Utilizing site-directed mutagenesis in combination with chemical modification of mutated residues, we have studied the roles of cysteine and arginine residues in the mitochondrial citrate transport protein (CTP) from *Saccharomyces cerevisiae*. Our strategy consisted of the sequential replacement of each of the four endogenous cysteine residues with Ser or in the case of Cys73 with Val. Wild-type and mutated forms of the CTP were overexpressed in *Escherichia coli*, purified, and reconstituted in phospholipid vesicles. During the sequential replacement of each Cys, the effects of both hydrophilic and hydrophobic sulfhydryl reagents were examined. The data indicate that Cys73 and Cys256 are primarily responsible for inhibition of the wild-type CTP by hydrophilic sulfhydryl reagents. Experiments conducted with triple Cys replacement mutants (i.e. Cys192 being the only remaining Cys) indicated that sulfhydryl reagents no longer inhibit but in fact stimulate CTP function 2–3-fold. Following the simultaneous replacement of all four endogenous Cys, the functional properties of the resulting Cys-less CTP were shown to be quite similar to those of the wild-type protein. Finally, utilizing the Cys-less CTP as a template, the roles of Arg181 and Arg189, two positively charged residues located within transmembrane domain IV, in CTP function were examined. Replacement of either residue with a Cys abolishes function, whereas replacement with a Lys or a Cys that is subsequently covalently modified with (2-aminoethyl) methanethiosulfonate hydrobromide, a reagent that restores positive charge at this site, supports CTP function. The results clearly show that positive charge at these two positions is essential for CTP function, although the chemistry of the guanidinium residue is not. Finally, these studies: (i) definitely demonstrate that Cys residues do not play an important role in the mechanism of the CTP; (ii) prove the utility of the Cys-less CTP for studying structure/function relationships within this metabolically important protein; and (iii) have led to the hypothesis that the polar face of α-helical transmembrane domain IV, within which Arg181, Arg189, and Cys192 are located, constitutes an essential portion of the citrate translocation pathway through the membrane.

The mitochondrial citrate transport protein (CTP) from higher eukaryotes catalyzes an obligatory exchange of citrate plus a proton across the mitochondrial inner membrane for either another tricarboxylate + H⁺, a dicarboxylate, or phosphoenolpyruvate (1). The CTP plays an essential role in intermediary metabolism in that it supplies the cytoplasm with citrate, which can then function as a carbon source for fatty acid and sterol biosyntheses and can also generate NAD⁺ for use in glycolysis (2–5). Because of its importance, the CTP has been extensively characterized. Thus, it has been purified and reconstituted (6, 7), kinetically characterized (8), cloned (9), and overexpressed (10). More recently, the mitochondrial CTP from the yeast *Saccharomyces cerevisiae* has been identified via overexpression followed by functional reconstitution of the purified protein product (11).

