The mitochondrial citrate transport protein: Evidence for a steric interaction between glutamine 182 and leucine 120 and its relationship to the substrate translocation pathway and identification of other mechanistically essential residues

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Abstract

Previous examination of the accessibility of a panel of single-Cys mutants in transmembrane domain III (TMDIII) of the yeast mitochondrial citrate transport protein to the hydrophilic, cysteine-specific methanethiosulfonate reagent MTSES enabled identification of the water-accessible surface of this TMD. Further studies on the effect of citrate on MTS reagent accessibility, indicated eight sites within TMD III at which citrate conferred temperature-independent protection, thus providing strong evidence for participation of these residues in the formation of a portion of the substrate translocation pathway. Unexpectedly, citrate did not protect against inhibition of the Leu120Cys variant, despite its location on a water- and citrate-accessible surface of the TMDIII helix. This led to the hypothesis that in the 3-dimensional CTP structure, TMDIV packs against TMDIII in a manner such that the Leu120 side-chain folds behind the side-chain of Gln182. The present investigations addressed this hypothesis by examining the properties of the Gln182Cys single mutant and the Leu120Cys/Gln182Ala double mutant. We observed that in contrast to our findings with the Leu120Cys mutant, citrate did protect the Gln182Cys variant against MTSES-mediated inhibition. Importantly, truncation of the Gln182 side-chain to Ala enabled citrate to protect the Leu120Cys double mutant against inhibition. In combination these data support the idea that the Gln182 side-chain lines the transport path and sterically blocks access of citrate to the Leu120 side-chain. In a parallel series of investigations, we constructed 24 single-Cys substitution mutants that were chosen based on their hypothesized importance in substrate binding and/or translocation. We observed that substitution of Cys for residues E34, K37, K83, R87, Y148, D236, K239, T240, R276, and R279 resulted in ≥98% inactivation of CTP function, suggesting an essential structural and/or mechanistic role for these native residues. Superposition of this functional data onto a detailed 3-dimensional homology model of the CTP structure indicates that the side-chains of each of these residues project into the putative transport pathway. We hypothesize that a subset of these residues, in combination with four previously identified essential residues, define the citrate binding site(s) within the CTP.

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1. Introduction

The mitochondrial citrate transport protein (i.e., CTP) resides within the inner mitochondrial membrane and in higher eukaryotes catalyzes an obligatory exchange of the dibasic form of a tricarboxylic acid (e.g., citrate, isocitrate, or cis-aconitate) for either another tricarboxylate, a dicarboxylate, or phosphoenolpyruvate [1]. The CTP plays a major role in intermediary metabolism since following the efflux of citrate...
from the mitochondrial matrix and subsequent passive diffusion across the outer mitochondrial membrane through VDAC, the resulting cytoplasmic citrate provides the prime carbon source supplying the fatty acid, triacylglycerol, and cholesterol biosynthetic pathways [2–5]. Furthermore, via the sequential action of citrate lyase and malate dehydrogenase, cytoplasmic citrate provides for the production of NADH, a cofactor that is required for continuation of glycolysis.

Due to the importance of the CTP, our laboratory has extensively investigated its structure/function relationships in order to understand the molecular mechanism underlying its function [6–8]. In recent years, we have focused our experimental efforts on the yeast mitochondrial CTP, since following high-level expression, purification and functional reconstitution in liposomes, the transporter displays a high specific transport activity [9]. Accordingly we have: (i) constructed a Cys-less form of the transporter which displays native functional properties [10]; (ii) used Cys-scanning mutagenesis combined with labeling the resulting single-Cys mutants with different probes to identify the residues within TMDs III and IV that form elements of the translocation pathway [11–13]; (iii) developed a detailed 3-dimensional homology model of the CTP and superimposed our functional data to delineate the translocation pathway [14]; and (iv) shown that in detergent micelles both the Cys-less and wild-type CTP exist as homodimers [15].

