Journal of Bioenergetics and Biomembranes, Vol. 36, No. 5, October 2004 (© 2004)

Analysis of the Secondary Structure of the Cys-Less Yeast Mitochondrial Citrate Transport Protein and Four Single-Cys Variants by Circular Dichroism

Michael Cascio,[1] June A. Mayor,[2] and Ronald S. Kaplan[2,3]

Received May 11, 2004; accepted May 25, 2004

Utilizing cysteine scanning mutagenesis, with functional Cys-less citrate transport protein (CTP) serving as the starting template, we previously demonstrated that four single-Cys mutants located in transmembrane domains III and IV, rendered the CTP nonfunctional. The present investigations assess and quantify the secondary structure of the Cys-less CTP and the four single-Cys mutants, both in the absence and presence of citrate, via circular dichroism (CD) spectroscopy. In detergent micelles, highly purified Cys-less CTP contained ~50% α-helix and ~20% β-sheet. The CD spectra of the G119C, E122C, R181C, and R189C mutants in detergent micelles were virtually superimposable with that of the functional Cys-less CTP, thereby suggesting that the wild-type residues, rather than affecting structure, may assume important mechanistic roles. Exogenously added citrate caused a significant change in the CD spectra of all solubilized CTP samples. Analyses of the spectra of the Cys-less CTP indicated an ~10% increase in its α-helical content in the presence of citrate. The conformational changes effected by the addition of substrate were less pronounced with the single-Cys mutants. Studies of the Cys-less CTP reconstituted in liposomes indicated that while the CD spectra was red-shifted, the net secondary structure of the reconstituted carrier is approximately equivalent to that of the transporter in detergent micelles, and displayed a response to added citrate. In combination, the above studies indicate that purified Cys-less CTP in either sarkosyl micelles or in liposomes, and the four inactive single-Cys mutants in sarkosyl micelles, retain native-like structure, and thus represent ideal material for detailed structural characterization.

KEY WORDS: Citrate transporter; mitochondria; circular dichroism; secondary structure; liposomes; membrane proteins.

INTRODUCTION

The mitochondrial citrate transport protein (CTP) carries out an obligatory exchange of anions across the mitochondrial inner membrane. In higher eukaryotes the CTP catalyzes the outward movement of citrate plus a proton across the inner membrane in exchange for either another tricarboxylate-H^+, a dicarboxylate, or phosphoenolpyruvate (Palmieri *et al*., 1972). Citrate then passively diffuses across the outer membrane through the voltage-dependent anion selective channel into the cytoplasm where it serves as the prime carbon source fueling both fatty acid and lipid biosyntheses, and generates NAD^+ for use in glycolysis (Brunengraber and Lowenstein, 1973; Conover, 1987; Endemann *et al*., 1982; Watson and Lowenstein, 1970). Because of the prominence of the CTP in eukaryotic cell intermediary metabolism, as well as its participation in the aberrant metabolism that characterizes certain diseases (Kaplan

[1] Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

[2] Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, The Chicago Medical School, 3333 Green Bay Road, North Chicago, Ilinois 60064.

[3] To whom correspondence should be addressed; e-mail: ronald.kaplan@rosalindfranklin.edu.

Key to abbreviations: CD, circular dichroism; CTP, citrate transport protein; MTS, methanethiosulfonate; NRMSD, normalized root mean standard deviation; sarkosyl, sodium *N*-lauroylsarcosinate; SE, standard error of the mean; and TMD, transmembrane domain.

et al., 1982, 1990b), the transporter has been extensively studied. Thus, the CTP has been purified in reconstitutively active form (Bisaccia *et al*., 1989; Kaplan *et al*., 1990a), kinetically characterized (Bisaccia *et al*., 1990), its sequence determined (Kaplan *et al*., 1993), and expressed in high abundance in *E. coli* (Xu *et al*., 1995).

More recently, our studies have focused on the yeast homologue of the higher eukaryotic protein, since following its overexpression, purification, and incorporation into liposomal vesicles, the yeast mitochondrial CTP displays a high specific transport activity (Kaplan *et al*., 1995; Xu *et al*., 2000). Thus, the yeast CTP represents ideal material with which to conduct a rigorous structure/function analysis. As part of this effort, we have previously demonstrated that the transporter exists as a homodimer in detergent micelles (Kotaria *et al*., 1999) and we have begun to identify those residues in transmembrane domains that form the citrate translocation pathway (Kaplan *et al*., 2000a; Ma *et al*., 2004). Furthermore, cysteine scanning mutagenesis coupled with MTS-labeling studies and functional assay have indicated that at least two of the transmembrane domains have periodicities consistent with α-helical structures.

The objective of the present study was to further our understanding of the secondary structure of the CTP, as well as the conformation of the previously observed inactivating Cys mutations. Accordingly we have analyzed the highly functional Cys-less CTP and four single-Cys mutants in TMDIII and TMDIV that are inactive, via circular dichroism (CD) spectroscopy in order to assign secondary structure and assess the responsiveness of different CTP variants to added citrate. CD spectroscopy is a useful tool for examining the structure of proteins since it is extremely sensitive to small changes in the folding of the peptide backbone and provides quantitative information on the net secondary structure (Johnson, 1988). We sought to address three questions: (i) Do the four single-Cys CTP mutations in TMDs III and IV which completely inactivate CTP function maintain a secondary structure similar to that of the Cys-less CTP; (ii) Does the Cys-less CTP in sarkosyl display a similar secondary structure to transporter incorporated into liposomal vesicles; and (iii) Does the presence of citrate, its natural substrate, cause significant conformational changes in the Cys-less CTP either in vesicles or in solution.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of CTP Variants