In the present paper we report the construction and optimization of a cysteine-less (i.e. Cys-less) yeast mitochondrial CTP, which upon overexpression and functional reconstitution displays properties that are quite similar to the wild-type transporter. We also report novel effects of sulfhydryl reagents on the functioning of single, double, and triple Cys replacement mutants that were sequentially constructed during the development of the final Cys-less CTP. Finally, we report the use of the Cys-less CTP to probe the roles of Arg181 and Arg189 in the transport mechanism. This work not only demonstrates that cysteines are not essential to the CTP translocation mechanism but importantly: (i) provides insight as to which cysteines are responsible for sulfhydryl reagent-mediated inhibition; (ii) demonstrates the suitability of the Cys-less CTP for a variety of structure/function analyses; and (iii) provides important new information regarding the role of positive charge and Cys192 within transmembrane domain IV of the CTP.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis, Overexpression, and Isolation of the Citrate Transport Protein Mutants—** Mutant CTP genes were prepared utilizing the PCR Site-Directed Mutagenesis System (Life Technologies, Inc.). This system combines the use of PCR to introduce the desired mutation followed by uracil DNA glycosylase cloning. The PCR amplifications and the subsequent cloning steps were carried out according to the manufacturer's instructions. Mutagenic primers were designed to yield the following codon replacements. All wild-type cysteines (i.e. residues 28, 73, 192, and 256) were encoded by TCT and were replaced with the TGC codon for Ser. In later experiments, the Ser192 codon (TCT) was further mutated to the Val codon (GTG). The Arg181 and Arg189 codons (AGA) were mutated to either the Cys encoding TGT codon (TCT) was further mutated to the Val codon (GTG). The Arg181 and Arg189 codons (AGA) were mutated to either the Cys encoding TGT codon...
or the Lys encoding AAA. All mutagenic primers were analyzed with Oligo 5.0 software and were purchased commercially. PCR amplifications, DpnI treatment, the addition of the pAM21 cloning vector, and the subsequent transformation of Max Efficiency DH5α competent cells (Life Technologies, Inc.) were carried out exactly as detailed by the manufacturer. The resulting recombinant plasmids were amplified in cells via direct colony PCR and restriction digestion of purified plasmid DNA. Plasmids of positive clones were purified with the Wizard MiniPrep DNA purification system (Promega) and restriction digested with NdeI and BamHI. The excised fragment was then purified from an agarose gel using the GeneClean II kit (Bio 101, Inc.) and directionally ligated into a similarly treated pBluescript plasmid. Mutagenized CTP genes were subsequently subcloned into the pET-21a vector (Novagen) and expression (i.e. BL21(DE3)) was confirmed by sequencing both strands. The overexpressed, isolated wild-type and mutant CTPs were overexpressed in Escherichia coli, and were subsequently extracted from isolated inclusion bodies with the detergent sarkosyl and then functionally reconstituted in a liposomal system. We have previously described this methodology in detail (10, 11) and have shown that it results in abundant quantities of wild-type CTP with a purity of approximately 75% which is highly functional when incorporated into liposomal vesicles (i.e. it displays essentially native kinetic parameters, substrate specificity, and inhibitor sensitivity). Third, the effects of both hydrophilic and hydrophobic sulfhydryl reagents on the function of each mutant were determined.

**Effect of Sulfhydryl Reagents on the Function of the Cys Replacement Mutants**—During construction of the Cys-less CTP, as Cys residues were sequentially replaced with Ser, we examined the ability of sulfhydryl reagents to inhibit CTP function. As depicted in Table I, both hydrophilic, relatively membrane-impermeant sulfhydryl reagents (i.e. mersalyl, pCMBS, MTSEA, MTSET, and MTSES) (12, 17, 21) and pCMB, a more hydrophilic membrane-permeable sulfhydryl reagent (17, 21) totally inhibited the function of the wild-type CTP, as well as the C28S variant. Thus, it is reasonable to conclude that Cys is not primarily responsible for sulfhydryl reagent-mediated inhibition of CTP function in the wild-type transporter. It should be pointed out that with our transport assays, greater than 100% inhibition simply indicates that a given reagent is more effective than 1,2,3-benzene tricarboxylate (i.e. BTC; the classical, defining inhibitor of the mitochondrial citrate carrier (1, 22)) at inhibiting citrate/citrate exchange. This is not surprising because BTC is a competitive inhibitor and thus, under the conditions of this assay (i.e. approximately a 10-fold excess of BTC over citrate), an agent that effectively inhibits the CTP by chemical modification would be expected to yield slightly greater inhibition than that observed with BTC. In fact, the BTC-inhibited inhibition of the CTP is approximately 80–85% of the inhibition observed with the most effective of the sulfhydryl reagents tested.