With this background in mind, the present investigations sought to address the following two issues. First, we examined our hypothesis that the reason for the unexpected inability of citrate to protect against MTSES-mediated inhibition of the Leu120Cys CTP variant [13], arises from steric blockage by the Gln182 side-chain which we proposed packs against the Leu120 side-chain and resides more proximal to the translocation pathway. Second, we examined the effect of Cys substitution at 24 locations, which were chosen based on their predicted importance in the CTP mechanism. In combination, the data obtained from these studies support our hypothesized Gln182–Leu120 steric interaction and indicate an important subset of residues that are essential for CTP function. Furthermore, superposition of this additional data onto the 3-dimensional CTP homology model provides new insight into the potential function of these essential residues in the CTP structure-based mechanism.

2. Materials and methods

2.1. Construction, overexpression, and isolation of CTP mutants

Single-Cys CTP variants were prepared exactly as previously described [12]. The Leu120Cys/Gln182Ala double mutant was prepared using the Leu120Cys CTP sequence in pET-21a(+) as the starting template during PCR amplification.

2.2. Incorporation of CTP mutants into phospholipid vesicles and determination of their kinetic properties

Overexpressed, detergent-solubilized and partially purified Cys-less and other CTP variants were reconstituted into liposomes via the freeze–thaw–sonication technique as previously described [6,9]. The kinetic properties (Km and Vmax) of the Cys-less, Gln182Cys, Leu120Cys, and Leu120Cys/Gln182Ala double mutant CTP variants were determined as previously detailed [13]. Briefly, immediately prior to the transport assay, a sample was thawed, sonicated on ice, and extraliposomal citrate was removed by chromatography on a Dowex column in a Pasteur pipette. Proteoliposomes (45 μl) were preincubated with 3.5 μl of either buffer [6] (experimental sample) or 200 mM BTC (control sample) for 10 min and were then further incubated with 3.5 μl of deionized/distilled water for an additional 10 min. The transport reaction was triggered via the addition of 21.5 μl of varying concentrations of [1,5-14C]citrate (Amersham Biosciences). Typically, 10 different citrate concentrations ranging from 0.08 to 2 mM, which bracket the Km value, were employed. Following transport incubations that ranged from 12 s to 2.5 min (depending on the intrinsic activity of a given CTP mutant), the experimental sample was quenched by the addition of 3.5 μl of 200 mM BTC. The control sample received an equal volume of buffer. All the transport reactions were conducted at room temperature (21 °C). Intraliposomal radiolabeled citrate was then separated from the external radiolabel via chromatography on small Dowex columns in Pasteur pipettes. The eluted (i.e. intraliposomal) radiolabel was quantified via liquid scintillation counting. The BTC-sensitive transport rate was calculated by subtracting the control value from the experimental value at each substrate concentration. For these studies, care was taken to ensure that initial rates and were measured.

2.3. Determination of the extent of substrate protection against MTSES-mediated inhibition of citrate transport

The assay for substrate protection against MTSES-mediated inhibition of citrate transport was carried out as follows. Proteoliposomes (125 μl) were incubated with water (10 μl) versus water (7 μl) plus MTSES (3 μl) versus varying concentrations of unlabeled citrate (7 μl) plus MTSES (3 μl) for a period of time (i.e., 30 s to 2 min) at room temperature (i.e., 21 °C). The concentration of MTSES and the length of the incubation interval were determined by an initial series of experiments with each CTP variant. When present, citrate was added immediately prior to MTSES. Following incubation with MTSES with or without citrate, the reactions were stopped via the addition of 6 ml of ice-cold buffer (120 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.4). Unreacted MTSES and citrate were removed by ultracentrifugation at approximately 412,000 × g for 25 min at 6 °C. The proteoliposomal pellets were washed with 6 ml of ice-cold buffer and subsequently resuspended gently in 115 μl of buffer. Transport reactions were conducted in duplicate with each resuspended pellet as previously described [13]. For the cases where unlabeled citrate protected against MTSES-mediated inhibition of citrate function, the percentage of control activity versus citrate concentration curves were fitted to a modified one-site binding equation: Y = A + [(B × (S/EC50 + S)) × 100] / (S + 100), where Y is the percentage of control activity remaining in the presence of MTSES plus a given citrate concentration S, A is the percent activity remaining when only MTSES was present, B is the maximal percent control activity obtained via substrate protection, EC50 is the effective concentration of citrate that yields one half-maximal protection against MTSES-mediated transport inhibition.