The Cys-less CTP and the four single-Cys mutants were overexpressed in *E. coli* and the inclusion body fraction was isolated as previously described (Kaplan *et al*., 1995; Xu *et al*., 1995). CTP was solubilized from the inclusion bodies by incubation with 1.2% sarkosyl and following ultracentrifugation was stored at -80°C. Each CTP variant was purified by sequential chromatography on MonoQ and Sephacryl S-300 exactly as previously detailed (Kaplan *et al*., 2000b) with the exception that 1 mM DTE was included in all buffers. In the present studies an additional chromatographic step was conducted on a Sephacryl S-300 Hi Prep (16/60) column that had been equilibrated with 10 mM Tris–HCI, pH 7.6, 150 mM NaCI, 1 mM DTE, 0.09% (w/v) sarkosyl in order to reduce the quantity of free sarkosyl in the final purified material. The CTP eluted as a single symmetrical peak at approx. 62 mL. The CTP was then concentrated in a Millipore centrifugal filter and stored at -80°C until assay.

Incorporation of CTP Variants Into Phospholipid Vesicles for CD Analysis

The purified, concentrated CTP was desalted on Micro Biospin Columns (BioRad) in order to remove DTE and was incorporated into pre-formed asolectin vesicles via the freeze-thaw-sonication technique as previously described (Kaplan *et al*., 1990a, 1995). The resulting proteoliposomes were diluted and centrifuged at $314{,}000 \times g$ (max) for 45 min at 6°C in order to remove unincorporated protein. The pellet was rinsed and then resuspended on ice in 120 mM Hepes, 50 mM NaCI, 1 mM EDTA, pH 7.4. Protein quantitation was performed via the method of Kaplan and Pedersen (1985). The concentration of free sarkosyl was determined by thin layer chromatography combined with laser densitometry via a procedure recently described in detail (Eriks *et al*., 2003). Since HEPES is an inappropriate buffer for CD studies due to its strong absorption, immediately prior to the CD analysis, proteoliposomes were diluted in excess 10 mM KP_i buffer (pH 7.4) and pelleted by ultracentrifugation for 1 h at $150{,}000 \times g$. The pelleted proteoliposomes were then washed, and gently resuspended in 10 mM KPi buffer (pH 7.4) to provide a final protein concentration of approx. 0.1 mg/mL.

Circular Dichroism Studies

Purified Cys-less CTP and single-Cys variants had a wide range of protein and free sarkosyl concentrations. Samples were diluted such that the final buffer conditions were 5 mM Tris–HCI, pH 7.6, 75 mM NaCI, 0.5 mM DTE, and 0.05% (w/v) sarkosyl (with a final protein concentration of approx. 0.1 mg/mL). All measurements were made using an Aviv 62DS spectropolarimeter and were

taken over the wavelength range from 280 to as low as 198 nm, with a 1 nm step size, at room temperature using a 0.1 cm pathlength quartz cell (Hellma). The presence of sarkosyl, lipids, and salts (and CTP) precluded measurement of CD signals in the lower UV region due to elevated absorbance of the sample. All samples and blanks were also measured in the presence of 10 mM citrate. At least 10 reproducible spectra were collected for each preparation, averaged, and smoothed using a Savitzky-Golay filter (Savitzky and Golay, 1964). All reported spectra were baseline corrected (by subtraction of similarly collected, averaged, and smoothed baselines of appropriate micelles or vesicles identically prepared, except without purified protein). Blank vesicles without protein were prepared in an identical manner to proteoliposomes, except that detergent-containing buffer was added in place of the CTP. The protein-free liposomes were treated equivalently for use as blanks. Given that the determination of protein concentration for membrane proteins has an associated error of approx. 10%, the magnitude of the CD spectra of CTP variants in the absence of citrate were normalized relative to the averaged minimum mean residue ellipticity of all spectra. Unless otherwise noted, the reported spectra are the average of two independently prepared protein purifications. To ensure that the effects of citrate were not due to altered protein concentration or protein degradation over time, aliquots of a 400 mM citrate stock were diluted 40-fold by direct addition to the cuvette immediately after scanning of the matched sample in the absence of citrate, mixed thoroughly, and an additional data set collected. CD signals were appropriately scaled to reflect the slight ($\sim$2.5%) dilution of protein concentration.

The CD spectra of the protein in the near UV region were analyzed using DICHROWEB (Lobley *et al.*, 2002), an interactive website for the analyses of protein secondary structure. Given the constraints in the data range of our collected spectra, analyses of the detergent-solubilized CTP samples were limited to the neural network program K2D (Andrade *et al.*, 1993). For all secondary structure determinations, a normalized root mean standard deviation (NRMSD) value was provided as a measure of the fit of the calculated curve to the experimental data (Mao *et al.*, 1982).

RESULTS

Purification and Characterization of the Cys-Less CTP and the Single-Cys Mutants

In previous studies (Xu *et al.*, 2000), we demonstrated that the Cys-less CTP catalyzes a high magnitude inhibitor-sensitive [^{14}C]citrate/citrate exchange when incorporated into liposomal vesicles. Moreover, we showed that of the 50 single-Cys CTP variants that we constructed by mutating residues in transmembrane domains III and IV, one at a time to cysteine, all but four demonstrated significant function upon incorporation into liposomes (Kaplan *et al.*, 2000a; Ma *et al.*, 2004). Many displayed near normal reconstituted specific transport activity values. However, four of the single-Cys mutants (i.e., G119C, E122C, R181C, and R189C) displayed little or no transport function (i.e., <0.1% residual function). One question we sought to address via CD analysis was whether the lack of function catalyzed by these four mutants arose as a consequence of a profound alteration in their secondary structure.