With the C256S mutant, nearly complete inhibition is observed with certain reagents (i.e. mersalyl, pCMBS, and MTSEA), but inhibition by MTSET, MTSES, or a low concentration of pCMBS (i.e. the most hydrophilic of the reagents tested)
is considerably reduced. This finding lends support to our contention that Cys\textsuperscript{256} is located in a hydrophilic interhelical domain (Fig. 1), which is accessible to the aqueous medium and is significantly, but not exclusively, responsible for the inhibition mediated by hydrophilic sulfhydryl reagents of the wild-type transporter.

Upon subsequent construction of the C28S/C73S/C192S/C256S double replacement mutant, we observed a drastic reduction in the ability of a low concentration of pCMBS (0.1 mM) to inhibit function relative to the C28S single replacement construct. However, a high concentration of this reagent (2 mM) still yielded total inhibition. We also observed a moderate decrease in the sensitivity of the transporter to MTSET and MTSES. Thus we conclude that Cys\textsuperscript{73} confers sensitivity to a low concentration of pCMBS and is partially responsible for inhibition observed with MTSET and MTSES. Interestingly, because Cys\textsuperscript{73} is located within \&eta;-helical transmembrane domain II (Fig. 1), our findings suggest that this residue resides within a water-accessible face of this helix. It is tempting to speculate that this domain may constitute a portion of the substrate translocation pathway through the CTP. Finally, it is important to note that the binding of mersaryl, pCMBS, MTSEA, or a high concentration of the reagent tested (i.e. Cys\textsuperscript{192} and Cys\textsuperscript{256}) still yielded total inhibition. Cys-less construct was observed independent of the charge or the hydrophobicity of the replacement residue.

Upon subsequent replacement of a third Cys (i.e. Cys\textsuperscript{256}) with Ser, a most remarkable finding was obtained. As depicted in Table II, sulfhydryl reagents no longer inhibit the function of the C28S/C73S/C256S triple Cys replacement mutant, but in fact caused a 2–3-fold stimulation in CTP activity. The effect was observed independent of the charge or the hydrophobicity of the reagent tested. To examine whether the use of Ser as a replacement residue was responsible for this effect, we substituted Ala at position 256. Again sulfhydryl reagents were found to cause a substantial stimulation of function. Thus, it is the removal of Cys\textsuperscript{256} \textit{per se} rather than the characteristics of the replacement Ser residue that is responsible for the observed stimulation. Related to this, it is important to note that because the $V_{\text{max}}$ of the triple mutant is considerably reduced compared with the wild-type value (see below), the interaction of sulfhydryl reagents with the single remaining Cys in this construct (i.e. Cys\textsuperscript{192}) in effect restores a portion of the original wild-type activity.

\textbf{Functional Characterization of the Triple Cys Replacement Mutant—}We thought our observation that the binding of sulfhydryl reagents to Cys\textsuperscript{192} (in the triple Cys replacement mutant) resulted in a stimulation in CTP function was of sufficient interest to warrant further characterization. Accordingly we determined the kinetic properties of this construct both before and after modification of Cys\textsuperscript{192} with pCMBS. As depicted in Table III, prior to modification with pCMBS, the mutant displays $K_m$ and $V_{\text{max}}$ values that are substantially reduced compared with the wild-type transporter (i.e. $K_m$ of 0.10 versus 0.33 mM; $V_{\text{max}}$ of 0.26 versus 2.74 $\mu$mol/min/mg; triple Cys replacement mutant versus wild-type CTP; note that wild-type values are presented in Table IV). The addition of pCMBS to the triple Cys replacement mutant caused a 2.2-fold increase in the $K_m$ and a 2.5-fold increase in the $V_{\text{max}}$ values, leaving the $V_{\text{max}}/K_m$ ratio essentially unaltered but rendering the kinetic properties of the transporter somewhat closer to the wild-type values.