3. Results

3.1. Kinetic characterization of the Cys-less, L120C, Q182C, and L120C/Q182A CTP variants

In order to address the issue of whether the side-chain of TMD IV residue Q182 sterically blocks the approach of citrate to the side-chain of TMD III residue L120, we conducted a detailed
analysis of the kinetic properties of each single-Cys mutant as well as the L120C/Q182A double mutant. As depicted in Table 1, the three Cys substitution mutants displayed modest decreases in their apparent $K_m$ values. The $K_m$ of the Q182C variant displays a clear statistically significant decrease, whereas the decreased $K_m$ of the L120C and the L120C/Q182A variants were of borderline statistical significance. The $V_{max}$ values of the three mutants ranged from 3 to 19% of the Cys-less value. While the reduced $K_m$ values partially compensated for the sharply reduced $V_{max}$ values, the resulting catalytic efficiencies (i.e., $V_{max}/K_m$) were decreased to 7–32% of the control Cys-less value.

3.2. Substrate protection against inhibition of each CTP variant by MTSES

In previous studies [11,12] we had demonstrated that MTSES effectively inhibits the L120C and the Q182C single mutants. In the present investigation effective inhibition was observed with the L120C/Q182A double mutant. For example, a 2 min incubation with 10 mM MTSES yielded 86% inhibition of CTP function. We then examined the ability of citrate to protect against the MTSES-mediated inhibition of each of these CTP variants. For these studies, in order to optimize the possibility of observing substrate protection, we chose MTSES concentrations and incubation times that yielded partial (i.e., 45–85%) inhibition and tested the ability of various concentrations of citrate to protect against inhibition of CTP function. As depicted in Fig. 1, and consistent with our previous observations [13], citrate offered no significant protection of the L120C mutant. In contrast, citrate afforded a high level of protection against the MTSES-mediated inhibition of the Q182C mutant, with an EC$_{50}$ value of 1.40 mM. This is consistent with our prediction that Q182 is more proximal to the transport pathway than is Leu120, and in fact directly lines the pore. Importantly, with the double mutant in which we have truncated the side-chain at position 182 (thereby removing the proposed steric block at this position) the Cys at position 120 is highly accessible to citrate such that this substrate affords excellent protection against MTSES modification. An EC$_{50}$ value of 0.90 mM was observed. This value is reasonably close to the $K_m$ value for this double mutant suggesting a possible involvement of cysteine at position 120 in substrate binding under these conditions.

![Fig. 1](image.png)

Fig. 1. Quantitative evaluation of the extent of substrate protection against MTSES-mediated inhibition of CTP function with mutations at residues Leu120 and/or Gln182. Proteoliposomes reconstituted with a given CTP variant were incubated with MTSES with or without varying concentrations of unlabeled citrate at 21 °C. Following dilution and removal of extraliposomal citrate plus unreacted MTSES, transport incubations were conducted and analyzed as described under Materials and methods. The specific activity values for the MTSES-inhibited Leu120Cys, Gln182Cys, and Leu120Cys/Gln182Ala mutants, in the absence of external citrate, were 198.5, 5.3, and 22.0 nmol/min/mg protein, respectively. Data depict means from at least eight incubations±S.E.

Table 1

<table>
<thead>
<tr>
<th>CTP variant</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$V_{max}/K_m$ (nmol/min/mg/mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysless</td>
<td>0.479±0.071</td>
<td>2369±83</td>
<td>4946 (100%)</td>
</tr>
<tr>
<td>Leu120Cys</td>
<td>0.290±0.014</td>
<td>456±26</td>
<td>1572 (31.8%)</td>
</tr>
<tr>
<td>Gln182Cys</td>
<td>0.162±0.011</td>
<td>60±4</td>
<td>370 (7.5%)</td>
</tr>
<tr>
<td>Leu120Cys/Gln182Ala</td>
<td>0.296±0.010</td>
<td>103±8</td>
<td>346 (7.0%)</td>
</tr>
</tbody>
</table>

Transport reactions and calculation of $K_m$ and $V_{max}$ were conducted as described under Materials and methods. Data represent means±S.E. from at least four $V_i$ versus $[S]$ profiles employing ten different citrate concentrations that bracketed the $K_m$ value.

a Values are $p<0.075$.

b Values are $p<0.01$ from a two-tailed student’s $t$ test between the Cysless CTP and individual CTP mutants.
sterically blocking the Cys side-chain at position 120 and thereby preventing access to it by citrate. Panel C indicates that as expected when Gln182 is mutated to Cys, the Cys residue is readily accessible to citrate. Finally, Panel D indicates that when the Gln182 side-chain is truncated by mutation to Ala, the Leu120Cys mutated side-chain is now accessible to the transport path (denoted by *).

