Site-Directed Mutagenesis and Chemical Modification of the Six Native Cysteine Residues of the Rat Mitochondrial Carnitine Carrier: Implications for the Role of Cysteine-136†

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ABSTRACT: By use of site-directed mutagenesis in combination with chemical modification of mutated proteins, the role of the six Cys residues in the transport function of the rat mitochondrial carnitine carrier (CAC) was studied. Several CAC mutants, in which one or more Cys residues had been replaced with Ser, were overexpressed in *Escherichia coli*, purified, and reconstituted in liposomes. The efficiency of incorporation into liposomes of the reconstituted proteins was lower for all constructs lacking Cys-23. Single, double, and quadruple replacement mutants showed V_{max} comparable to that of the wild type. On the basis of the values of internal and external transport affinities (K_m) for carnitine and of their comparison with those measured in mitochondria, the recombinant CAC is oriented unidirectionally in the liposomes, right side out compared to mitochondria. Substitution of Cys-136 with Ser caused a nearly complete loss of sensitivity of the CAC to *N*-ethylmaleimide, (2-aminoethyl)methanethiosulfonate hydrobromide (MTSES), and other hydrophilic SH reagents but not to the very hydrophobic *N*-phenylmaleimide. The wild-type CAC and the mutants containing Cys-136 showed substrate protection against NEM and MTSES inhibition and against NEM labeling. The data show that none of the native cysteines is essential for the transport mechanism and that Cys-136 is the major target of SH reagents and raise the hypothesis that Cys-136 is accessible from the external medium and is located at, or near, the substrate binding site. A model of the CAC is proposed in which the matrix hydrophilic loop containing Cys-136 protrudes into the membrane between the transmembrane domains of the protein.

The inner mitochondrial membrane contains a specific carrier protein, known as the carnitine/acylcarnitine or carnitine carrier (CAC), that catalyzes an electroneutral exchange of cytosolic acylcarnitines for mitochondrial carnitine. The CAC plays a central role in the translocation of fatty acids into the mitochondria, where they are oxidized (*1*). It has been purified (*2*), reconstituted into liposomes (see ref *3* for references), and sequenced (*4*). It is encoded in man by the gene CACT that maps to chromosome 3p21.31 (*5*) and in yeast by the gene CRC (*6*) and belongs to the mitochondrial carrier family (for a review, see ref *7*). On the basis of its hydropathy profile, its tripartite structure, and its relation to other mitochondrial carriers, a secondary structure has been proposed in which the carrier has six α-helical transmembrane domains connected by hydrophilic loops (*4*). However, little is known about the spatial organization of the helices and the loops nor about the molecular mechanism of carnitine and acylcarnitine translocation through the CAC protein. One approach for obtaining more information on the molecular mechanism of a carrier system is to investigate the functional groups and their role in substrate transport. Recently, we have overexpressed the CAC in *Escherichia coli* and reconstituted it into liposomes (*8*), which opens the way to using site-directed mutagenesis to elucidate the structure−function relationships of this metabolically important transporter.

In this work, by using site-directed mutagenesis, we aimed (i) to determine the importance of the six Cys residues present in the rat CAC protein for its transport function, (ii) to identify which Cys residue(s) is (are) responsible for the transport inhibition by SH reagents, and (iii) to investigate the relationship between this/these residue(s) and the substrate binding site. This was achieved by constructing several CAC mutants in which one or more Cys residues were replaced by Ser. The results show that none of the native cysteines is irreplaceable and that Cys-136 is the principal target for SH reagents in the native protein. It is also hypothesized that Cys-136, located in the matrix hydrophilic loop between transmembrane domains III and IV, is accessible from the external side and may be located in, or near, the substrate binding site.

MATERIALS AND METHODS

Materials. Bio-Beads SM-2 were purchased from Bio-Rad, Celite 535 was from Serva, Sephadexes G-50, G-75, and

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G-200 were from Pharmacia, L-[*methyl*-^{3}H]carnitine was from Amersham, [^{14}C]NEM[1] was from Dupont de Nemours, MTSEA and MTSES were from Fluorescent Dyes Inc., and egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), Pipes, Triton X-100, cardiolipin, L-carnitine, and *N*-dodecanoylsarcosine (sarcosyl) were from Sigma. All other reagents were of analytical grade.