Finally, a series of preliminary experiments (data not shown) indicated that prior to modification, the triple Cys replacement mutant displays a very strict specificity for citrate (much stricter than the wild-type transporter) such that even isocitrate is only moderately effective at inhibiting the \textsuperscript{14}C citrate/citrate exchange. Subsequent covalent modification of Cys\textsuperscript{192} with pCMBS caused a relaxation of the substrate specificity to values much more similar to those observed with the wild-type transporter. In summary, modification of Cys\textsuperscript{192} with sulfhydryl reagents causes an increase in the $K_m$ and $V_{\text{max}}$ values of the transporter and a relaxation of its substrate specificity. These results, together with our proposed topography model (Fig. 1), are consistent with the notion that Cys\textsuperscript{192} resides either within or near the entrance to the CTP translocation pathway and that introduction of charge and/or mass at this site causes an important (possibly local) conformational change.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\multicolumn{2}{|c|}{Sulfhydryl reagent} & \multicolumn{3}{c|}{Inhibition of citrate transport} \\
\hline & Concentration & Wild-type & C28S & C256S & C28S/C256S \\
\hline & mM & % & % & % & % \\
\hline Mersaryl & 2 & 102 & 117 & 103 & 121 \\
pCMBS & 2 & 104 & 119 & 103 & 123 \\
pCMBS & 2 & 99 & 101 & 92 & 98 \\
MTSEA & 0.1 & 78 & 68 & 39 & 9 \\
MTSET & 2 & 111 & 108 & 103 & 113 \\
MTSES & 1 & 116 & 109 & 29 & 63 \\
MTSES & 9 & 119 & 112 & 59 & 72 \\
\hline
\end{tabular}
\caption{Effect of sulfhydryl reagents on wild-type and mutated CTP function}
\end{table}
we directly measured the quantity of added wild-type and Cys-less CTP that actually incorporated into the phospholipid vesicles. This was accomplished by subjecting the final proteoliposomes to a high speed centrifugation followed by assay of the supernatant and pellet fractions for protein. Our data indicate that with the wild-type and Cys-less CTPs $85\pm61\%$ and $80\pm61\%$ (mean of four independent experiments $\pm$ S.E.) of the added protein incorporated into the liposomes, respectively. Thus, we conclude that both CTPs incorporate to a similar extent.

The substrate specificity of the Cys-less CTP was examined by measuring the ability of a high concentration (20 mM) of different external anions to compete with the $[14C]$citrate/citrate exchange. As depicted in Table V, our results indicate that the Cys-less CTP displays a very similar specificity compared with the wild-type CTP. Thus, citrate and isocitrate effectively inhibited the citrate/citrate exchange catalyzed by both constructs and thus function as the primary substrates of this carrier. In contrast, other anions assayed, such as $\alpha$-ketoglutarate, succinate, ADP, and pyruvate display some ability to compete with $[14C]$citrate/citrate exchange with the wild-type transporter, they display no such ability with the Cys-less carrier.

We also examined whether the Cys-less transporter maintains a strict requirement for intraliposomal citrate as has been previously observed with the wild-type carrier (11). Accordingly, the CTP was incorporated into liposomes both in the

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**TABLE II**

Effect of sulfhydryl reagents on the function of CTP triple Cys replacement mutants

| Sulfhydryl reagent | Concentration | Citrate transporter activity |
|-------------------|---------------|-----------------------------|
|                   |               | C28S/C73S/C256S | C28S/C73S/C256A |
| Mersalyl          | 2.0          | 208                   | 195               |
| pCMB              | 2.0          | 199                   | 203               |
| PCMBBS            | 2.0          | 217                   | 202               |
| Mersalyl          | 0.1          | 224                   | 174               |
| pCMB              | 0.1          | 216                   | 191               |
| PCMBBS            | 0.1          | 270                   | 203               |
| MTSEA             | 2.3          | 216                   | 205               |
| MTSET             | 0.9          | 210                   | 152               |