3.3. Transport activities of a panel of single-cys substitution mutants hypothesized to participate in substrate binding and/or translocation

Based on our modeling studies we constructed 24 single-Cys substitution mutants that were chosen based on their hypothesized importance in substrate binding and/or translocation. These residues were localized throughout the homology-modeled CTP structure. As depicted in Fig. 3, we found that Cys substitution for E34 and K37 (TMDI), K83 and R87 (TMDII), D140 and Y148 (M2), D236 and K239 (TMDV), T240, and Q243 (M3), and R276 and R279 (TMDVI) was highly disruptive of CTP function (i.e., ≥96% inhibition of function). (Note: certain of the above residues are quite close to the membrane/matrix loop interface and thus assignment to a given domain cannot be made with certainty at present). In contrast, substitution for selected residues in M1 (i.e., T38, R39, D44, K45, S47, and K48), TMDII (F76), M2 (i.e., K141, Q142, S143) and TMDV (T228 and V229) was reasonably well-tolerated.

Fig. 4 depicts the superposition of the specific activity data of those mutants that were most disruptive of CTP function (i.e., defined as causing ≥98% inactivation of CTP function) onto Fig. 3. Specific citrate transport activities of single-cysteine CTP variants. Overexpressed, solubilized Cys-less and single-Cys CTP variants were reconstituted into phospholipid vesicles. Transport incubations were performed at room temperature and BTC-sensitive [14C]citrate/citrate exchange was measured. Specific activity data represent the means of triplicate incubations±S.E. The specific transport activity of the Cys-less CTP was 1375 nmol/min/mg protein and represents the 100% value. Other conditions were as described under Materials and methods.
the 3-dimensional CTP homology modeled structure. A view into the transport pathway from the cytosolic surface of the bilayer is presented. Residues that display less than 2% of the starting Cys-less CTP function are depicted as ball and stick models. Blue denotes positively charged residues, red denotes negatively charged residues, and yellow denotes neutral residues.

Fig. 4. Depiction of essential residues within the 3-dimensional CTP homology modeled structure. A view into the transport pathway from the cytosolic surface shows the steric blockage by the Gln182 side-chain of the Leu120 side-chain in the Cys-less transporter (Panel A), and of the Cys side-chain in the L120C single mutant (Panel B). Furthermore, the side-chain of either a Gln or a Cys at position 182 (i.e., Panels A–C) resides in close contact with the transport path and sterically blocks access of citrate to the side-chain at position 120. However, truncation of the Gln side-chain to an Ala at position 182 (Panel D), removes the steric block and now a Cys at position 120 is fully accessible to citrate from the transport path. Also, note that a comparison of Panels B versus D in Fig. 2 shows the cysteine side-chain at position 120 existing in different conformations, which reflects the increased conformational flexibility that arises from truncation of the Gln182 side-chain to an alanine. In summary, we believe these experimental results and interpretations provide important new information regarding both the packing of α-helical TMDs III and IV in this critical region and the make-up of this portion of the transport pathway. Furthermore, our data provide additional evidence in support of the accuracy and the usefulness of our CTP homology model for experimental design.

The second issue we sought to address, was the identification of additional residues that are essential for CTP function. To this end, we constructed 24 single-Cys mutants, and following overexpression, solubilization, and reconstitution in liposomal vesicles, measured their specific citrate transport activities (Fig. 3). It should be noted that these CTP variants were selected based on our modeling studies which pointed to their potential involvement in substrate binding and/or translocation. These mutagenesis investigations resulted in the identification of 10 residues within the CTP that upon mutation to Cys cause a loss of ≥98% of CTP specific activity. Based on this criteria we consider