Site-Directed Mutagenesis, Overexpression, and Isolation of the CAC Proteins. The coding region for the rat CAC was amplified by PCR from total rat liver cDNA as described previously (*8*). The single Cys/Ser replacements were constructed with complementary mutagenic primers using the overlap extension PCR method (*9*). The PCR products were purified by the Gene Clean Kit (La Jolla), digested with *Nde*I and *Hin*dIII (restriction sites added at the 5′ end of forward and reverse primers, respectively), and ligated into the pMW7 expression vector. The double mutant C136S/C155S was prepared by the same method using the plasmid pMW7/C155S as vector. Since five out of six Cys residues are located within the first 165 amino acids, the cysteine-less (Cys-less) version of the CAC was prepared using a cassette of 496 bp containing the mutations C23S, C58S, C89S, C136S, and C155S. This cassette was digested with *Nde*I and *Bam*HI, the *Bam*HI site being located at 496 bp in the coding sequence of the rat CAC cDNA (*4*), and ligated into the pMW7 vector containing the 3′ portion of the C283S CAC cDNA using nucleotide 496. The five replacement mutants C23/58/89/136/283S and C23/58/89/155/283S were prepared using the cassette of 496 bp containing the mutations C23S, C58S, and C89S. All mutations were verified by DNA sequencing, and except for the desired base changes, all of the sequences were identical to that of rat CAC cDNA. The resulting plasmids were transformed into *E. coli* CO214 (*8*). Bacterial overexpression, isolation of the inclusion body fraction, solubilization, and purification of the wild-type CAC and mutant CAC proteins were performed as described (*8*). Each mutant of about 32.5 kDa molecular mass, identified by its reaction with a rabbit antiserum raised against the CAC isolated from rat liver mitochondria, was more than 90% pure.

Reconstitution of CAC and CAC Mutants into Liposomes and Transport Measurements. The recombinant proteins were reconstituted into liposomes in the presence of 13 mM carnitine, as described previously (*10*). The external substrate was removed from proteoliposomes on Sephadex G-75 columns. Transport at 25 °C was started by addition of [^{3}H]-carnitine to proteoliposomes and terminated by the addition of 1.5 mM NEM (*10*). In controls, the inhibitor was added at the beginning, together with the labeled substrate. Finally, the external substrate was removed from quenched samples on Sepadex G-50 columns, and the radioactivity in the liposomes was measured (*10*). The experimental values were corrected by subtracting control values. The initial transport rate was calculated in millimoles per minute per gram of protein from the radioactivity taken up by the proteoliposomes after 2 min (in the initial linear range of substrate uptake). All of the transport activities were determined by taking into account the efficiency of reconstitution (i.e., the share of successfully incorporated protein). For efflux measurements, the internal carnitine pool of the proteoliposomes was made radioactive by carrier-mediated exchange equilibration (*10*) with 0.1 mM [^{3}H]carnitine added at high specific radioactivity. After 20 min, the residual external radioactivity was removed by passing the proteoliposomes again through a column of Sephadex G-75. Efflux was started by addition of unlabeled external carnitine (backward exchange) and terminated by the addition of NEM (*3*). The decrease in radioactivity inside the liposomes was fitted to the equation $\alpha = 100(1 - e^{-kt})$ (where α is the percentage of isotopic equilibrium). The rates were expressed as apparent velocities, i.e., the product of *k* and the substrate concentration inside the liposomes, and they are directly proportional to the actual transport rate (*10*).

Other Methods. SDS−PAGE was performed according to Laemmli (*11*) as described previously (*8*). The amount of recombinant protein was estimated on Coomassie blue-stained SDS−PAGE gels by the Bio-Rad GS-700 imaging densitometer equipped with the software Bio-Rad Multi-Analist, using bovine serum albumin as standard. The extent of incorporation of the recombinant protein into liposomes was determined as described in Phelps et al. (*12*), with the following modifications: the proteoliposomes were passed through Sephadex G-200 columns (20 × 0.75 cm) before ultracentrifugation, and this was performed at 110000*g* for 90 min at 4 °C. [^{14}C]NEM labeling of reconstituted wild-type CAC and CAC mutants was detected by liquid scintillation counting of Coomassie blue-stained SDS−PAGE gels (*13*).