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**TABLE III**

Effect of pCMBS on the kinetic properties of the CTP triple mutant construct

The overexpressed yeast mitochondrial CTP triple mutant construct (C28S/C73S/C256S) was extracted from inclusion bodies and functionally reconstituted in liposomes. Following a preincubation with either 0.5 mM pCMBS or water for 10 min, initial rates of BTC-sensitive citrate transport were then measured at 30°C in the presence of varying concentrations of external $[14C]$citrate (i.e. 0.1–1.0 mM). Data obtained from four independent measurements at each of nine different substrate concentrations were averaged and analyzed via Lineweaver-Burk plot, and a best fit line was constructed based on linear regression analysis via the method of least squares.

| CTP variant | Condition | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|-------------|-----------|-------|-----------|---------------|
|             |           | mM    | $\mu$mol | $\mu$mol/min/mg|
|             |           | min/mg| $mg/\mu$mol$^{-1}$|
| C28S/C73S/C256S | pCMBS | 0.10  | 0.26     | 2.6           |
| C28S/C73S/C256S | + pCMBS | 0.22  | 0.64     | 2.9           |
The overexpressed yeast mitochondrial wild-type and Cys-less CTPs were extracted from inclusion bodies and functionally reconstituted in liposomes in the presence of 48 μM citrate. Transport reactions were carried out for 10–30 s at 30°C in the presence of 0.1–1.0 mM external [14C]citrate as described under "Experimental Procedures." Six to eight substrate concentrations were employed for each mutant. Data obtained from 8 to 12 incubations with each substrate concentration (except for the C28S/C73V/C192S/C256S, for which data was obtained from duplicate incubations at each substrate concentration) were averaged and analyzed via Lineweaver-Burk plot, and a best fit line was constructed based on linear regression analysis via the method of least squares.

### TABLE IV
Comparison of the kinetic parameters of the wild-type and three different Cys-less CTP constructs

| CTP variants     | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | Wild-type activity % | $V_{max}$/$K_m$ (μmol/min/mg·mM$^{-1}$) |
|------------------|------------|-------------------------|----------------------|-----------------------------------------|
| Wild-type CTP    | 0.33       | 2.74                    | 100                  | 8.3                                     |
| C28S/C73V/C192S/C256S (i.e. initial Cys-less construct) | 0.12       | 0.17                    | 6                    | 1.4                                     |
| C28S/C73V/C192S/C256S | 0.15       | 1.14                    | 42                   | 7.6                                     |
| C28S/C73V/C192S/C256S (i.e. final Cys-less construct) | 0.25       | 1.71                    | 63                   | 6.8                                     |

### TABLE V
Comparison of the substrate specificity of wild-type versus Cys-less CTPs

| Competing anion       | Wild-type Activity | Cys-less Activity | % Activity remaining |
|-----------------------|--------------------|-------------------|----------------------|
| Citrate               | 13 ± 3             | 11 ± 2            | 100                  |
| Isocitrate            | 33 ± 5             | 25 ± 5            | 100                  |
| Phosphoenolpyruvate   | 71 ± 8             | 80 ± 3            | 100                  |
| α-ketoglutarate       | 82 ± 6             | 100 ± 4           | 100                  |
| Succinate             | 91 ± 3             | 110 ± 4           | 100                  |
| ADP                   | 85 ± 7             | 100 ± 6           | 100                  |
| Pyruvate              | 92 ± 6             | 107 ± 5           | 107                  |
| Malonate              | 98 ± 4             | 107 ± 4           | 107                  |
| Malate                | 100 ± 9            | 96 ± 5            | 96                   |

