Identification of the Substrate Binding Sites within the Yeast Mitochondrial Citrate Transport Protein*

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The objective of the present investigation was to identify the substrate binding site(s) within the yeast mitochondrial citrate transport protein (CTP). Our strategy involved kinetically characterizing 30 single-Cys CTP mutants that we had previously constructed based on their hypothesized importance in the structure-based mechanism of this carrier. As part of these studies, a modified transport assay was developed that permitted, for the first time, the accurate determination of $K_m$ values that were elevated >100-fold compared with the Cys-less control value. We identified 10 single-Cys CTP mutants that displayed sharply elevated $K_m$ values (i.e. 5 to >300-fold). Each of these mutants displayed $V_{\text{max}}$ values that were reduced by $\geq 98\%$ and resultant catalytic efficiencies that were reduced by $\geq 99.9\%$. Importantly, superposition of this functional data onto the three-dimensional homology-modeled CTP structure, which we previously had developed, revealed that nine of these ten residues form two topographically distinct clusters. Additional modeling showed that: (i) each cluster is capable of forming numerous hydrogen bonds with citrate and (ii) the two clusters are sufficiently distant from one another such that citrate is unlikely to interact with all of these residues at the same time. We deduced from these findings that the CTP contains at least two citrate binding sites per monomer, which are located at increasing depths within the translocation pathway. The identification of these sites, combined with an initial assessment of the citrate-amino acid side-chain interactions that may occur at these sites, substantially extends our understanding of CTP functioning at the molecular level.

The mitochondrial citrate transport protein (CTP)3 is located within the inner mitochondrial membrane and catalyzes an obligatory exchange of the dibasic form of tricarboxylic acids (e.g. citrate and isocitrate) for other tricarboxylic acids or in higher eukaryotes for dicarboxylic acids (e.g. malate and succinate) or phosphoenolpyruvate (1). Once in the cytoplasm, the transported citrate serves as the prime carbon source fueling fatty acid, triacylglycerol, and cholesterol biosyntheses (2–5). In addition, the concerted action of citrate lyase and malate dehydrogenase enables the generation of NADH+, a cofactor that is essential for the glycolytic pathway. Based on these roles, the CTP is considered essential for eukaryotic cell metabolism.

Because of the prominent role of the CTP in cellular bioenergetics, our laboratory has conducted extensive investigations with the aim of elucidating its structure-based mechanism. Thus we have cloned (6), overexpressed (7, 8), and purified (9, 10) the functional form of this transporter. Recently, employing a Cys-less yeast mitochondrial CTP construct that displays native functional properties (11) as the template, we have: (i) demonstrated that the transporter exists as a homodimer in detergent micelles (12); (ii) utilized cysteine-scanning mutagenesis combined with probing the accessibility of single-Cys mutants to MTS reagents, in both the absence and presence of citrate, to identify those residues in transmembrane domains III and IV that line the substrate translocation pathway (13–15); (iii) developed a detailed homology model of the three-dimensional structure of the CTP (16) based on the crystal structure of the mitochondrial ADP/ATP carrier (17); and (iv) superimposed our functional data onto this homology model to delineate substantial portions of the translocation pathway within the structure (15, 16).

With this background in mind, the current studies focused on characterizing the kinetic properties of a panel of single-Cys mutants that we had previously constructed based on their predicted importance in the CTP mechanism (11, 14, 18). We identified ten mutations that display both substantially elevated $K_m$ values and reduced $V_{\text{max}}$ values, nine of which localize to two topographically distinct clusters within the CTP structure. Modeling studies indicate that, although citrate is capable of numerous hydrogen bonding interactions with each cluster, one molecule of citrate cannot simultaneously interact with all members of both clusters. These findings have led us to propose a model, wherein the CTP contains at least two discrete substrate binding sites per monomer. The details of these sites and the implications of this model are discussed in detail.
EXPERIMENTAL PROCEDURES

Construction, Overexpression, and Isolation of Single-Cys CTP Variants—Single-Cys CTP variants were prepared as previously described (14). Briefly, single-Cys CTP genes were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenic primers were analyzed with Oligo 5.0 software. PCR amplifications and subsequent cloning steps were conducted according to the manufacturer’s instructions. The Cys-less yeast mitochondrial CTP gene in pET-21a (+) was utilized as the starting template (11). Transformants were screened for the presence of inserts via restriction digestion of plasmid DNA with NdeI and BamHI. The DNA from positive clones was then partially sequenced to assure the presence of the desired mutation. Plasmids scoring positively for the mutation of interest were then subcloned into the storage (Novagen) and expression (BL21(DE3)) hosts. Mutations were constructed using the QuikChange site-directed mutagenesis kit previously described (14). Briefly, single-Cys CTP genes were incorporated into liposomal vesicles, in the presence of 48 mM citrate, via the freeze-thaw sonication procedure, intraliposomal radiolabeled citrate was separated from the external radiolabel via chromatography on short (i.e. 4 cm) Dowex columns in Pasteur pipettes. However, with those incubations containing a high external citrate concentration, we employed longer (i.e. 8 cm) Dowex columns to ensure the effective removal of extraliposomal citrate. The eluted (i.e. intraliposomal) radiolabel was quantified via liquid scintillation counting. The 1,2,3-benzenetricarboxylate-sensitive transport rate was calculated by subtracting the control value from the experimental value.

Identification of Substrate Binding Sites within the CTP

The measurement of the kinetic parameters following MTS labeling of the single-Cys mutants was conducted in a manner very similar to that described above. Consequently, only the methodological differences are described below. Proteoliposomes (45 μl) were preincubated with 3.5 μl of either buffer (experimental sample) or 200 mM 1,2,3-benzenetricarboxylate (control sample) for 10–12 min and were then further incubated with 3.5 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 [1,5-14C]citrate (Amersham Biosciences). Typically, ~10 different external citrate concentrations bracketing the Km values were employed. However, with several CTP variants that displayed extremely high Km values (i.e. K83C, R87C, R189C, and R276C) substrate concentrations that were greater than the Km value could not be utilized because 50 mM external citrate was the highest concentration that we could employ and still maintain a specific radioactivity that was sufficiently high to enable accurate measurement of transport activity. Also, with very high Km mutants, the [14C]citrate source was concentrated by evaporation under vacuum prior to making up the stock solutions. Following transport incubations that ranged from 12 s to 2.5 h (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 1,2,3-benzenetricarboxylate. The control sample received an equal volume of buffer. Transport reactions were conducted at room temperature (21 °C). Following all incubations, extraliposomal radiolabeled citrate was separated from the external radiolabel via chromatography on short (i.e. 4 cm) Dowex columns in Pasteur pipettes. With the eluted (i.e. intraliposomal) radiolabel was quantified via liquid scintillation counting. The 1,2,3-benzenetricarboxylate-sensitive transport rate was 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 Km and Vmax values for each single-Cys CTP mutant, before and after modification with MTS reagents (see below), were calculated by taking the mean of the best fit Km and Vmax values derived from each separate V versus S profile.