RESULTS

Functional Analysis of the Mutant CAC Proteins. To verify whether any of the six Cys residues were critical to the transport function and to identify the target(s) of transport inhibition by SH reagents (*3*), mutants of CAC were constructed by replacing one or more Cys with Ser, which has steric and chemical properties similar to those of Cys. This approach has been previously applied to several other transport proteins (*14*−*20*). Each mutant was overexpressed in *E. coli* and reconstituted into liposomes. The efficiency of reconstitution (i.e., the share of successfully incorporated protein) of the wild-type CAC, of the double replacement C136/155S mutant, and of five of the single replacement mutants ranged from 30% to 38% (Table 1). The other mutants containing C23S (C23S, C23/58/89/283S, C23/58/89/136/283S, C23/58/89/155/283S, and the Cys-less CAC) showed a lower reconstitution efficiency, ranging from 13% to 20%.

The majority of the mutants transported carnitine with very significant activities, between 46% and 100% or more compared to the wild-type CAC (Table 1). Only mutants in which more than four Cys residues were substituted (C23/58/89/136/283S, C23/58/89/155/283S, and Cys-less) exhibited markedly diminished activities (<10% of the wild type). To gain further insight into the role of Cys residues in the CAC translocation mechanism, the kinetic characteristics of the mutated proteins were compared to those of the wild-type carrier, which was previously shown to be virtually

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; NEM, *N*-ethylmaleimide; *p*-CMBS, *p*-(chloromercuri)benzenesulfonate; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Table 1: Transport and Liposome Incorporation of CAC Mutants[a]

CAC mutants	efficiency of reconstitution (%)	specific transport activity [μmol (10 min)$^{-1}$ (g of protein)$^{-1}$]
wild type	38 ± 9	1445 ± 545
C23S	17 ± 4	1010 ± 251
C58S	37 ± 10	901 ± 232
C89S	33 ± 9	1614 ± 572
C136S	33 ± 8	859 ± 277
C155S	38 ± 10	1234 ± 455
C283S	30 ± 7	1592 ± 537
C136/155S	40 ± 10	657 ± 212
C23/58/89/283S	20 ± 6	898 ± 239
C23/58/89/136/283S	18 ± 4	138 ± 81
C23/58/89/155/283S	17 ± 5	105 ± 69
Cys-less	13 ± 3	122 ± 54

[a] Recombinant protein (6 μg) was added to the reconstitution mixture. The percent efficiency of reconstitution into liposomes represents the percentage of protein incorporated into liposomes. Specific transport activity was measured as 0.1 mM [^{3}H]carnitine uptake into proteoliposomes containing 13 mM internal carnitine in 10 min and per gram of incorporated protein. The data are means ± SD of four independent experiments.

Table 2: Kinetic Constants of CAC Mutants[a]

CAC mutants	V_{max} [mmol min^{-1} (g of protein)$^{-1}$]	K_m (mM)
wild type	1.8 ± 0.5	0.48 ± 0.09
C23S	1.7 ± 0.4	2.05 ± 0.35
C58S	1.5 ± 0.5	1.85 ± 0.07
C89S	2.2 ± 0.7	0.69 ± 0.16
C136S	1.7 ± 0.5	1.90 ± 0.16
C155S	1.6 ± 0.4	1.20 ± 0.28
C283S	2.3 ± 0.6	0.31 ± 0.03
C136/155S	1.4 ± 0.3	2.33 ± 0.47
C23/58/89/283S	1.5 ± 0.4	2.39 ± 0.39

[a] The values were calculated from double reciprocal plots of the rate of [^{3}H]carnitine uptake versus substrate concentration. Radioactive carnitine (0.08−4.0 mM) was added to proteoliposomes reconstituted with the CAC mutants and containing 13 mM carnitine. Reaction time: 2 min. The data are means ± SD of four independent experiments. The kinetic constants of the mutants C23/58/89/136/283S, C23/58/89/155/283S, and Cys-less were not determined due to low activity.