### TABLE VI
Effect of sulfhydryl reagents on wild-type versus Cys-less CTP function

| Reagent     | Concentration (mM) | Wild-type Activity (μmol/min/mg) | Cys-less Activity (μmol/min/mg) | % Inhibition |
|-------------|--------------------|----------------------------------|--------------------------------|--------------|
| Mersalyl    | 0.5                | 96 ± 1                           | 1 ± 3                          |              |
| pCMB        | 0.5                | 102 ± 1                          | 3 ± 1                          |              |
| pCMBS       | 0.5                | 90 ± 1                           | 0 ± 1                          |              |
| MTSEA       | 2.5                | 103 ± 2                          | -8 ± 2                         |              |
| MTSET       | 1.0                | 113 ± 3                          | 0 ± 2                          |              |
| MTSES       | 10.0               | 118 ± 2                          | 6 ± 1                          |              |

Overall, this suggests that positive charge at this site may play a role that is essential for the functioning of members of this family. Third, in contrast to Arg$^{181}$, Arg$^{189}$ is conserved among CTPs from several organisms of moderate evolutionary distance (11) but is not conserved among other mitochondrial anion carriers,$^2$ suggesting that this residue carries out a function unique to the CTP. With these two residues of interest, the following experiments were carried out. First, each residue was replaced with Cys. As depicted in Table VII upon the replacement of either Arg with Cys, CTP function is totally lost, suggesting that some property of each arginine is essential to CTP function. Upon replacement of either Arg with Lys, significant function is retained. Most interestingly, upon replacement of either Arg with Cys followed by covalent modification of the Cys with MTSEA, a positively charged hydrophilic cysteine-specific reagent, a degree of function is restored. There is in fact precedence for this type of effect in studies carried out with another metabolite transporter (i.e. the lac permease (23)). In contrast when the larger reagent MTSET or the negatively charged MTSES is employed, CTP function is not restored. Taken together, the data indicate that the chemistry of the guanidinium group is not essential for CTP function. However, positive charge at both of these two sites is essential. Moreover, it appears that the precise three-dimensional orientation of the positive charge and/or the bulk of the side chain is important (see "Discussion").

Finally, experiments were conducted with the R189C transporter in which the ability of alkylamines of varying size to rescue transporter function was examined. The idea behind these experiments was that noncovalent placement of positive charge throughout much of the CTP might be able to effectively

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$^2$ Optimized alignment of mitochondrial transporter sequences carried out by A. M. Dean (personal communication).
neutralize a potentially unpaired residue with which Arg<sup>189</sup> would normally have interacted (24). We observed no significant rescue of CTP function by the alkylamines (data not shown).

**DISCUSSION**

The main goals of the present investigation were 3-fold, namely: (i) a determination of whether any of the four endogenous cysteine residues are critical to the CTP transport mechanism, as well as which residues are responsible for the well documented sulfhydryl reagent-mediated inhibition of CTP function; (ii) the construction of a cysteine-less CTP that displays wild-type functional properties; and (iii) the subsequent use of the Cys-less construct to probe the roles of Arg<sup>189</sup> and Arg<sup>189</sup> in CTP function. In the process of attaining these goals significant new information concerning molecular aspects of CTP function has been obtained as detailed below.

Our data concerning the effects of hydrophobic and hydrophilic sulfhydryl reagents on the function of the various Cys replacement mutants permit several interesting conclusions. First, based on our observation that the double C28S/C73S mutant is considerably less sensitive to inhibition by hydrophobic sulfhydryl reagents (e.g. MTSET, MTSES) and a low concentration of pCMBS) compared with the single C28S mutant (Table I), we conclude that Cys<sup>73</sup> is likely located in a water-accessible domain within transmembrane domain II and that its modification is partly responsible for the observed inhibition of the wild-type transporter with hydrophilic sulfhydryl reagents. Although it is tempting to speculate that Cys<sup>73</sup> may actually reside on a face of a-helical transmembrane domain II that constitutes a portion of the citrate translocation pathway, an unambiguous determination of this will clearly require further study. It should also be noted that our observation that the C28S/C73S double mutant can still be completely inhibited by other hydrophilic reagents (i.e. mersalyl, PCMB, MTSEA, and a high concentration of pCMBS) clearly implicates involvement of one or both of the remaining two cysteines (i.e. Cys<sup>192</sup> and/or Cys<sup>256</sup>) in the sulfhydryl reagent-mediated inhibition observed with the wild-type transporter. The data presented in Table I support involvement of the latter residue. Thus, with the C256S single replacement mutant, we also observe a considerable diminution of the inhibition observed with the hydrophilic sulfhydryl reagents. As depicted in Fig. 1, Cys<sup>256</sup> is predicted to reside within an interhelical loop that is likely to reside in a hydrophilic milieu. It is noteworthy that the presence of a Cys in the loop connecting helices V and VI appears to be characteristic of other members of the mitochondrial anion carrier family (e.g. the ADP/ATP translocase (25) and the uncoupling protein (26)), suggesting that it may play an important structural role. Taken together, our data suggest that residues Cys<sup>73</sup> and Cys<sup>256</sup> are primarily responsible for inhibition observed with the hydrophilic sulfhydryl reagents.