With this goal in mind, for the present investigations, the overexpressed Cys-less CTP, as well as the four inactive single-Cys CTP variants were further purified via a combination of ion exchange and gel filtration chromatography (see "Experimental Procedures"). As depicted in Fig. 1, CTP variants prepared via this method are quite pure (i.e., approx. 96 $\pm$ 1% purity; mean $\pm$ SE; based on laser densitometric analysis). Thus, we are able to state with confidence that the CD analyses described below do in fact characterize the secondary structural elements of the CTP rather than of a contaminant protein(s).

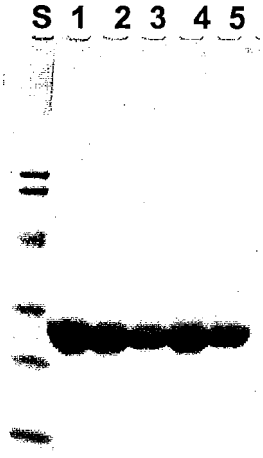

Fig. 1. Coomassie-stained SDS-polyacrylamide gel depicting the purity of the Cys-less CTP and the four single-Cys CTP mutants following purification. Proteins were separated on a precast 14% polyacrylamide gel (Invitrogen) in Tris–glycine buffer. Lane S, 5μL Bio-Rad prestained SDS-PAGE standards: phosphorylase B (108,000), bovine serum albumin (90,000), ovalbumin (50,700), carbonic anhydrase (35,500), soybean trypsin inhibitor (28,600), and lysozyme (21,200). Lanes 1–5, 2 μL (i.e., 5–10 μg protein) of Cys-less CTP, and the R181C, R189C, G119C, and E122C mutants, respectively. Each CTP was purified as described under "Experimental Procedures."

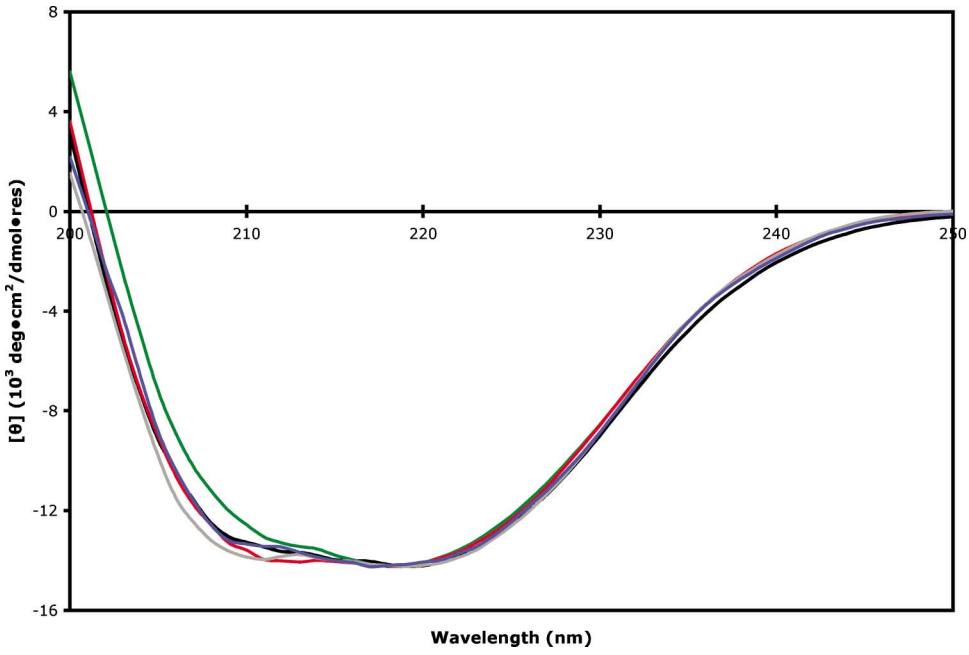

Fig. 2. Comparison of the CD spectra of Cys-less CTP and single-Cys mutants in detergent micelles. Detergent and buffer conditions of purified protein samples were matched by dilution as described in Experimental Procedures and measured over the 280–200 nm range. The spectra of Cys-less CTP (black), G119C (red), E122C (green), R181C (blue), and R189C (gray) in detergent micelles are qualitatively similar and suggest that the net secondary structures of these variants are essentially equivalent, thus indicating that the single-Cys mutations do not appreciably affect the conformation of the CTP.

CD Analysis of the Cys-Less CTP and the Single-Cys CTP Variants in Detergent Solution

In sarkosyl micelles, the Cys-less CTP, as well as the G119C, E122C, R181C, and R189C mutants all displayed qualitatively similar CD spectra (Fig. 2) strongly suggesting they all share a common native-like fold. Spectral deconvolution indicated that these samples contained 43–50% α-helix (Table I). These measurements represent the first information regarding the secondary structure of the CTP in micelles and indicate that the four inactive single-Cys mutants do not display substantive alterations in secondary structure.

