicting the effect of 792949 on the citrate transport activity. The citrate/citrate exchange (0.05-14mM external/48mM internal) was determined in the absence (purple line) and in the presence of 792949 (green, 0.3mM; blue, 0.9mM; and red, 2mM lines) as described under Materials and Methods. Non-linear regression analysis of v vs. [S] data was used to calculate Vmax (apparent) and Km (apparent) for each inhibitor concentration. These lines were then plotted on a double reciprocal plot. (Inset) – Showing the y-intercept (1/Vmax) of each line. The Vmax (apparent) does not change with increasing concentrations of the inhibitor 792949 while Km (apparent) increases. Panel B: Global fit analysis of v vs. [S] data (in the presence and absence of inhibitor) fitted simultaneously according to the full non-linear expression for competitive inhibition. Panel C: Replot of 1/Vmax (apparent) versus inhibitor (I) concentration. The slopes of the 792949 and the BTC best fit lines are 2.1 ± 3.2 x 10⁻⁵ and 14.2 ± 1.8 x 10⁻⁵, respectively. The dashed lines depict an ideal competitive inhibitor (i.e., 0 slope) using the Vmax obtained in the absence of either 792949 (red dashed line) or BTC (blue dashed line). Note: the observed difference in 1/Vmax in the absence of inhibitor reflects differences in assay conditions and protein preparations.
Figure 7. **Important binding interactions for the docked conformation of ZINC compound 792949 with the mitochondrial CTP model.** Residues K83 and R87 are part of substrate site 1, and K37, R181, R276, and R279 are part of substrate site 2, as described previously (Ma et al., 2007). A, CTP backbone shown as a green ribbon, with transmembrane domains identified using Roman numerals, with interacting side-chains shown as stick structures colored by atom type, and 792949 shown as a stick structure with carbon atoms colored orange. Ion-pair and ionic hydrogen bond interactions are shown using magenta dotted lines. Portions of helices I and II have been removed for clarity. The inhibitor and important CTP side-chains are represented as stick structures, with N = blue, O = red, sulfur = yellow, chlorine = green; CTP carbon = gray, and inhibitor carbon = orange. B, Schematic representation with ion-pair and ionic hydrogen bond interactions shown as dotted lines.
Table 1

Effect of cysteine substitution mutation of the CTP substrate binding site residues on the $K_m$ for citrate transport and the $K_{ic}$ (the competitive inhibition constant) of BTC binding to CTP. Transport reactions and calculations of $K_m$ and $K_{ic}$ were conducted as described under “Materials and Methods”. $K_m$ values (± S.E.) were obtained from at least duplicate v versus [S] profiles employing at least 10 different substrate concentrations that bracketed the $K_m$ value. $K_{ic}$ values (± S.E.) were extracted from $(K_m/V_{max})_{app}$ versus [BTC] plots (see Supplementary Data, Figure 2).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$K_m$ (mM)</th>
<th>Fold $\uparrow$ in $K_m$ compared to Cys-less</th>
<th>$K_{ic}$ of BTC (mM)</th>
<th>Fold $\uparrow$ in $K_{ic}$ compared to Cys-less</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-less</td>
<td>0.62±0.04</td>
<td></td>
<td>0.124±0.015</td>
<td></td>
</tr>
<tr>
<td>Substrate binding site 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K83C</td>
<td>28.2±7.2</td>
<td>45</td>
<td>24.1±3.7</td>
<td>194</td>
</tr>
<tr>
<td>R87C</td>
<td>31.1±4.7</td>
<td>50</td>
<td>25.0±4.6</td>
<td>202</td>
</tr>
<tr>
<td>R189C</td>
<td>14.5±1.7</td>
<td>23</td>
<td>15.1±2.2</td>
<td>122</td>
</tr>
<tr>
<td>Substrate binding site 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K37C</td>
<td>2.3±0.6</td>
<td>4</td>
<td>7.7±0.8</td>
<td>62</td>
</tr>
<tr>
<td>R181C</td>
<td>25.8±5.2</td>
<td>42</td>
<td>15.9±2.5</td>
<td>128</td>
</tr>
<tr>
<td>K239C</td>
<td>30.9±10.9</td>
<td>50</td>
<td>13.2±1.2</td>
<td>106</td>
</tr>
<tr>
<td>R276C</td>
<td>16.4±3.3</td>
<td>26</td>
<td>32.4±5.4</td>
<td>261</td>
</tr>
<tr>
<td>R279C</td>
<td>19.5±5.4</td>
<td>31</td>
<td>18.6±2.4</td>
<td>150</td>
</tr>
</tbody>
</table>
Figure 2

Panel A: Lineweaver-Burk Plot

Panel B: Mixed Inhibition-Global Fit Analysis
Figure 3
Figure 4

![Graph showing the relationship between ΔΔG (Kcal/mol) and log (Km'/Km). The graph includes points for Cys-less, R87C, R276C, R279C, R189C, K239C, K83C, and R181C.]
Figure 5

- **792949** 85%
- **4180643** 79%
- **236104** 76%
- **854962** 67%
- **1571200** 63%
- **3884917** 62%
- **1049456** 56%
- **386175** 55%
- **4028828** 53%
- **3884698** 50%

These compounds represent different molecular structures with varying yields.
Figure 6

Panel A: Lineweaver Burk Plot

Inhibitor (mM)
- 0
- 0.3
- 0.9
- 2

Panel B: Competitive Inhibition-Global Fit Analysis

Panel C: Intercept Replot

Inhibitor (mM)
- 0
- 0.3
- 0.9
- 2
Figure 7

A. 

B.