The observation that the triple Cys replacement mutants C28S/C73S/C256S and C28S/C73S/C256A were no longer inhibited but in fact could be stimulated by sulfhydryl reagents was quite unexpected. This increase was observed with both hydrophobic as well as hydrophilic reagents and was independent of the charge of the reagent. Thus, although sulfhydryl reagents typically inhibit transporter function via a steric blockage of either substrate binding and/or the translocation pathway, it appears that with these mutants the modification of Cys<sup>192</sup> has actually enhanced the V<sub>max</sub> of the transporter. Further investigation indicated that both the K<sub>m</sub> and V<sub>max</sub> are proportionally increased and that the substrate specificity is relaxed slightly. As depicted in Fig. 1, Cys<sup>192</sup> is thought to reside near the entrance of transmembrane domain IV into the lipid bilayer. We postulate that this residue may reside near the entrance of the translocation pathway such that a small conformational change in this region can substantially alter transporter function. Two related points merit comment. First, modification of the triple Cys replacement mutant renders its function more similar to the wild-type transporter than prior to its modification. Thus, it appears that the addition of charge and/or mass to Cys<sup>192</sup> partially compensates for the deleterious effects of multiple Cys replacements by Ser. Second, there is an example of a similar type of effect in the literature; namely, with the mitochondrial phosphate carrier, it has been shown that modification of a single Cys causes the transporter to convert from an antiport to a uniport mode (19).

The data presented in Tables IV–VI clearly demonstrate that upon simultaneous replacement of all four endogenous cysteines with either Ser or Val, the final Cys-less CTP displays functional properties (i.e. K<sub>m</sub>, V<sub>max</sub>, substrate specificity, requirement for intraliposomal substrate) that are very similar to those observed for the wild-type transporter. Thus, for all intents and purposes the Cys-less CTP function mimics wild-type transporter function, and we therefore conclude that structure/function relationships determined for the Cys-less transporter are likely to be directly applicable to the wild-type carrier. This conclusion provides the foundation for the studies presented in the next portion of this paper, as well as for much of our future mutagenesis program. Our results also clearly demonstrate that none of the four endogenous cysteines are required for CTP function. Thus, the well documented observation that cysteine-specific reagents abolish wild-type CTP activity (Table I) likely arises as a consequence of a steric effect but certainly is not due to the functional importance of the cysteinyl residue in either the secondary structure or the transport mechanism of the CTP. In this regard, the CTP is similar to several other carriers (e.g. the lac permease (16), the UhpT anion exchange protein (17), the mitochondrial phosphate transporter (19), and the uncoupling protein (20) that have been studied in this manner.

It is interesting that Cys<sup>73</sup> is quite sensitive to the nature of the replacement residue such that Val or Ala are much less disruptive of CTP function compared with Ser (Table IV). Thus placement of a residue with increased H bonding capacity and hydrophilicity compared with the starting Cys in transmem-
brane α-helix II is not well tolerated. Although the exact cause for this deleterious effect is not clear at this time, there is precedence for this type of effect with other transport proteins (e.g. the lac permease (16) and the UhpT anion exchange protein (17)).