Table I. Calculated Secondary Structures of Cys-Less CTP and Single-Cys CTP Mutants in Sarkosyl Micelles

Sample	α-helix	β-sheet	Random	NRMSD
Cys-less CTP	49	18	33	0.096
G119C	50	17	33	0.097
E122C	46	23	31	0.117
R181C	43	20	34	0.090
R189C	46	18	36	0.090
Cys-less CTP + citrate	58	8	34	0.145
G119C + citrate	45	23	31	0.100
E122C + citrate	45	23	31	0.131
R181 C + citrate	46	23	31	0.114
R189C + citrate	43	22	35	0.111

The Effect of Added Citrate on the CD Spectra of CTP in Detergent Micelles

We next examined the effect of the addition of substrate (i.e., citrate) to the Cys-less CTP, as well as to the inactive single-Cys mutants, on transporter secondary structure via CD analysis. As depicted in Fig. 3, exogenously added citrate significantly changed the net spectra of the Cys-less CTP. The spectra showed a substantial reduction in the shoulder at ~210 nm (primarily due to a π to π^* peptide transition) relative to a more pronounced minima at ~222 nm (the n to π^* peptide transition) that was slightly red-shifted relative to the minima observed in the absence of citrate. These changes corresponded to an~10% increase in the calculated α-helical content of the Cys-less CTP (Table I).

As depicted in Figs. 4 and 5, each of the inactivating single-Cys mutations (i.e., G119C, E122C, R181C,

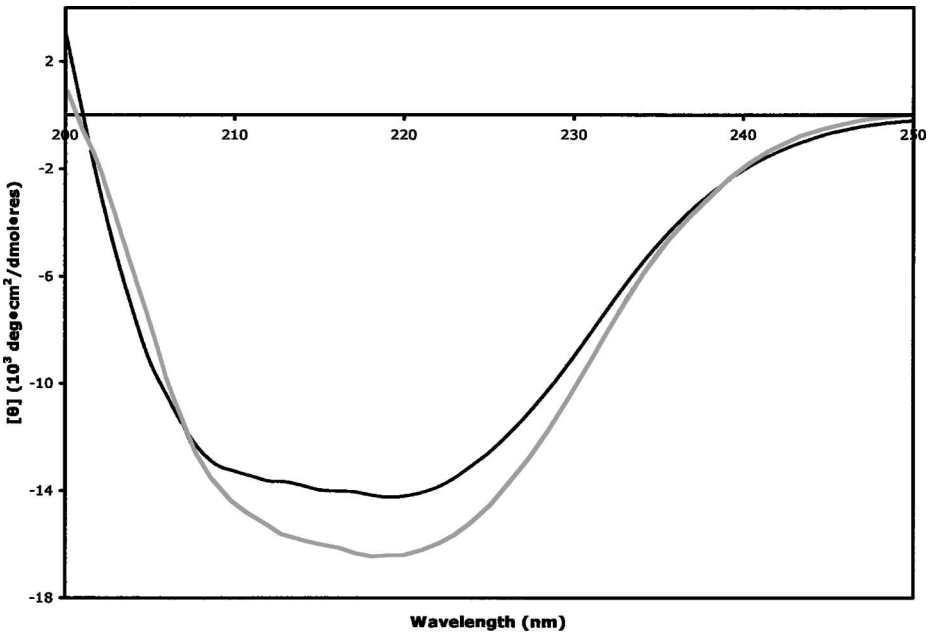

Fig. 3. Effects of added substrate on the CD spectra of Cys-less CTP in detergent micelles. The addition of 10 mM citrate to the Cys-less CTP in sarkosyl micelles (gray) resulted in significant changes compared to the spectra of the protein in the absence of citrate (black), suggesting that interaction of substrate with the CTP results in a change in the net secondary structure of the transporter.

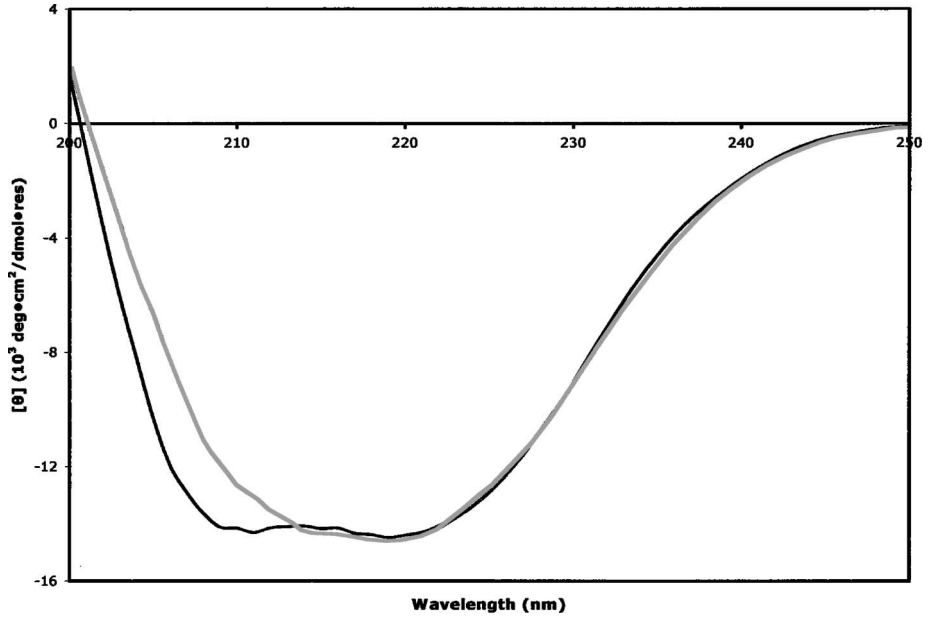

Fig. 4. Effect of added substrate on the CD spectra of the R181C CTP mutant in detergent micelles. Comparison of the CD spectra of the R181C single-Cys CTP mutant in sarkosyl micelles in the presence (gray) and absence (black) of added 10 mM citrate indicates that the interaction of substrate with this CTP variant results in a change in the transporter's net secondary structure. Given the small variations in spectral response upon the addition of citrate amongst the Cys mutants and between preparations of any given mutant, the representative spectra shown are the baseline corrected average spectra from a single preparation.