Determination of the Kinetic Parameters of CTP Variants before and after Modification with MTS Reagents—The kinetic parameters (Km and Vmax) of the Cys-less and the single-Cys CTP variants before modification with MTS reagents were determined as follows (15). Proteoliposomes (45 μl) were preincubated with 3.5 μl of either buffer (experimental sample) or 200 mM 1,2,3-benzenetricarboxylate (control sample) for 10–12 min and were then further incubated with 3.5 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 [1,5-14C]citrate (Amersham Biosciences). Typically, ~10 different external citrate concentrations bracketing the Km values were employed. However, with several CTP variants that displayed extremely high Km values (i.e. K83C, R87C, R189C, and R276C) substrate concentrations that were greater than the Km value could not be utilized because 50 mM external citrate was the highest concentration that we could employ and still maintain a specific radioactivity that was sufficiently high to enable accurate measurement of transport activity. Also, with very high Km mutants, the [14C]citrate source was concentrated by evaporation under vacuum prior to making up the stock solutions. Following transport incubations that ranged from 12 s to 2.5 h (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 1,2,3-benzenetricarboxylate. The control sample received an equal volume of buffer. Transport reactions were conducted at room temperature (21 °C). Following all incubations, extraliposomal radiolabeled citrate was separated from the external radiolabel via chromatography on short (i.e. 4 cm) Dowex columns in Pasteur pipettes. With the eluted (i.e. intraliposomal) radiolabel was quantified via liquid scintillation counting. The 1,2,3-benzenetricarboxylate-sensitive transport rate was 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 Km and Vmax values for each single-Cys CTP mutant, before and after modification with MTS reagents (see below), were calculated by taking the mean of the best fit Km and Vmax values derived from each separate V versus S profile.

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Identification of Substrate Binding Sites within the CTP

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Molecular Modeling of the CTP—Molecular modeling was carried out using version 5 of DOCK (20), obtained from the University of California at San Francisco, and version 2005.06 of Molecular Operating Environment (MOE), obtained from Chemical Computing Group, Montreal. The homology-modeled CTP structure was constructed as described previously (16). Citrate, modeled in multiple conformations as a diion, was initially docked into the CTP model using DOCK. In DOCK, five different scoring grids, centered at varying depths in the transport path, were utilized to ensure that all regions...
were thoroughly examined. The resulting docking poses were found to be clustered in two distinct regions of the translocation pathway. The first cluster was located about 10 Å into the pathway, centered around Lys-83, Arg-87, and Arg-189. The second cluster was located about 20 Å into the pathway, centered around Lys-37, Arg-181, Lys-239, Arg-276, and Arg-279. In the modeled CTP structure, these were found to be spread over too large an area to constitute a single binding site; even with side chains fully extended, the most distant residues are at least 20 Å apart. In contrast, a fully extended citrate molecule is <9 Å long. This result strongly suggested that there must be two citrate binding sites. We examined this possibility by mutating each of the above residues, as well as additional residues in the vicinity of these binding sites, to cysteine, one at a time. After kinetic analysis of these mutants verified the likely location of the above residues at a given citrate binding site, the two-binding site model was subjected to further refinement as follows. With all residues within 4.5 Å of the citrate, alternate side-chain conformations were explored using MOE. Models were then minimized using the CHARMM27 force field (21) in MOE. For each cluster, the most energetically favorable orientation was selected, producing two final proposed binding sites as described below.

RESULTS

Kinetic Characterization of a Panel of Single-Cys CTP Substitution Mutants Hypothesized to Participate in Substrate Binding and/or in Other Aspects of the Transport Mechanism—The present investigations focused on the kinetic characterization of a panel of 30 single-Cys substitution mutants that were previously constructed (11, 14, 18) utilizing the Cys-less CTP as the template. Mutations of four groups of residues were characterized. The first group consisted of eight arginine and lysine residues, which, based on our molecular modeling results (see “Experimental Procedures”), cluster in two topographically distinct regions within the transport pathway. Residues Lys-83, Arg-87, and Arg-189 constitute one cluster and were predicted to form one binding site, and residues Lys-37, Arg-181, Lys-239, Arg-276, and Arg-279 comprise the second cluster and were predicted to form a second binding site. Each cluster appeared capable of forming multiple ionic hydrogen bonds with citrate. The second group consisted of residues that were predicted to reside nearby a given substrate binding site but were thought unlikely to be its prime determinants. Thus, Leu-116, Gly-119, Leu-120, Ser-123, Gln-182, Asn-185, and Gln-186 were predicted to be near binding site one and residues Glu-34, Phe-76, Glu-131, Lys-134, Thr-228, Val-229, Asp-236, Thr-240, and Gln-243 were predicted to be near binding site two. The effect of Cys substitution at several of these locations had been characterized previously as part of our studies into the role of TMDs III and IV in CTP function (15, 18). The third group consisted of selected residues in matrix loops A (i.e. Thr-38, Arg-39, Asp-44, Lys-45, Ser-47, and Lys-48) and C (Asp-140, Lys-141, Gln-142, Ser-143, and Tyr-148). The rationale for exploring the role of these residues in CTP function was based upon findings with the mitochondrial ADP/ATP carrier (22, 23), which suggested that, rather than project into the matrix compartment, these loops may in fact project into the translocation pathway during portions of the transport cycle and possibly assume an important role in the translocation mechanism. Finally, we characterized the mutation of Glu-122 because our previous studies indicated that this residue was essential for function. As depicted in Fig. 1 and Table 1, the mutated residues are located throughout the CTP structure.

The strategy that we used was as follows. Each single-Cys CTP variant was overexpressed in Escherichia coli, solubilized from the isolated inclusion body fraction with Sarkosyl, and then functionally reconstituted in proteoliposomes. In the past, we had utilized this approach to extensively study the effects of mutating residues in transmembrane domains III and IV on CTP function (13, 14). In the present studies, we determined the kinetic properties of each of the 30 CTP variants described above. Conducting these studies necessitated substantial modification of our standard transport assay to accurately determine \( K_m \) values that were markedly elevated compared with the Cys-less control values. The principal modifications, which are described in detail under “Experimental Procedures,” entailed: (i) concentrating the \([^{14}C]\)citrate source so that we could attain large enough specific radioactivity values, even in the presence of the high concentrations of citrate needed when dealing with a mutant that displayed a substantially elevated \( K_m \) to enable the accurate measurement of transport rates; (ii) increasing the intraliposomal citrate concentration; and (iii) increasing the quantity of Dowex utilized to effectively bind and remove extraliposomal (i.e. non-transported) \([^{14}C]\)citrate. The modified assay enabled the determination of \( K_m \) values that were increased at least 100-fold over the Cys-less CTP control value. An example of the high quality data obtained with both our standard and modified assays is shown in Fig. 2. Fig. 2A depicts a \( V \) versus \( S \) plot obtained with the Cys-less CTP employing our standard assay. Fig. 2B depicts the plot obtained with the R181C variant utilizing our modified assay. Notice the similar and high \( R^2 \) values obtained with both assays (i.e. \( R^2 = 0.92 \) and \( R^2 = 0.93 \) for the standard and modified assays, respectively) indicating the high quality fit of the non-linear regression line with the data points.