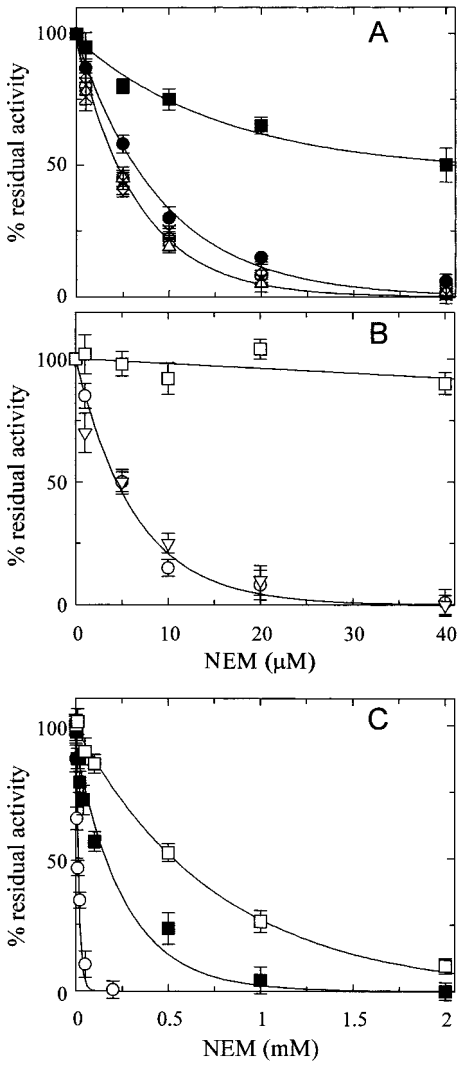

FIGURE 1: Dependence on NEM concentration of carnitine transport catalyzed by reconstituted CAC mutants. Proteoliposomes were preincubated for 1 min with 0−40 μM (A and B) or 0−2.0 mM (C) NEM. Transport was started by the addition of 0.1 mM [^{3}H]-carnitine and stopped after 5 min. Results are expressed as percent residual activity, i.e., percent of the activity in the presence of SH reagent with respect to the control value without inhibitor. The data represent means ± SD of at least three independent experiments in duplicate. Symbols: wild type (○), C23S (+), C58S (◇), C89S (△), C136S (■), C155S (●), C283S (×), C136/155S (□), and C23/58/89/283S (▽).

identical to the native protein with regard to its functional properties (*8*). Table 2 shows that the V_{max} values of single, double, and quadruple replacement mutants were similar to that of the wild-type protein. The half-saturation constants (K_m) of the CAC mutants were 2−5-fold higher than that of the wild type (Table 2), except those of the single replacement mutants C89S and C283S, which were close to that of the wild type. We conclude that none of the native Cys is irreplaceable.

Identification of the SH Reagent Target. To identify the Cys residues targeted by SH reagents, carnitine transport catalyzed by the CAC mutants in liposomes was titrated by NEM in a micromolar concentration range and in the absence of external substrate. To compare the data related to different mutants, the percent of residual activity was plotted vs inhibitor concentration. As shown in Figure 1A, the single mutants C23S, C58S, C89S, and C283S were completely inhibited by 40 μM NEM with a kinetics virtually coincident with that of the wild type (IC_{50} 4.7 ± 0.7 μM). The C155S mutant showed a small, but reproducible shift of the inhibition kinetics toward higher NEM concentrations (IC_{50} 6.5 ± 0.8 μM). By contrast, the C136S mutant was poorly inhibited, showing less than 50% inhibition at 40 μM NEM. These data indicate that Cys-136 is a major target for NEM in the native protein. The residual small inhibition observed with C136S suggests an involvement of other Cys residue-(s) in the CAC inhibition by NEM. Cys-155 is the best candidate for this inhibition due to the above-mentioned shift of the C155S inhibition kinetics toward higher concentrations of NEM. As shown in Figure 1B, the C136/155S double replacement mutant was virtually insensitive to NEM up to 40 μM. On the other hand, the presence of both Cys-136 and Cys-155 in the quadruple replacement mutant C23/58/89/283S conferred a sensitivity to NEM that was virtually identical to that of the wild-type protein. All of these data strongly indicate that the inhibition of CAC by micromolar concentrations of NEM is primarily due to the interaction of the alkylating agent with Cys-136 and only partly to the interaction with Cys-155. None of the other Cys residues