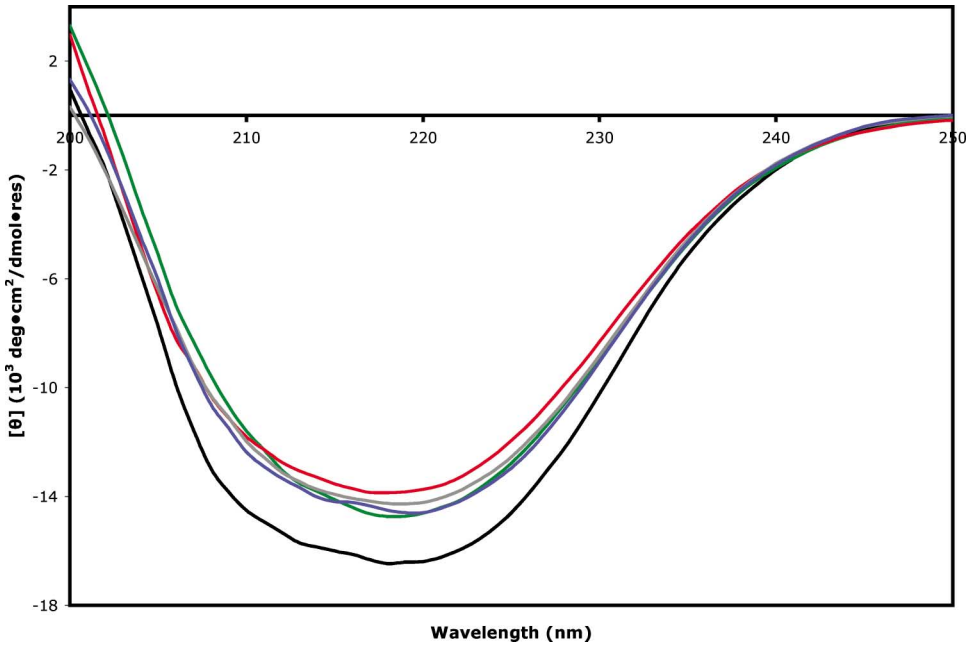

Fig. 5. Effects of substrate on the CD spectra of the Cys-less CTP and single-Cys CTP mutants in detergent micelles. In the presence of 10 mM citrate, the spectra of G119C (red), E122C (green), R181C (blue), and R189C (gray) in detergent micelles were qualitatively similar, but did not show the pronounced effects observed upon the addition of citrate to the Cys-less CTP (black) (as shown in Fig. 3).

R189C) also showed small, but significant, changes in the spectra upon the addition of citrate suggesting that citrate either binds to the CTP in solution and/or occupies the transport pathway. Similar to the changes observed in the spectra of Cys-less CTP (Fig. 3), in the presence of 10 mM citrate the spectra of the single-Cys mutants showed a reduction of the minima of the peptide absorption centered around ~210 nm (relative to the minima at 222 nm), such that the minima which had a pronounced bilobed characteristic in the absence of citrate (Fig. 2), appears to consist of a single minima with a reduced shoulder at the lower wavelength in the presence of citrate (for clarity, a representative spectrum of R181C CTP ± citrate from a single preparation is shown in Fig. 4). It is noteworthy, that upon the addition of citrate there was a less pronounced change in the magnitude of the minima at 222 nm with the single-Cys mutants compared with the Cys-less CTP (e.g., see Figs. 3 and 4), suggesting that the helical content of the mutant forms of CTP remained relatively unchanged (see Table I for calculated secondary structures). Thus, while the significant change in the CD spectra upon addition of citrate suggests that these Cys mutants do interact with citrate with a concomitant change in their structure, the effects of citrate binding on the net secondary structure of the transporter appears to be reduced relative to the effects calculated for the Cys-less CTP.

CD Spectral Analysis of Cys-Less CTP in Liposomes and the Effect of External Citrate

In order to examine whether a native-like structure is retained when the CTP is solubilized, the averaged CD spectra of the Cys-less CTP incorporated into liposomes was compared to that of the Cys-less CTP in detergent micelles. As depicted in Fig. 6, the spectra of the Cys-less CTP in liposomes is qualitatively similar to that of the protein in sarkosyl micelles except that it appears to be relatively red-shifted. This red-shifting is consistent with the difference in the dielectric constants of the protein's microenvironment in liposomes as compared to micelles, since similar effects have been observed with other peptides and proteins where the different absorption peaks giving rise to the CD spectra (the n to π^* transition, as well as the parallel and perpendicular π to π^* transitions of the peptide bond) are shifted to differential degrees (Cascio and Wallace, 1995). In the absence of red-shifting, it appears that the two spectra are similar, if not identical. Given that the Cys-less CTP is fully functional in liposomes, the data suggest that the net secondary structure of the protein in both liposomes, as well as in sarkosyl micelles, exists in a native-like conformation. Unfortunately, the increased absorbance of the proteoliposome samples prevented measurement of the CD signal below 205 nm. This limitation