Table 1 depicts the kinetic characteristics of each of the 30 single-Cys CTP mutants. Also included in Table 1 are data obtained from our previous studies with 6 single-Cys mutants (15, 18) that neighbor a given site. In total, we observed that of the 36 mutants for which data are presented, 21 displayed reductions in both their \( K_m \) and \( V_{\text{max}} \) values. In each of these cases, the reduction in \( V_{\text{max}} \) was of greater magnitude than the decrease in \( K_m \), resulting in decreased catalytic efficiencies that ranged from 1 to 93% of the Cys-less control value. In contrast, 10 mutants displayed both \( K_m \) values that were increased 5-fold to 328-fold relative to the Cys-less control and \( V_{\text{max}} \) values that were reduced by \( \approx 98\% \), resulting in catalytic efficiencies that were reduced by \( \approx 99.9\% \). With the K83C and R276C mutants, a precise \( K_m \) could not be determined due to the fact that a nearly linear \( V \) versus \( S \) plot was obtained over the entire range of substrate concentrations tested. However, we can conclude with certainty that in both cases the \( K_m \) values exceeded 100 mM. Furthermore, the catalytic efficiencies (i.e. \( V_{\text{max}}/K_m \)) of these two mutants were determined by taking the reciprocal of the slope obtained in a Lineweaver-Burk analysis of the data.
We are therefore also able to conclude that, although we cannot precisely determine the $V_{\text{max}}$ values for these two mutants (denoted as ND in Table 1), clearly these values will be quite small. Finally, we previously had shown (24), via CD analyses of a subset of these essential residues, that in detergent micelles their CD spectra were virtually superimposable with that of the Cys-less control CTP, indicating that no detectable difference in secondary structure occurred upon mutation and, thereby, suggesting that the wild-type residues, rather than affecting structure, instead may have assumed important mechanistic roles.

Identification of the CTP Citrate Binding Sites via Superposition of Functional Data onto a Three-dimensional Homology Modeled CTP Structure—The functional data generated with the single-Cys mutants revealed the identity of the residues that may play prominent roles in substrate binding within the CTP, namely, those residues whose mutation to a Cys imparted a substantially elevated $K_m$ value to the CTP combined with a sharply reduced $V_{\text{max}}$ and catalytic efficiency. These results provided experimental support for our initial predictions, which had been based on a molecular modeling approach. Thus, upon mapping the $K_m$-sensitive residues onto our CTP homology model it is apparent that nine of the ten $K_m$-sensitive residues reside in two topographically distinct clusters within the CTP and that a plausible citrate binding site could be formed by each cluster. Furthermore, these residues extend over a sufficiently large distance within the CTP such that it is impossible for a single citrate molecule to interact with all of the implicated residues simultaneously. Based on these findings, we deduce a two-binding site model. Following experimental support for the predicted binding site residues, we then refined our model by exploring alternate side chain conformations of all residues within 4.5 Å of a given binding site, followed by energy minimization of the structure (see “Experimental Procedures”). For each binding site the most energetically favorable orientation was selected, resulting in the two final proposed binding sites described below. The atomic detail of each of these binding sites is presented in Figs. 3–5.

Fig. 3 depicts the location of the two citrate binding sites, which reside at different depths within the homology-modeled three-dimensional CTP structure as viewed from the plane of the membrane. Fig. 4 presents the details of citrate binding site one. Panel A portrays the direct interaction of residues Arg-189, Arg-87, and Lys-83 with a molecule of citrate. Panel B shows these residues plus additional neighboring residues of interest residing within 4.5 Å of the citrate. We propose that this site may bind citrate via a total of six ionic hydrogen bonds. Fig. 5 depicts the atomic details of citrate binding site two. Panel A portrays residues Arg-276, Arg-279, Arg-181, Lys-239, and Lys-37 interacting with a molecule of citrate. Panel B shows these residues plus additional neighboring residues of interest residing within 4.5 Å of the citrate. We propose that site two binds citrate via a total of eight ionic hydrogen bonds. The putative citrate-amino acid side-chain interactions that occur at each site are explored in detail under “Discussion.”

Effect of Charge Restoration via Chemical Modification of Single-Cys CTP Variants with MTS Reagents on CTP Function—Of the 10 mutants that displayed large increases in their $K_m$ values, concurrent with large decreases in both their $V_{\text{max}}$ values and their catalytic efficiencies, 9 involved replacement of

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**Identification of Substrate Binding Sites within the CTP**

*JUNE 8, 2007 • VOLUME 282 • NUMBER 23 • JOURNAL OF BIOLOGICAL CHEMISTRY* 17213

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**FIGURE 1.** Transmembrane topography of the Cys-less yeast mitochondrial citrate transport protein monomer. The proposed model has been developed based on hydropathy analysis and our CTP homology model (16). Six α-helical domains are connected by five hydrophilic loops (A–E). Helical structures were derived from the homology model and may extrude into the aqueous phase. The rectangles (I–VI) depict the likely transmembrane domains. Whether the hydrophilic loops (A–E) are external to, or instead partially penetrate the membrane, remains an open question. Positively charged residues are denoted in red, negatively charged residues are in black, and the wild-type cysteines, which have been replaced with Ser or Val in the Cys-less CTP construct, are in orange. The residues that were studied in the present investigation are highlighted with squares.
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**TABLE 1**

Effect of cysteine substitution on the CTP kinetic parameters

Transport reactions and calculation of $K_m$ and $V_{max}$ were conducted as described under “Experimental Procedures.” Data represent means ± S.E. from at least duplicate $V$ versus $[S]$ profiles employing at least 10 different substrate concentrations that bracketed the $K_m$ value with a few exceptions. Cysteine substitutions that cause profound increases in $K_m$ and decreases in $V_{max}$ (i.e., $K_m$ increases ≥5-fold and $V_{max}$ reductions of ≥98%) are depicted in bold. TMD (I–VI) and loop (A–E) nomenclature is defined in the legend to Fig. 1. The predicted topographical location of a given residue was based upon initial modeling results. However, with Gly-119, although it was initially predicted to be near site one, based on the large $K_m$ increase and $V_{max}$ decrease of the GI19C mutant, it has been included as a key site one residue.