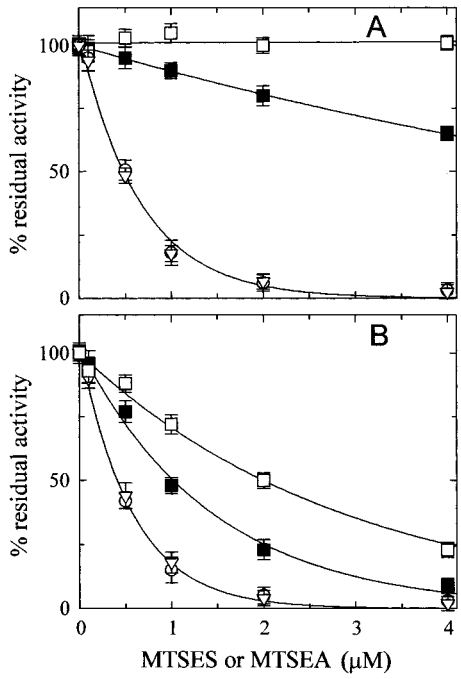

FIGURE 2: Dependence on MTSES or MTSEA concentration of carnitine transport catalyzed by reconstituted CAC mutants. Proteoliposomes, reconstituted with wild type (○), C136S (■) C136/155S (□), and C23/58/89/283S (▽), were preincubated for 1 min with 0−4.0 μM MTSES (A) or 0−4.0 μM MTSEA (B). Other conditions as in Figure 1.

seem to be involved in the inactivation of the carrier by low concentrations of NEM.

When the concentration of NEM was increased to 2 mM, mutants C136S and C136/155S also became inhibited by the alkylating reagent (Figure 1C). Nearly full inhibition of C136S and C136/155S was obtained at 1 and 2 mM NEM, respectively. These results clearly indicate that NEM can also bind other Cys residues of the CAC in addition to Cys-136 and Cys-155, but at higher concentrations.

Comparison of the Sensitivity of Reconstituted CAC Mutants toward Various SH Reagents. Since NEM is a known hydrophobic membrane-permeable thiol reagent, we extended our investigation to MTSES and MTSEA, which are charged, hydrophilic, and cysteine-specific reagents (*21*). They both form a mixed disulfide via addition of $-SCH_2-CH_2X$ to the reduced sulfydryl of cysteine, where X is SO_3^- or NH_3^+ for MTSES and MTSEA, respectively (*15*, *21*−*26*). However, MTSES is impermeable to a lipid bilayer in the absence of a transport or channel protein (*21*, *27*, *28*), whereas in marked contrast MTSEA is permeable to some extent (*28*). As illustrated in Figure 2, both MTSES and MTSEA efficiently inhibited the wild type, the quadruple replacement mutant C23/58/89/283S, and (not shown) the single mutants C23S, C58S, C89S, and C283S with about the same IC_{50}. However, C136/155S was unaffected by MTSES (Figure 2A) but inhibited by MTSEA (Figure 2B),; and C136S was very poorly affected by MTSES but strongly inhibited by MTSEA. In addition, in other experiments (not shown) we found that MTSEA inhibits C155S as efficiently as the wild type, whereas MTSES inhibits C155S somewhat less than the wild type similarly to NEM (see Figure 1A). Therefore, MTSES reacts mainly with Cys-136 and only poorly with Cys-155, whereas MTSEA also reacts with the other Cys of the CAC. The high reactivity of Cys-136 to