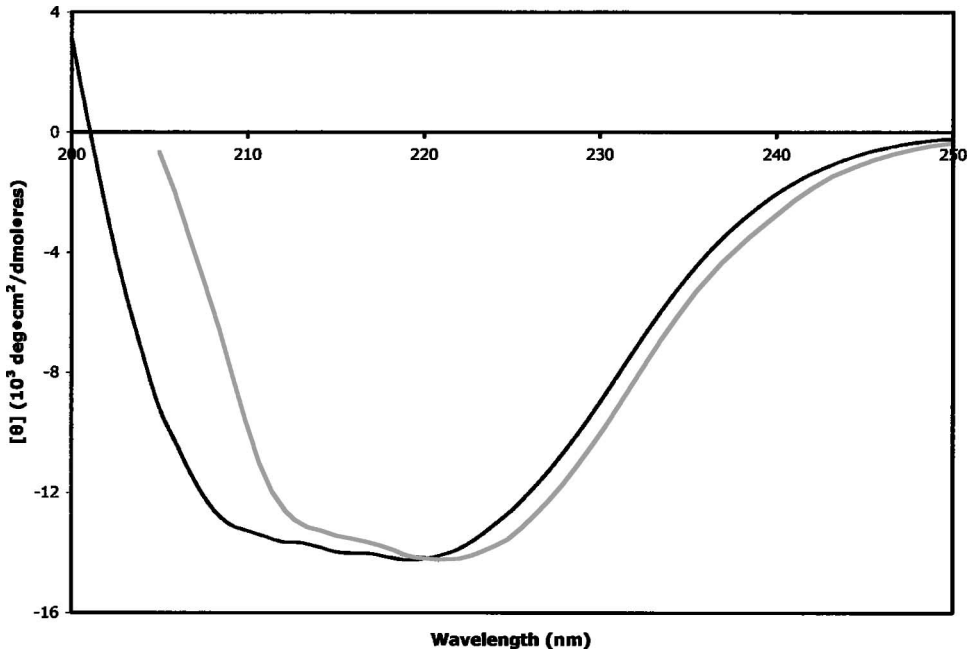

Fig. 6. Comparison of the CD spectra of the Cys-less CTP in detergent micelles and reconstituted in proteoliposomes. Comparison of the spectra of the Cys-less CTP in sarkosyl micelles (black) and reconstituted in liposomes (gray).

in the data range precluded accurate quantitation of the net secondary structure in liposomes by available algorithms.

Similar to the spectra in micelles, in liposomes a bilobed minima is observed at ~215 and 222 nm in the absence of citrate (Fig. 6). The effects of the addition of external citrate to the proteoliposomes are depicted in Fig. 7. Similar to the effects observed upon citrate addition to CTP variants in micelles, upon addition of citrate to the proteoliposomes containing the Cys-less CTP there is a reduction in the minima at the lower wavelength, resulting in a spectra that has a similar waveform as that of the CTP with added citrate in micelles. However, in the presence of citrate there was also a reduction in the magnitude of the minima centered ~222 nm for the transporter in liposomes (in contrast to the effects observed with the Cys-less CTP in detergent micelles; see Fig. 3). While these pronounced changes upon addition of citrate to the proteoliposomes are easily observed in the spectra, as mentioned above we cannot quantitate the associated changes in the net secondary structure due to the reduced wavelength range of these studies. However these changes suggest a decrease in the α-helical content upon interaction of citrate with the Cys-less CTP in liposomes (i.e., via substrate binding and/or occupancy of the transport pathway). Finally, it should be noted that even if the collected data range could be expanded, it remains uncertain whether the changes effected by citrate binding could be accurately quantitated since the reference databases typically used in the analyses of CD data are often inappropriate for determining the structures of membrane proteins in vesicles (Wallace *et al.*, 2003).

DISCUSSION

The present investigations have resulted in the first comprehensive analysis of the secondary structure of the mitochondrial citrate transport protein from any source. These studies were conducted with *highly purified* Cys-less CTP, and four single-Cys CTP mutants, enabling us to conclude with a high degree of confidence that the secondary structural information obtained is not influenced by the presence of contaminant protein. Several important conclusions arise from our data which we now discuss in detail.

First, as expected the α-helix is clearly the dominant secondary structural element in the mitochondrial CTP. Thus with the Cys-less CTP we observe 49% α-helix in detergent solution. This value increased to 58% when the substrate, citrate, is added. On the basis of initial topography predictions of six α-helical TMDs, assuming an average length of approx. 24 residues per TMD (Foster *et al.*,

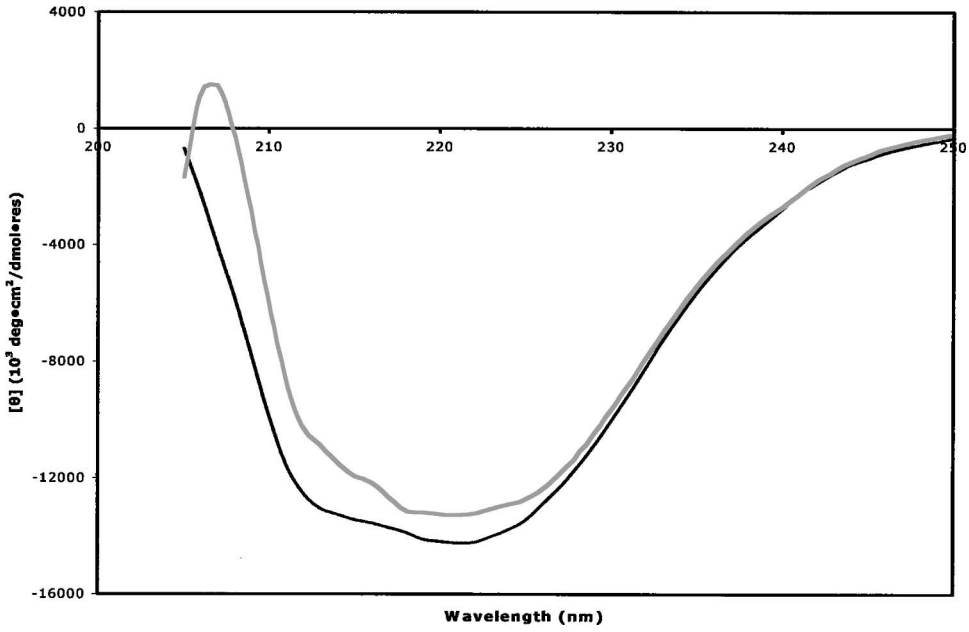

Fig. 7. Effect of added substrate on the CD spectra of the Cys-less CTP in liposomes. Comparison of the CD spectra of Cys-less CTP reconstituted in liposomes in the presence (gray) and absence (black) of added 10 mM citrate.