| Mutation    | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $V_{max}/K_m$ (%) | Predicted topographical location |
|-------------|------------|--------------------------|-------------------|----------------------------------|
| Cys-less    | 0.461 ± 0.053 | 2305.0 ± 78.2          | 5000.0 (100)      | TMD II, binding site one         |
| K83C        | ≥100        | ND                       | 0.0 (0.0)         | TMD II, binding site one         |
| R87C        | 87.613 ± 58.863$^a$ | 0.2 ± 0.1$^e$         | 0.0 (0.0)         | TMD II, binding site one         |
| G119C       | 5.624 ± 1.224$^a$ | 0.3 ± 0.0$^e$         | 0.1 (0.0)         | TMD III, binding site one        |
| R189C       | 151.385 ± 59.715$^a$ | 2.4 ± 0.8$^e$         | 0.7 (0.0)         | TMD IV, binding site one         |
| K37C        | 2.817 ± 0.103$^a$ | 1.9 ± 0.1$^e$         | 0.4 (0.0)         | TMD IV, binding site two         |
| R181C       | 24.085 ± 5.455$^a$ | 1.8 ± 0.1$^e$         | 0.4 (0.0)         | TMD IV, binding site two         |
| K239C       | 7.843 ± 2.405$^a$ | 43.3 ± 11.3$^e$       | 5.5 (0.1)         | TMD V, binding site two          |
| R276C       | >100        | ND                       | 0.0 (0.0)         | TMD VI, binding site two         |
| R279C       | 3.721 ± 0.758$^a$ | 0.1 ± 0.0$^e$         | 30.4 (0.6)        | TMD V, binding site two          |
| E34C        | 0.381 ± 0.035 | 11.6 ± 0.3$^e$         | 12.9 (0.3)        | TMD I, near binding site two     |
| T38C        | 0.110 ± 0.018$^a$ | 307.2 ± 9.4$^e$       | 2792.7 (55.9)     | TMD I/Matrix Loop A              |
| K39C        | 0.373 ± 0.003 | 1732.0 ± 94.5$^e$     | 4643.4 (92.9)     | TMD I/Matrix Loop A              |
| D44C        | 0.296 ± 0.065 | 360.7 ± 11.8$^e$       | 12186.0 (24.4)    | Matrix Loop A                    |
| K45C        | 0.234 ± 0.030 | 903.4 ± 25.4$^e$       | 3860.7 (72.2)     | Matrix Loop A                    |
| S47C        | 0.367 ± 0.011 | 1102.0 ± 9.5$^e$       | 3002.7 (60.1)     | Matrix Loop A                    |
| K48C        | 0.297 ± 0.042 | 1312.0 ± 63.5$^e$     | 4417.5 (88.4)     | Matrix Loop A                    |
| F76C        | 0.670 ± 0.194 | 509.4 ± 2.2$^e$       | 753.6 (15.1)      | Matrix Loop A                    |
| L116C       | 0.228 ± 0.008$^a$ | 487.1 ± 22.8$^e$     | 2136.4 (71.8)$^a$ | TMD II, near binding site two    |
| L120C       | 0.290 ± 0.014$^a$ | 455.5 ± 26.1$^e$       | 1570.7 (31.8)$^a$ | TMD III, near binding site one   |
| E122C       | 2.298 ± 0.764$^a$ | 1.7 ± 0.3$^e$         | 0.70 (0.0)        | TMD III, dimer interface         |
| S123C       | 2.502 ± 0.928$^a$ | 103.4 ± 14.5$^e$      | 41.3 (14.9)       | TMD III, near binding site one   |
| E131C       | 0.170 ± 0.014$^a$ | 12.3 ± 0.3$^e$         | 72.4 (2.4)$^e$    | TMD III, near binding site two   |
| K134C       | 1.339 ± 0.168$^a$ | 55.8 ± 11.7$^e$       | 41.7 (1.4)$^e$    | TMD III, near binding site two   |
| D140C       | 0.064 ± 0.002$^a$ | 49.9 ± 2.9$^e$         | 779.7 (15.6)      | Matrix Loop C                    |
| K141C       | 0.714 ± 0.002$^a$ | 1434.0 ± 125.5$^e$  | 2002.8 (40.1)     | Matrix Loop C                    |
| Q142C       | 0.304 ± 0.016 | 1341.0 ± 101.5$^e$   | 4411.2 (88.2)     | Matrix Loop C                    |
| S143C       | 0.281 ± 0.034 | 798.0 ± 31.8$^e$      | 2839.9 (56.8)     | Matrix Loop C                    |
| Y148C       | 0.070 ± 0.000$^a$ | 26.4 ± 0.5$^e$         | 377.1 (7.5)       | Matrix Loop C                    |
| Q182C       | 0.162 ± 0.011$^a$ | 60.4 ± 3.7$^e$         | 652.8 (13.5)      | TMD IV, near binding site one    |
| N185C       | 0.574 ± 0.072 | 372.8 ± 15.0$^e$      | 6450.0 (129.9)    | TMD IV, near binding site one    |
| K186C       | 0.302 ± 0.037 | 109.8 ± 5.0$^e$       | 363.6 (7.3)       | TMD IV, near binding site one    |
| T228C       | 0.137 ± 0.019$^a$ | 431.8 ± 20.8$^e$       | 3151.8 (63.0)     | TMD V, near binding site one     |
| V229C       | 0.084 ± 0.005$^a$ | 174.7 ± 25.0$^e$      | 2079.8 (41.6)     | TMD V, near binding site two     |
| D236C       | 0.323 ± 0.049 | 12.4 ± 0.3$^e$        | 38.4 (0.8)        | TMD V, near binding site two     |
| T240C       | 0.102 ± 0.036$^a$ | 53.4 ± 2.9$^e$         | 523.5 (10.5)      | TMD V, near binding site two     |
| Q243C       | 0.089 ± 0.001$^a$ | 62.8 ± 1.7$^e$         | 705.6 (14.1)      | TMD V/E, near binding site two   |

$^a$ ND, not determined.

$^b$ Values are $p < 0.05$ from a two-tailed Student’s t test between Cys-less and individual single-Cys mutants.

$^c$ Values are $p < 0.001$.

$^d$ Values are $p < 0.01$.

$^e$ Data adapted from Ref. 15. The $V_{max}/K_m$ percentage values were calculated for these mutants utilizing the control value presented in the cited publication.