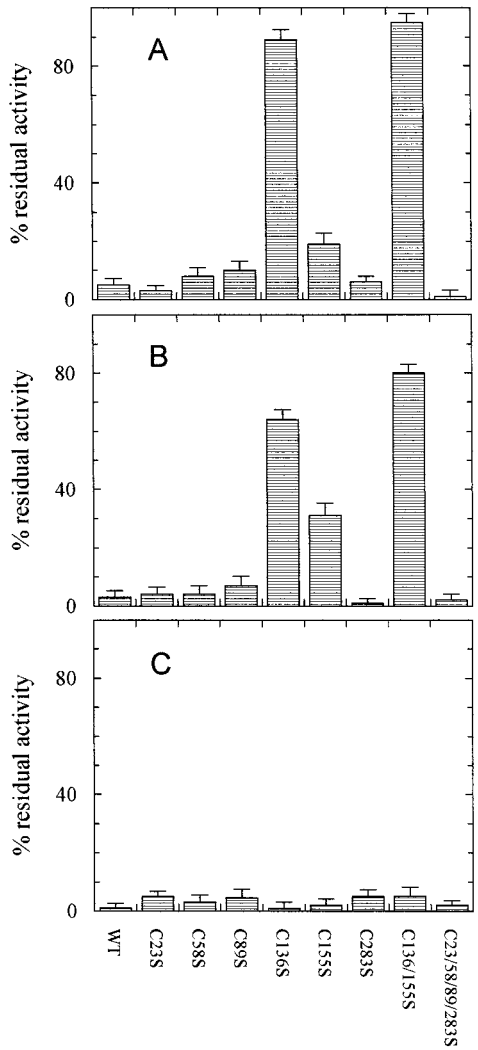

FIGURE 3: Effect of *p*-CMBS, $HgCl_2$, and *N*-phenylmaleimide on carnitine transport catalyzed by reconstituted CAC mutants. Proteoliposomes, reconstituted with the indicated proteins (wild type or mutant CAC), were preincubated for 1 min with 0.5 μM *p*-CMBS (A), 0.5 μM $HgCl_2$ (B), or 1 μM *N*-phenylmaleimide (C). Other conditions as in Figure 1.

the membrane-impermeable MTSES suggests that this residue may be exposed to the external side of the proteoliposomes and located in a hydrophilic environment.

In a further set of experiments, the effectiveness of other SH reagents in inhibiting the reconstituted transport activity of the wild-type CAC and of the CAC mutants was compared. *p*-CMBS (0.5 μM) (Figure 3A) and (not shown) mersalyl, which are impermeable reagents at low concentrations, inhibited the wild-type CAC and the CAC mutants similarly to NEM in the micromolar concentration range. $HgCl_2$ (0.5 μM) (Figure 3B) almost completely inhibited the wild-type and the CAC mutants, except those in which Cys-136 or Cys-155 was substituted, i.e., C136S, C155S, and C136/155S. However, C136S was somewhat more sensitive and C155S less sensitive to $HgCl_2$ than to NEM, MTSES, *p*-CMBS, and mersalyl, suggesting that $HgCl_2$ reacts with Cys-155 more than the above-mentioned SH reagents. In contrast to all the other cysteine-specific reagents tested, the very hydrophobic reagent *N*-phenylmaleimide (at a concentration of 1 μM) inhibited all of the mutants tested.

Substrate Protection of the CAC Mutants against Inhibition by NEM and MTSES. To investigate whether the substrate

Table 3: Influence of Substrate on the Inhibition of CAC Mutants by NEM and MTSES[a]

	with NEM				with MTSES			
CAC mutants	NEM (μM)	% residual activity −carnitine	% residual activity +carnitine	protected activity (%)	MTSES (μM)	% residual activity −carnitine	% residual activity +carnitine	protected activity (%)
wild type	10	35 ± 12	83 ± 21	74	0.5	43 ± 12	88 ± 21	79
C23S	10	40 ± 11	65 ± 13	42	0.5	40 ± 8	69 ± 18	38
C58S	10	38 ± 8	61 ± 15	37	0.5	50 ± 18	83 ± 25	66
C89S	10	31 ± 6	73 ± 8	61	0.5	48 ± 15	75 ± 20	52
C136S	250	35 ± 15	38 ± 17	5	5.0	65 ± 12	67 ± 18	6
C155S	10	35 ± 10	62 ± 14	42	0.5	38 ± 13	75 ± 19	60
C283S	10	31 ± 13	89 ± 25	84	0.5	49 ± 21	91 ± 23	82
C136/155S	500	64 ± 14	64 ± 16	0	nd	nd	nd	nd
C23/58/89/283S	10	36 ± 10	87 ± 19	80	0.5	58 ± 19	87 ± 22	69