1983; Fu and Maloney, 1997) leads to a predicted α-helical content of 144/299 residues or approx. 48%. Recently, we have developed a homology modeled structure of the wild-type CTP (Walters and Kaplan, 2004) based on the high resolution X-ray structure of the mitochondrial ADP/ATP carrier (Pebay-Peyroula *et al*., 2003). This analysis predicts an α-helical content of 69% for the CTP arising from the presence of 6-TMDs, some of which are longer than the average of 24 residues/TMD plus the existence of several α-helical domains within the hydrophilic loops. Clearly the α-helical content observed in the present study resides within the range defined by these two predictions.

In the present analysis, the CTP secondary structural properties were obtained by comparison of the CTP spectrum with a CD reference spectrum obtained from a set of well-studied globular proteins. While there is still some debate regarding the accuracy of this comparison with membrane proteins (Fu and Maloney, 1997), as recently pointed out by Douette *et al*. (2004), the α-helical content estimated by CD analysis of several membrane proteins has been quite similar to the values ultimately obtained when their structures were solved.

Along these lines, it is of interest to compare the CTP α-helical content with that of other membrane transporters. Thus with two other mitochondrial carriers that have been well-studied, values of 68% and 66% were reported for the uncoupling protein (Douette *et al*., 2004) and the ADP/ATP carrier (Douette *et al*., 2004; Pebay-Peyroula *et al*., 2003), respectively. With transporters from other superfamilies, values of 60–70% have been reported for the oxalate transporter from *Oxalobacter formigenes* (Fu and Maloney, 1997), 82% for the human erythrocyte glucose transporter (in proteoliposomes) (Chin *et al*., 1987), and 85% for the lac permease from the cytoplasmic membrane of *E. coli* (Foster *et al*., 1983). Thus, as with the CTP, the α-helix is clearly the dominant secondary structural element in a variety of membrane transporters.

It is noteworthy, that the four single-Cys mutants which render the CTP nonfunctional, display secondary structural properties that are nearly identical to that of the Cys-less CTP. Thus we hypothesize that the corresponding wild-type residues, rather than affecting structure, likely assume important mechanistic roles, possibly including a facilitation of the CTP conformational changes required for transport. This interpretation is supported by our finding that while the Cys-less CTP significantly changes conformation in response to the addition of citrate (i.e., the α-helical content increases by approx. 10%), the magnitude of the response of the single-Cys mutants to citrate is reduced. Importantly, the similar secondary structure and the likely reduction in conformational change of the mutants in response to substrate, suggest the suitability of these mutants for crystallization trials since their conformational heterogeneity is likely to be reduced.

Comparison of the spectra of the Cys-less CTP incorporated into liposomes versus the transporter in sarkosyl micelles (i.e., Fig. 6) reveals a very similar net secondary structure with the caveat that the spectrum with the vesicles was red-shifted as is often seen when proteins are placed in a low dielectric environment such as a lipid bilayer (Cascio and Wallace, 1994, 1995). Moreover, some similar qualitative responses to the addition of citrate were observed. Since our transport assays indicate that the Cys-less CTP displays a high specific transport activity in proteoliposomes, one can conclude that the transporter maintains a native-like structure in both the liposomes, as well as in the sarkosyl micelles, and the latter therefore represents an ideal system for subsequent structural characterization. Interestingly, other transporters and channels that have been studied in both detergent solution as well as in reconstituted proteoliposomes [e.g., the lac permease (Foster *et al*., 1983) and the CHIP28 water channel (Van Hoek *et al*., 1993)] have also displayed similar secondary structure in both environments, thereby lending credence to the conclusion that data obtained in detergent solution is likely representative of the structure assumed in the native membrane.

Future studies are planned to supplement the current investigation and provide quantitation of the secondary structure of the CTP in liposomes. More appropriate spectral databases that will provide for more accurate determination of secondary structure of membrane proteins are not currently available, but are under development (Wallace *et al*., 2004). The utilization of these databases, when coupled with enhanced data collection (via empirically optimized sample conditions) will aid in elucidating the mechanism of citrate transport by the CTP.

In conclusion, the similarity in secondary structure between the CTP in sarkosyl micelles compared with the CTP in proteoliposomes, combined with the transporter's responsiveness to substrate in both conditions, suggests that the solubilized CTP retains native-like properties and thus represents an ideal system with which to conduct a detailed structural analysis. Furthermore, the observation that the four inactive single-Cys mutants display native-like structure in detergent solution, as well as reduced conformational change in response to substrate, suggest that they may be locked in a conformation(s) that represents a discrete step(s) in the transport cycle. With these ideas in mind, extensive crystallization trials are currently underway with both the purified Cys-less CTP, as well as with the inactive single-Cys mutants, with the aim of extending our understanding of the structure-based mechanism of this metabolically important transporter to the atomic level.