$^f$ Data adapted from Ref. 18. The $V_{max}/K_m$ percentage values were calculated for these mutants utilizing the control value presented in the cited publication.

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**FIGURE 2.** $V$ versus $S$ plot obtained with the Cys-less CTP and the R181C variant utilizing our standard and modified transport assays, respectively. A, a non-linear regression analysis of $V$ versus $S$ data obtained with the Cys-less CTP utilizing our standard transport assay. B, the identical plot of data obtained with the R181C CTP variant utilizing our modified transport assay. Transport reactions and $K_m$ and $V_{max}$ calculations were carried out as described under “Experimental Procedures.”
Identification of Substrate Binding Sites within the CTP

FIGURE 3. Two citrate binding sites within the CTP transport pathway viewed in the plane of the membrane bilayer. Two citrate binding sites, viewed in the plane of the membrane bilayer (i.e. a side view) are presented. The backbone of the CTP is represented as a green ribbon. Important side chains are shown as stick structures, and citrate molecules are shown as space-filling structures. Citrate oxygens are red. In site one, the citrate carbons and the directly interacting side chains are magenta; in site two, the citrate carbons and the directly interacting side chains are cyan. The distance between the two sites (measuring at the central carbon atoms of the two citrates) is 9.2 Å. Portions of TMDs I, II, and VI have been cut away for clarity. Black horizontal lines at right indicate the approximate boundaries of the bilayer, and the atomic ruler at the left indicates approximate dimensions.

FIGURE 4. Depiction of citrate binding site one within the CTP homology-modeled structure in the presence of citrate. The CTP backbone is represented as a green ribbon, whereas the amino acid side chains that form the binding site are represented as sticks, and citrate is depicted as a ball-and-stick model. Citrate carbons are numbered in yellow. Ionic hydrogen bonds are depicted by gray dots. Hydrogen atoms are omitted for clarity. The site is viewed in the plane of the membrane, and only portions of those TMDs that participate in formation of the binding site are depicted. A, key residues that form ionic hydrogen bonds to citrate. B, all residues that form this citrate binding site and reside within ~4.5 Å from citrate.

a charged residue with cysteine. To examine the role of charge versus other properties of the native residue in the CTP translocation mechanism, we examined the effect of restoring charge via chemical modification of the substituted Cys with MTS reagents. As depicted in Table 2, chemical modification of either the K37C or the K239C mutants with MTSEA completely restored the $K_m$ to its normal value but had little effect on the $V_{\text{max}}$, and only marginal effects on the catalytic efficiencies of these CTP variants. Similarly, modification of the K83C, R87C, R181C, R189C, R276C, and R279C mutants with MTSEA caused a substantial reduction in the $K_m$ value (i.e. 4-fold to 128-fold reductions in $K_m$ were noted), although not a total restoration of the native value. Although in most cases chemical modification caused slight increases in the $V_{\text{max}}$ values, these were sufficiently unremarkable such that the catalytic efficiencies of these mutants remained quite low (i.e. <1% of the Cys-less control value).

With the E122C CTP variant, we noted that neither restoration of the negative charge via modification with MTSES, nor addition of a positive charge via modification with MTSEA or MTSET reduced the $K_m$ value and in fact modest additional increases in this parameter were observed. Furthermore, none of these modifications caused substantive increases in the $V_{\text{max}}$ value. Interestingly, based on our homology-modeled CTP structure, we hypothesize that the side chain of Glu-122 does not reside within the citrate binding sites but instead projects into the monomer-monomer interface of the homodimeric transporter. We also examined whether addition of charge to the G119C mutant had any restorative effect on the function of this mutant. As expected, none was observed.

DISCUSSION

The objective of the present investigation was to identify the residues that comprise the substrate binding site(s) within the yeast mitochondrial CTP. To pursue this objective we kinetically characterized a panel of single-Cys CTP mutants that we had previously constructed (11, 14, 18). A novel and critical aspect of these studies is that they represent one of the few examples where $K_m$ values have been determined with mitochondrial transporter mutants that are substantially inactivated, thereby providing insight into the effect of a given mutation on the transporter’s affinity for substrate versus its $V_{\text{max}}$.

With respect to our contention that the $K_m$ of the CTP primarily reflects binding affinity, we note the following two observations. First, kinetic analyses of the reconstituted rat liver mitochondrial citrate carrier (25), as well as two other members of the mitochondrial carrier family (26, 27), indicate that substrate binding occurs via a rapid-equilibrium mechanism; in such kinetic mechanisms, it is well known that the $K_m$ approx-
Identification of Substrate Binding Sites within the CTP

**FIGURE 5. Depiction of citrate binding site two within the CTP homology-modeled structure in the presence of citrate.** The CTP backbone is represented as a green ribbon, whereas the amino acid side chains that form the binding site are represented as sticks, and citrate is depicted as a ball-and-stick model. Citrate carbons are numbered in yellow. Ionic hydrogen bonds are depicted by gray dots. Hydrogen atoms are omitted for clarity. The site is viewed in the plane of the membrane and only portions of those TMDs that participate in formation of the binding site are depicted. A, key residues that form ionic hydrogen bonds to citrate. B, all residues that form this citrate binding site and reside within $\sim 4.5$ Å from citrate.

Estimates of the $K_m$ as in the original Michaelis-Menten derivation (28). Second, the mutants identified in this study as containing replacements for proposed binding site residues not only display large $K_m$ values but also display significantly reduced $V_{max}$ values and catalytic efficiencies. Thus, the $k_{cat}$ for transport is severely depressed while concomitantly the $K_m$ is highly elevated. Even in most reasonable, simple steady-state models, an increase in $K_m$ is likely caused by an increase in $k_{cat}$ or an increase in $K_p$. Given that our data show a profound decrease in $V_{max}$, we reason that the increase in $K_m$ therefore results from an increase in $K_p$. In combination, these observations provide support for our contention that the observed $K_m$ value for a given mutant approaches its $K_p$ value and therefore primarily reflects the affinity of the transporter for substrate. For residues that line the transport pathway, mutations leading to a combination of a marked elevation in $K_m$ and reduction in the $V_{max}$ indicate likely participation in the substrate binding sites. However, it is also important to note that, given the complex sequence of events comprising the transport mechanism that we are proposing (i.e. citrate binding at site one followed by transfer to and binding at site two, and then subsequent release from site two and continued movement across the bilayer), it is possible that $K_m$ reflects other mechanistic steps in addition to binding affinity and thus changes in this parameter must be interpreted with caution.