4. Discussion

The present investigations sought to address two questions. First, we sought to understand the molecular basis for our earlier observation that even though substitution of a Cys at position 120 places it on the water-accessible surface of TMD III, and that such a Cys is moderately accessible to modification by MTSES [12], nonetheless citrate was unable to protect against this modification [13]. This lack of citrate protection strongly suggested that in the wild-type transporter Leu120 does not have direct access to the translocation pathway. This conclusion was somewhat unexpected given that other residues on this face of the TMD III helix can be protected by citrate and thus do in fact directly access the transport path. This observation, in combination with the detailed 3-dimensional CTP homology model that we recently developed [14], which derived from the excellent work of Pebay-Peyroula et al. [16] wherein they determined the crystal structure of the mitochondrial ADP/ATP carrier, led to the hypothesis that the molecular explanation for the unexpected inability of citrate to protect against MTSES-mediated inhibition of the Leu120Cys CTP variant arises from the packing arrangement of TMDs III and IV in the CTP structure [13]. Thus, our model suggests that TMD III packs against TMD IV at a 30° angle and that this packing arrangement places the Leu120 side-chain behind the side-chain of Gln182. This potential explanation led to the testable hypotheses that: (i) a Cys substituted for Gln182 should be highly accessible to MTSES in a citrate protectable manner [13]; and (ii) truncation of the Gln182 side-chain to Ala, should then render the Leu120Cys mutation accessible to MTSES in a citrate-protectable manner. The data presented in Fig. 1 fully support these hypotheses. Thus, substrate protection against MTSES-mediated inhibition of the CTP function is not observed with the L120C mutant, but is readily evident with the Q182C single and the L120C/Q182A double mutants. It is intriguing that the EC50 value obtained with the double mutant (0.90 mM) is reasonably close to the Km observed with this mutant (i.e., 0.30 mM) thereby suggesting the possibility that in this CTP variant the cysteine at position 120 may play some role in substrate binding. However, clearly the lack of substrate protection with the L120C single mutant, suggests that this is not the case in the presence of the Q182 side-chain which serves to sterically limit the access of citrate from the translocation path to the Leu120 side-chain. Fig. 2 depicts the above explanation of the substrate protection data in the context of our CTP homology model. Accordingly, a view down into the transport path from the cytosolic surface shows the steric blockage by the Gln182 side-chain of the Leu120 side-chain in the Cys-less transporter (Panel A), and of the Cys side-chain in the L120C single mutant (Panel B). Furthermore, the side-chain of either a Gln or a Cys at position 182 (i.e., Panels A–C) resides in close contact with the transport path and sterically blocks access of citrate to the side-chain at position 120. However, truncation of the Gln side-chain to an Ala at position 182 (Panel D), removes the steric block and now a Cys at position 120 is fully accessible to citrate from the transport path.
these residues to be essential. Residues in this category include E34, K37, K83, R87, Y148, D236, K239, T240, R276, and R279. Data from our previous investigations indicate that G119, E122, R181, and R189 are also included in this category [10,12]. As depicted in Fig. 4, inspection of the location of each of these essential residues in the context of our CTP homology modeled structure, indicates that with the single exception of E122, each of these residues project their side-chains into the substrate translocation pathway. Furthermore, our current studies in combination with our earlier investigations [11,12] indicate that single-Cys replacements for S123, E131, K134, D140, Q182, and Q243 result in 96–98% inactivation of CTP function and thus these residues, while perhaps not essential, are nonetheless clearly important for CTP function. Inspection of the location of the side-chains of this latter group of amino acids, indicates that they too project into the transport path (data not shown; Note: the side-chain of D140 is present in a loop and can likely adopt multiple conformations some of which project into the pathway and others of which do not). We hypothesize that subsets of the mutation-intolerant residues are involved in different functions. For example, one subset will likely be involved in salt bridge formation in order to maintain CTP native conformation and/or facilitate conformational changes associated with transport. A second set will likely comprise the lining of the transport path, and a third subset will directly participate in substrate binding. With respect to E122, an essential residue whose side-chain projects away from the transport path, we have proposed that it may be involved in the coordination of the presumed conformational changes that must occur in each monomer of the homodimer during a transport cycle [14]. Furthermore, the flexible G119 can likely be accessed from both the transport pathway as well as the dimer interface and we have hypothesized may represent an important component of the latter (G119) [12]. Experimental efforts are currently underway in our laboratory which seek to define the precise role of each of the essential residues in the structure-based mechanism of the CTP.

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