ACKNOWLEDGMENTS

The authors thank Dr. Rusudan Kotaria and Mr. Chunlong Ma for expert assistance with the protocols involving protein overexpression, quantification, and purity analysis. We also acknowledge Shared Instrumentation grant 1S10RR11998 from the NIH/NSF that provided support for the circular dichrometer facilities. Furthermore, we thank Dr. B. A. Wallace and her colleagues for the development of DICHROWEB. This work was supported by National Institutes of Health Grant GM-054642 to R.S.K.

REFERENCES

Andrade, M. A., Chacon, P., Merelo, J. J., and Moran, F. (1993). *Protein Eng.* **6**, 383–390.
Bisaccia, F., De Palma, A., and Palmieri, F. (1989). *Biochim. Biophys. Acta* **977**, 171–176.
Bisaccia, F., De Palma, A., Prezioso, G., and Palmieri, F. (1990). *Biochim. Biophys. Acta* **1019**, 250–256.
Brunengraber, H., and Lowenstein, J. M. (1973). *FEBS Lett.* **36**, 130–132.
Cascio, M., and Wallace, B. A. (1994). *Protein Pept. Lett.* **1**, 136–140.
Cascio, M., and Wallace, B. A. (1995). *Anal. Biochem.* **227**, 90–100.
Chin, J. J., Jung, E. K. Y., Chen, V., and Jung, C. Y. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4113–4116.
Conover, T. E. (1987). *Trends Biochem. Sci.* **12**, 88–89.
Douette, P., Navet, R., Bouillenne, F., Brans, A., Sluse-Goffart, C., Matagne, A., and Sluse, F. E. (2004). *Biochem. J.* **380**, 139–145.
Endemann, G., Goetz, P. G., Edmond, J., and Brunengraber, H. (1982). *J. Biol. Chem.* **257**, 3434–3440.
Eriks, L. R., Mayor, J. A., and Kaplan, R. S. (2003). *Anal. Biochem.* **323**, 234–241.
Foster, D. L., Boublik, M., and Kaback, H. R. (1983). *J. Biol. Chem.* **258**, 31–34.
Fu, D., and Maloney, P. C. (1997). *J. Biol. Chem.* **272**, 2129–2135.
Johnson, W. C. J. (1988). *Ann. Rev. Biophys. Biophys. Chem.* **17**, 145–166.
Kaplan, R. S., Mayor, J. A., Brauer, D., Kotaria, R., Walters, D. E., and Dean, A. M. (2000a). *J. Biol. Chem.* **275**, 12009–12016.
Kaplan, R. S., Mayor, J. A., Gremse D. A., and Wood, D. O. (1995). *J. Biol. Chem.* **270**, 4108–4114.
Kaplan, R. S., Mayor, J. A., Johnston, N., and Oliveira, D. L. (1990a). *J. Biol. Chem.* **265**, 13379–13385.
Kaplan, R. S., Mayor, J. A., Kotaria, R., Walters, D. E., and Mchaourab, H. S. (2000b). *Biochemistry* **39**, 9157–9163.
Kaplan, R. S., Mayor, J. A., and Wood, D. O. (1993). *J. Biol. Chem.* **268**, 13682–13690.
Kaplan, R. S., Morris, H. P., and Coleman, P. S. (1982). *Cancer Res.* **42**, 4399–4407.
Kaplan, R. S., Oliveira, D. L., and Wilson, G. L. (1990b). *Arch. Biochem. Biophys.* **280**, 181–191.
Kaplan, R. S., and Pedersen, P. L. (1985). *Anal. Biochem.* **150**, 97–104.
Kotaria, R., Mayor, J. A., Walters, D. E., and Kaplan, R. S. (1999). *J. Bioenerg. Biomembr.* **31**, 543–549.
Lobley, A., Whitmore, L., and Wallace, B. A. (2002). *Bioinformatics* **18**, 211-212.
Ma, C., Kotaria, R., Mayor, J. A., Eriks, L. R., Dean, A. M., Walters, D. E., and Kaplan, R. S. (2004). *J. Biol. Chem.* **279**, 1533–1540.
Mao, D., Wachter, E., and Wallace, B. A. (1982). *Biochemistry* **21**, 4960–4968.

Palmieri, F., Stipani, I., Quagliariello, E., and Klingenberg, M. (1972). *Eur. J. Biochem.* **26**, 587–594.
Pebay-Peyroula, E., Dahout-Gonzalez, C., Kahn, R., Trezeguet, V., Lauquin, G. J.-M, and Brandolin, G. (2003). *Nature* **426**, 39–44.
Savitzky, A., and Golay, M. J. E. (1964). *Anal.Chem.* **36**, 1627–1639.
Van Hoek, A. N., Wiener, M., Bicknese, S., Miercke, L., Biwersi, J., and Verkman, A. S. (1993). *Biochemistry* **32**, 11847–11856.
Wallace, B. A., Lees, J. G., Orry, A. J., Lobley, A., and Janes, R. W. (2003). *Protein Sci.* **12**, 875–884.
Wallace, B. A., Wien, F., Miles, A. J., Lees, J. G., Hoffmann, S. V., *et al.* (2004). *Faraday Discuss.* **126**, 237–243; discussion 45–54.
Walters, D. E., and Kaplan, R. S. (2004). *Biophys. J.* **87**, 907–911.
Watson, J. A., and Lowenstein, J. M. (1970). *J. Biol. Chem.* **245**, 5993–6002.
Xu, Y., Kakhniashvili, D. A., Gremse, D. A., Wood, D. O., Mayor, J. A., Walters, D. E., and Kaplan, R. S. (2000). *J. Biol. Chem.* **275**, 7117–7124.
Xu, Y., Mayor, J. A., Gremse, D., Wood, D. O., and Kaplan, R. S. (1995). *Biochem. Biophys. Res. Commun.* **207**, 783–789.