Employing both our standard and modified transport assays, the latter enabling accurate analysis of high $K_m$ mutants (Fig. 2), we identified 10 single-Cys CTP variants that displayed substantially elevated $K_m$ values (i.e. 5- to >300-fold) and $V_{max}$ values that were reduced by $\approx 98\%$, resulting in catalytic efficiencies that were reduced by $\approx 99.9\%$. The unique significance of this group of residues is highlighted by our observation that, of 53 mutants studied in the current and previous investigations (15, 18), only these 10 display this level of alteration. In fact most residues studied, even those that line the transport path but nonetheless do not participate in the binding sites, show no elevation in the $K_m$ value and, therefore, can be eliminated as being directly involved in citrate binding. Importantly, localization of these 10 functionally essential mutants within the three-dimensional CTP homology model, which we previously developed (16) based on the crystal structure of the mitochondrial ADP/ATP carrier (17), revealed that nine of these ten residues form two topographically distinct clusters that reside within the transport pathway (see Fig. 3). Additional modeling revealed that: (i) each cluster is capable of forming multiple hydrogen bonds with citrate and (ii) there is sufficient distance between the two clusters such that citrate cannot interact with all of these residues concurrently (i.e. even with side chains fully extended, the most distant residues comprising these sites are at least 20 Å apart; furthermore, the distance between the C-3 carbon of citrate modeled into site one versus site two is 9.2 Å). We interpret these findings as indicating that the CTP contains at least two citrate binding sites per monomer that are located at increasing depths within the translocation path. The identification of these sites is a novel finding of critical importance that substantially extends our knowledge of CTP functioning at the molecular level.

The atomic details of these sites are as follows. Lys-83, Arg-87, and Arg-189 form binding site one (Fig. 4A) with Lys-83 forming an ionic hydrogen bond to the C6–COOH of citrate, Arg-87 forming a pair of ionic hydrogen bonds to the C5–COO$^-$ of citrate, and Arg-189 forming a pair of ionic hydrogen bonds to the C1–COO$^-$ of citrate. At this citrate binding site, other nearby residues, that is residues located within 4.5 Å of the bound citrate (Fig. 4B), include: Asn-185, which forms an ionic hydrogen bond to the C1–COO$^-$ of the citrate; Leu-120 and Gln-186, which reside within a distance of 4.5 Å, but fold behind Gln-182 and Leu-116, respectively. With regard to the neighboring residues, Gly-119 appears to be particularly important, because mutation to cysteine causes a >10-fold increase in the $K_m$ and a >99.9% reduction in the $V_{max}$. Accordingly, in Table 1 we have identified it as one of the key residues of binding site one. We posit that the addition of bulk and/or the reduction of flexibility that occurs...
Lys-134, which forms an ionic hydrogen bond to the C1–COO\(^{-}\) site, Lys-37, Arg-181, and Arg-276 form ionic hydrogen bonds to both the -OH and the C6–COOH of citrate, and Arg-279 forms an ionic hydrogen bond to the C5–COO\(^{-}\) of citrate. Other neighboring residues (Fig. 5A) display its own unique subset of CTP–citrate bonds, the sum of which yields the alternating accessibility that is the essence of a complete transport cycle.

We had kinetically characterized several residues in our previous studies (15) \(i.e.\) Val-124, Val-127, and Thr-128) with side chains projecting into the portion of translocation pathway that resides \textit{between} site one and site two. It is noteworthy that, in each case, no significant elevation in \(K_{m}\) was observed. Thus, these residues serve as internal controls indicating that this general region within the CTP is not uniquely sensitive to upon placement of a cysteine at this location within the binding pocket is\(d\) responsible for the disruption in the functioning of this site. Ser-123 represents a second nearby residue of interest. Although in the modeled conformation this residue is just beyond hydrogen bonding distance to the C5–COO\(^{-}\) group of citrate, we speculate that it plays an important role in substrate recognition based on our finding that the S123C CTP variant displayed a \(K_{m}\) value that was elevated 5-fold and a \(V_{\max}\) value that was reduced to 7% of the control value. In contrast, other nearby residues (\(i.e.\) Leu-116, Leu-120, Gln-182, Asn-185, and Gln-186) appear to be less important to this binding site, because their \(K_{m}\) values are not elevated upon substitution. In summary, site one binds citrate via a total of six ionic hydrogen bonds and is located \(\sim 10\) Å into the transport pathway, away from the aqueous intermembrane space/bilayer interface.