With the Cys-less CTP in hand, our first mutagenesis experiments have focused on two positively charged residues, namely Arg¹⁸¹ and Arg¹⁸⁹. These were of particular interest because of their intramembranous location, plus the fact that at position 181 an Arg or Lys is found in many different mitochondrial anion transporters. In contrast, at position 189 an Arg is conserved among mitochondrial CTPs from several species but not among other mitochondrial anion carriers. Thus, both residues might be of mechanistic importance, 181 being involved in a function characteristic of most of the anion carriers and 189 being involved in a function specific for the CTP.

Utilizing a combination of site-directed mutagenesis and sulfhydryl group modification, our studies clearly indicate that although the chemistry of the guanidinium group is not required at either site, positive charge is absolutely required at both sites. Thus at either position, arginine, lysine, and Cys-MTSEA-modified Cys, all support CTP function to varying extents. As depicted in Fig. 2, within each of these side chains the positive charge is primarily distributed among several hydrogen atoms that reside approximately 5–10 angstroms from the peptide backbone. In contrast the MTSET-modified Cys, which does not support CTP function, displays a considerably more delocalized positive charge as well as a bulkier side chain. For example the molecular volumes of Arg, Lys, and Cys-MTSEA range from 106 to 125 Å³, as compared with the much larger Cys-MTSET (173 Å³). Thus it appears that a combination of charge distribution and/or steric size and shape may be important. Furthermore, the lack of CTP activity with the negatively charged, intermediately sized Cys-MTSES derivative suggests that the nature of the charge at this site is important, although a steric explanation is also plausible.

It is of interest to note that preincubation of proteoliposomes containing the R189C transporter with a high concentration of alkylamines (of varying size) did not rescue CTP function. The rationale for these studies originated from investigations with the lac permease (23), which showed that following the mutation of an intramembranous positively charged residue to a neutral residue and the resulting inactivation of transport because of the generation of an uncompensated charge, alkylamines could subsequently rescue transporter function by mimicking the missing charged side chain and providing the required charge neutralization. However, rescue occurred only when the initial residue (prior to mutation) was not essential or important to the transport mechanism. This observation, in combination with our findings that: (i) alkylamines did not rescue the function of the R189C CTP mutant and (ii) there is an absolute requirement for positive charge at position 189 in the CTP suggests an important mechanistic role for Arg¹⁸⁹ in CTP function.

A related point concerns the fact that the yeast mitochondrial phosphate transporter also contains two positively charged residues (i.e. Lys¹⁷⁹ and Lys¹⁸⁷) that reside within its fourth α-helical transmembrane domain (27). These residues...
are predicted to reside on the same face of an α-helix and similar to our findings with the CTP, amino acid replacements are tolerated as long as positive charge is maintained at each site.

Finally, we believe it important to point out that Arg₁₈₁, Arg₁₈₉, and Cys₁₉₂ are located on the same face of the α-helix comprising CTP transmembrane domain IV, and our data clearly indicate that mutation and/or covalent modification of any one of these residues causes important functional consequences. Taken together, our results support the importance of this helical domain in CTP function and have led to the formulation of a hypothesis, which states that: (i) the polar face of α-helical transmembrane domain IV constitutes a portion of the citrate translocation pathway through the membrane and (ii) Arg₁₈₁ and Arg₁₈₉ may be involved in binding other negatively charged intramembranous residues and/or citrate in a competitive manner, and such interactions constitute an essential step in the antiport reaction and possibly in CTP substrate discrimination. A determination of which negatively charged residues are involved and the potential effect of citrate on such interactions will be the subject of future investigations.

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Mutagenesis of the Mitochondrial Citrate Transporter