[a] Proteoliposomes were preincubated for 2 min with NEM or MTSES at the concentrations indicated in the presence or absence of 10 mM carnitine. After removal of unbound reagent and external substrate by Sephadex G-75 chromatography, transport was initiated by addition of 0.1 mM [^{3}H]carnitine and terminated after 10 min. The data are means ± SD. of three independent experiments. Percent protected activity represents the percentage of the activity that is protected by substrate. nd = not determined.

has any protective effect on NEM or MTSES inhibition of the wild-type CAC and CAC mutants, proteoliposomes were incubated with NEM or MTSES in the presence and absence of carnitine. In these experiments, the concentrations of NEM or MTSES were kept sufficiently low in order to prevent the inhibition from being complete. As shown in Table 3, all of the mutants tested except those in which Cys-136 was substituted, i.e., C136S and C136/155S, exhibited substantial substrate protection. This result suggests that the reaction of NEM or MTSES with Cys-136 is prevented by the presence of carnitine. It is noteworthy that the protective effect of carnitine was specific, since other chemically related compounds, such as choline and ornithine, which are neither substrates nor inhibitors of the CAC, exhibited no protection (data not shown). Furthermore, under the conditions used in the experiments shown in Table 3 with C89S, C283S, and the wild-type CAC, 50% protection was obtained at a carnitine concentration of about 0.8 mM, which is close to the transport affinity (K_m) of the reconstituted carrier for external carnitine (ref *29* and this study).

NEM Labeling of Cys-136. To address the question of the role and location of Cys-136 in the CAC protein more thoroughly, we applied direct labeling assays using radioactive NEM. In these experiments we also tested the most useful mutant for assessing the role of Cys-136, i.e., the single Cys-136 corresponding to C23/58/89/155/283S, which was of limited use for transport measurements due to its negligible activity. Figure 4 reports the radioactivity bound to the wild-type CAC and CAC mutants recovered from proteoliposomes treated with 20 μM [^{14}C]NEM in the presence and absence of carnitine or MTSES. The data of Figure 4 give direct evidence that Cys-136 is the main target of NEM, since the radioactivity associated with the single Cys-136 mutant is almost the same as that associated with the wild-type CAC and more than 3-fold higher than that associated with the mutant lacking Cys-136 but still containing the other five native Cys residues of the CAC. Furthermore, the results obtained show that carnitine protects the single Cys-136 mutant and the wild-type CAC against reaction with NEM, whereas it has no effect on the NEM labeling of the mutant in which Cys-136 was replaced by serine. The NEM labeling of the single Cys-136 mutant and of the wild-type CAC was also decreased by the presence of the membrane-impermeable MTSES (Figure 4), suggesting that Cys-136 is accessible from the external medium.

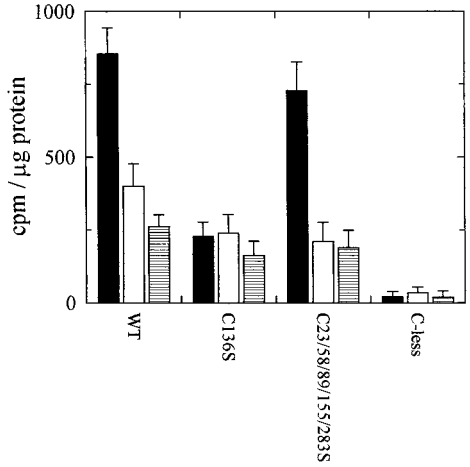

FIGURE 4: NEM labeling of wild-type CAC and CAC mutants. Proteoliposomes, reconstituted with the indicated proteins (wild type or mutant CAC), were preincubated with 10 mM carnitine (white columns), 2 μM MTSES (gray columns), or no addition (black columns) for 1 min and then incubated with 20 μM [^{14}C]NEM for a further 2 min at 25 °C. The reaction carried out at 25 °C was stopped by addition of 10 mM DTE. The data represent means ± SD of three independent experiments.