| TABLE 2                                                                | CTP variant | \(K_{m}\) | \(V_{\max}\) | \(V_{\max}/K_{m}\) | \(K_{m}\) reduction upon modification with MTS |
|------------------------------------------------------------------------|-------------|------------|---------------|---------------------|-----------------------------------------------|
| Mutants whose \(K_{m}\) values were completely restored by MTS modification | K37C        | 2.82 \(\pm\) 0.10\(^a\) | 1.9 \(\pm\) 0.1\(^a\) | 0.7 (0.0) | 8.6-fold                                    |
|                                                                        | K37C-MTSEA  | 0.33 \(\pm\) 0.02 | 1.5 \(\pm\) 0.0\(^a\) | 4.6 (0.1) |                                                |
|                                                                        | K299C       | 7.84 \(\pm\) 2.41\(^a\) | 43.3 \(\pm\) 11.3\(^a\) | 5.5 (0.1) |                                                |
|                                                                        | K299C-MTSEA | 0.52 \(\pm\) 0.03 | 21.6 \(\pm\) 0.0\(^a\) | 15.1 (0.1) |                                                |
| Mutants whose \(K_{m}\) values were substantially but not completely restored by MTS modification | K83C        | >100        | ND\(^d\)       | 0.0 (0.0) |                                                |
|                                                                        | K83C-MTSEA  | 1.46 \(\pm\) 0.05\(^a\) | 10.2 \(\pm\) 0.6\(^a\) | 7.0 (0.1) | >685-fold                                    |
|                                                                        | R87C        | 87.61 \(\pm\) 58.86\(^c\) | 0.2 \(\pm\) 0.1\(^a\) | 0.0 (0.0) |                                                |
|                                                                        | R87C-MTSEA  | 6.76 \(\pm\) 1.12\(^a\) | 0.5 \(\pm\) 0.0\(^a\) | 0.1 (0.0) | 13.0-fold                                    |
|                                                                        | R181C       | 24.09 \(\pm\) 5.46\(^a\) | 1.8 \(\pm\) 0.1\(^a\) | 1.0 (0.0) |                                                |
|                                                                        | R181C-MTSEA | 3.58 \(\pm\) 2.04\(^d\) | 23.9 \(\pm\) 11.0\(^c\) | 6.7 (0.1) | 6.7-fold                                    |
|                                                                        | R181C-MTSET | 4.02 \(\pm\) 0.25\(^a\) | 4.2 \(\pm\) 0.0\(^a\) | 1.0 (0.0) | 6.0-fold                                    |
|                                                                        | R189C       | 151.39 \(\pm\) 59.72\(^a\) | 2.4 \(\pm\) 0.8\(^a\) | 0.0 (0.0) |                                                |
|                                                                        | R189C-MTSEA | 1.18 \(\pm\) 0.06\(^d\) | 37.3 \(\pm\) 11.1\(^c\) | 31.6 (0.6) | 128.3-fold                                    |
|                                                                        | R276C       | >100        | ND\(^d\)       | 0.0 (0.0) | >14.6-fold                                    |
|                                                                        | R276C-MTSEA | 9.43 \(\pm\) 1.19\(^a\) | 0.5 \(\pm\) 0.1\(^a\) | 0.1 (0.0) |                                                |
|                                                                        | R279C       | 3.72 \(\pm\) 0.76\(^d\) | 0.1 \(\pm\) 0.0\(^a\) | 0.0 (0.0) | 3.6-fold                                    |
|                                                                        | R279C-MTSEA | 1.02 \(\pm\) 0.20\(^d\) | 0.8 \(\pm\) 0.0\(^a\) | 0.8 (0.0) |                                                |
| Mutants whose \(K_{m}\) values were not restored and in fact were increased by MTS modification | GI19C       | 5.62 \(\pm\) 1.12\(^a\) | 0.3 \(\pm\) 0.0\(^a\) | 0.1 (0.0) | −2.3-fold                                    |
|                                                                        | GI19C-MTSEA | 13.17 \(\pm\) 3.98\(^a\) | 0.3 \(\pm\) 0.1\(^a\) | 0.0 (0.0) | −1.7-fold                                    |
|                                                                        | GI19C-MTSES | 9.75 \(\pm\) 4.55\(^a\) | 0.1 \(\pm\) 0.1\(^a\) | 0.0 (0.0) | −1.7-fold                                    |
|                                                                        | E122C       | 2.30 \(\pm\) 0.76\(^c\) | 1.7 \(\pm\) 0.3\(^a\) | 0.7 (0.0) | −1.5-fold                                    |
|                                                                        | E122C-MTSEA | 3.37 \(\pm\) 1.02\(^a\) | 0.4 \(\pm\) 0.1\(^a\) | 0.1 (0.0) | −1.5-fold                                    |
|                                                                        | E122C-MTSET | 11.34 \(\pm\) 7.01\(^d\) | 3.7 \(\pm\) 2.0\(^a\) | 3.0 (0.0) | −4.9-fold                                    |
|                                                                        | E122C-MTSET | 8.55 \(\pm\) 1.58\(^a\) | 4.3 \(\pm\) 0.4\(^a\) | 0.5 (0.0) | −3.7-fold                                    |

\(^a\) Values are \(p < 0.001.\)

\(^b\) ND, not determined.

\(^c\) Values are \(p < 0.05\) from a two-tailed Student’s t test between Cys-less and individual single-Cys mutants.

\(^d\) Values are \(p < 0.1.\)
Identification of Substrate Binding Sites within the CTP

mutation, but rather only selective residues within this domain (i.e. the binding site residues) display marked enhancement of $K_m$ values upon mutation. Furthermore, the data presented in Table 1 indicate that, in contrast to suggestions with the ADP/ATP carrier (22, 23), at least the portions of CTP matrix loops A and C that we have examined do not appear to be involved in substrate binding.

Several additional points regarding sites one and two merit comment. First, we have modeled Gly-119 as forming part of the pocket into which citrate fits in binding site one. This is consistent with both modeling considerations and the large perturbation in the CTP $K_m$ value that occurs upon mutation of Gly-119 to Cys. However, due to the location of this residue within the TMDIII helix, as well as its intrinsic flexibility, it is also possible that the $\alpha$-carbon in Gly-119 faces away from the binding site, possibly toward the monomer-monomer interface. At present, we cannot clearly distinguish between these two possibilities, although we believe the binding site location to be more likely. Second, it is interesting to note that mutation of site one residues in general causes a larger increase in $K_m$ than does mutation of site two residues. This larger effect may be due to the fact that a reduced number of ionic hydrogen bonds are predicted for site one versus site two (i.e. 6 and 8, respectively). Consequently, mutation of a given residue in site one may be more disruptive and thus have a larger effect on the $K_m$ of the system than does mutation of a residue in site two. However, a second potentially more salient cause for this finding becomes apparent upon inspection of an alignment of the sequences of members of the yeast mitochondrial carrier family (29). Such alignment reveals that site one residues Lys-83, Arg-87, Gly-119, and Arg-189 are relatively unique to the CTP and in general are conserved among only a few closely related keto acid carriers. In contrast, core site two residues Lys-37, Arg-181, Lys-239, Arg-276, and Arg-279 are conserved throughout a greater number of mitochondrial carrier subfamilies, suggesting a more generalized role in substrate binding. Based on the above findings, we propose that site one may serve to confer a unique substrate specificity upon the CTP, which enables the efficient capture of citrate from the external media and a subsequent funneling of the substrate into the transport pathway in the correct orientation. Thus, perturbation of the residues forming site one severely disrupts CTP substrate recognition and consequently function. We further postulate that binding of external citrate to site one is required prior to citrate binding to site two and facilitates delivery of substrate to site two. Binding to the second, more widely conserved site may then trigger a change in conformation and presumably in accessibility via a mechanism that is conserved throughout this transporter family. Third, it is possible that, in addition to interacting with sites one and two in a discrete and sequential manner, citrate may also interact with a subset of residues contributed by both sites during the conformational changes that occur during the transport cycle. Fourth, although our data are consistent with the existence of at least two citrate binding sites within the CTP, they do not rule out the potential presence of additional sites that have yet to be identified. Thus, it will be of interest to determine in future studies whether an additional highly specific substrate binding site exists near the matrix side of the bilayer.

It is of interest to compare the substrate binding sites of the yeast mitochondrial CTP with a CTP of higher eukaryotic origin whose function has also been characterized in detail, such as is the case with the CTP from rat liver mitochondria (1, 7, 9). Thus, despite the fact that the two transporters exhibit a sequence identity of only 37.7%, a comparison of their binding site residues indicates considerable conservation. With respect to site one, all core residues are identical between the two transporters. Minor substitutions are observed with the more peripheral residues Leu-120 and Ser-123, wherein they are replaced by Val and Ala, respectively, in the rat liver transporter. Similarly, in site two, all core residues are conserved with the exception that Arg-181 in the yeast transporter is replaced with a Lys in the rat liver transporter, thus retaining positive charge at this location. With regard to the peripheral site two residues, all amino acids are conserved with the single exception that Met-233 is replaced by a Thr in the rat liver carrier. Hence, the two binding sites are highly conserved between the two orthologues. It therefore is intriguing to note that, despite this strong conservation, malate is a substrate for the rat liver transporter (1, 7), but not for the yeast CTP (8). Our modeling studies suggest that this alteration in substrate specificity is unlikely to be due to the modest changes in the binding site residues. Thus, the molecular basis for the altered substrate specificity of these two orthologues remains unknown at present.

A related point concerns a comparison of the two substrate binding sites within the yeast CTP with the hypothetical common mitochondrial carrier substrate binding site proposed by Robinson and Kunji (29). These authors followed an approach in which extensive sequence comparisons between mitochondrial carriers were combined with the application of distance and chemical constraints, as well as modeling onto the known ADP/ATP carrier crystal structure (17), to develop a proposed common substrate binding site for members of the mitochondrial carrier family. With the CTP, they propose that a single substrate binding site exists that is formed by residues Lys-83, Arg-181, Gln-182, Arg-276, and Arg-279. Although we believe that the power of their approach is demonstrated by their identification of several key residues that are consistent with our data (e.g. Lys-83, Arg-181, Arg-276, and Arg-279), the limitations of their strategy are also apparent. For example, data from our function-based approach clearly demonstrate that major shifts in $K_m$ occur upon mutation of several additional residues (i.e. Arg-87, Gly-119, Arg-189, Lys-37, and Lys-239) and thus implicate the involvement of these residues in substrate binding. Furthermore, our functional data are fully complemented by molecular modeling, which indicates that citrate, the transported substrate, can be effectively docked with two clusters of residues that are topographically distinct and can readily form two separate substrate binding sites. Although the existence of two binding sites was unexpected, we believe it is the conclusion most thoroughly supported by all of our data. Moreover, although our data indicate that Gln-182 may play a minor role in the shape of binding site one, it is not a major determinant of the site as proposed by Robinson and Kunji (29), because muta-
Identification of Substrate Binding Sites within the CTP

Three other points regarding the CTP substrate binding sites merit comment. First, our findings suggest that CTP sites one and two approximately flank the midpoint of the bilayer with site one residing at a depth approximately one-third of the way into the lipid bilayer and site two residing approximately two-thirds into the bilayer. This is in agreement with the depth of the hypothetical common mitochondrial carrier substrate binding site proposed by Robinson and Kunji (29), as well as the approximate midpoint location that has been proposed for the substrate binding site of the lactose permease (30) and the Na\(^+\)/Cl\(^-\)-dependent neurotransmitter transporter (31). Second, our proposal of multiple substrate binding sites within the CTP is not without precedence in other channel and transport proteins. For example, the K\(^+\) channel contains four K\(^+\) binding sites that span a distance of 12 Å (32). This arrangement of ion binding sites is thought to enable rapid conduction, because it creates the shortest path both for diffusion across the bilayer and for repulsion between sequential ions in line (32). Similarly, the bacterial Cl\(^-\)/H\(^+\) exchange transporter is thought to contain three anion binding sites (31). Third, a comparison of the CTP citrate binding sites with the citrate binding site within the enzyme citrate synthase from Pyrococcus furiosus (33) shows that, in the latter case, citrate binds via interaction with three arginine side chains, three histidine residues, and an aspartate, forming a total of seven hydrogen bonds. This agrees well with the six and eight hydrogen bonds thought to form in CTP sites one and two, respectively, and suggests the occurrence of a similar extent of ligand-protein interaction in all three cases.

An important finding from the present studies is that restoration of positive charge by chemical modification of Cys substitution mutants with MTSEA resulted in total (K37C and K239C) or substantial (K83C, R87C, R181C, R189C, R276C, and R279C) restoration of \(K_{\text{m}}\) values. The reduction in \(K_{\text{m}}\) values ranged from 4- to 128-fold (see Table 2). As depicted in Fig. 6, the distribution of partial positive charge among protons is roughly similar in Arg, Lys, and Cys-MTSEA. Moreover, the lengths and molecular volumes of these side chains are similar. Thus, addition of positive charge, distributed in a manner that more or less resembles the native residue, appears to be sufficient to restore the citrate binding function of the CTP. Interestingly, the \(V_{\text{max}}\) value is not substantially restored upon modification of any of the mutants. These results suggest that, although substrate can bind to the MTSEA-modified CTP, some characteristic of either the chemistry and/or the steric and torsional properties of the restored positive charge at a given site is insufficiently native-like to enable the triggering of the conformational changes that constitute the transport event. We also cannot rule out the possibility that, with the mutants, substrate may be bound in a non-native manner that is ineffective at triggering the required conformational changes. For example, this may include the inability to facilitate the movement of citrate from one site to the next. Suffice it to say that transport appears to be exquisitely sensitive to the precise characteristics of positive charge at these sites. We speculate that perhaps it is the introduction of a disulfide bond (formed between cysteine and the MTS reagent) in place of a carbon–carbon bond that somehow limits the required conformational changes, perhaps due to the more limited angular geometry of the former (34). The fact that the \(V_{\text{max}}\) is dramatically reduced upon mutation of each of the binding site residues raises the possibility that they all may be involved in the conformational changes that are captured in the \(V_{\text{max}}\) parameter. Relatedly, with another member of this transporter family (i.e. the mitochondrial ADP/ATP carrier), substrate binding also manifests a lower specificity than does transport (35). Thus, a variety of substrate analogues are recognized by the substrate binding site and can bind with high affinity but nonetheless are not transported. Again this suggests that effective triggering of the conformational changes comprising the transport event requires a much higher stereochemical specificity than does substrate binding.

In conclusion, analysis of the kinetics data obtained in the present investigations in the context of our homology-modeled CTP structure has provided the first function-based identification of the likely substrate binding sites within a mitochondrial metabolite carrier. We plan to continue the merging of functional and structural approaches as we attempt to identify the gate(s) of the translocation pathway, the conformational changes that occur during the transport cycle, a potential third

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Comparison of the properties of arginine, lysine, glutamate, and MTS-modified cysteine side chains. All side chains are shown in fully extended conformations. Volumes (in Å\(^3\)) were calculated for side chains starting with the β-carbon in MOE using standard van der Waals radii. Partial atomic charges of selected protons and oxygen atoms (calculated in MOE using MNDO parameters) are indicated with arrows, to indicate the degree to which MTS-modified side chains are similar to or different from the natural amino acids. Oxygen atoms are depicted in red, nitrogens in blue, sulfurs in yellow, carbons in dark gray, and hydrogens in light gray.}
\end{figure}
binding site that may confer substrate specificity upon approach from the opposite side (i.e., the matrix side) of the membrane, and the location of the monomer–monomer interface in this putatively dimeric transport protein.

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