Asymmetric Orientation of Recombinant CAC. When studying a reconstituted carrier, it is important to establish the orientation of the protein in the liposomal membrane. Since antibodies or side-specific inhibitors are not available for the CAC to discriminate the two faces of the reconstituted recombinant CAC, we investigated the existence of substrate binding sites on the internal and external surface of the membrane and their affinity constants for carnitine. This kinetic approach has successfully been applied before for the CAC purified from rat liver mitocondria (*29*) and other purified carriers in the reconstituted system (*30*−*34*). The intraliposomal half-saturation constant (transport affinity) K_m for carnitine/carnitine exchange was determined by measuring the efflux of [^{3}H]carnitine from prelabeled proteoliposomes (backward exchange), because this method provides a more convenient assay of transport kinetics in the presence of low intraliposomal substrate concentrations (*10*). The intraliposomal K_m value of recombinant CAC for carnitine/carnitine exchange was 10.2 ± 2.1 mM (four experiments) over a wide range of internal carnitine (0.15−12.0 mM) at a constant external concentration of 10 mM carnitine. Since

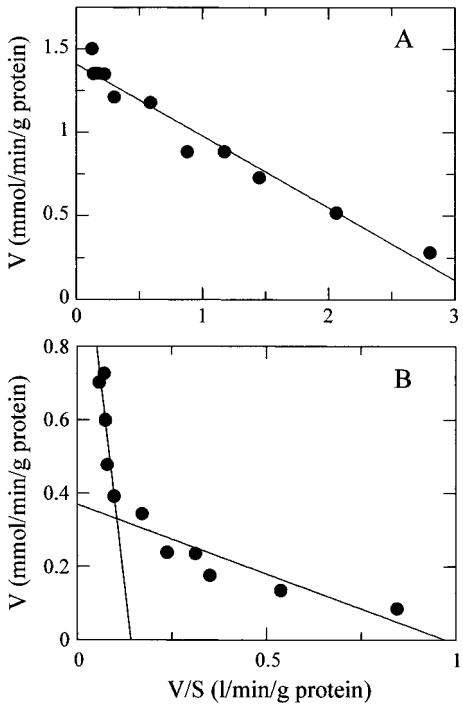

FIGURE 5: Eadie−Hofstee plot of carnitine transport of reconstituted wild-type CAC before (A) and after (B) freeze/thaw/sonication of proteoliposomes. Transport was initiated by the addition of 0.1−12 mM [^{3}H]carnitine to proteoliposomes containing 13 mM carnitine. In (B), the proteoliposomes were freezed, thawed, pulse sonicated for 3 min, and passed through Sephadex G-75 columns. The K_m values were calculated from the slope of the Eadie−Hofstee plots. S = external carnitine.

the external K_m (see Table 2) of recombinant CAC appeared to be several times lower than the internal K_m, we looked for a higher K_m on the external surface covering an external carnitine concentration range of 1−13 mM. The results obtained (external K_m of 0.52 ± 0.15 mM in three experiments) excluded the presence of a second outward-facing binding site with a lower affinity for carnitine. The presence of only one single K_m at each side of the liposomal membrane and the large difference between the inside and outside K_m values clearly show that no significant amount of the carrier population with high K_m ("internal K_m") was present on the outside and vice versa. To confirm or deny the existence of low-affinity binding sites at the internal surface of the carrier, the proteoliposomes, after formation, were frozen, thawed, and sonicated in order to scramble the orientation of the carrier molecules. After this procedure, we expected to find the high K_m also on the external face. Figure 5 shows an Eadie−Hofstee plot of the transport rate as a function of external carnitine (0.1−12.0 mM) before and after freeze/thaw/sonication of the proteoliposomes (13 mM carnitine inside). A nonlinear dependence became evident after sonication of proteoliposomes (Figure 5B). The two different K_m values observed, 0.34 and 9.0 mM, corresponded well to the previously determined external and internal K_m, respectively. The low-affinity and high-affinity kinetic components contributed to V_{max} in a ratio of 3.5:1. Obviously, the orientation of the membrane-inserted carrier molecules was substantially inverted by the freeze/thaw/sonication treatment. By comparison of the determined K_m values with the published data measured in intact mitochondria (*35*−*37*), a right-side-out orientation can be concluded for the recombinant CAC, as previously reported for the CAC purified from mitochondria (*29*).

DISCUSSION

Cys-23 Is Necessary for Proper Insertion of CAC into the Liposomal Membrane. In this work a series of site-directed Cys mutants replacing native Cys residues of the rat mitochondrial CAC with Ser were employed. For each protein, the extent of incorporation into phospholipid vesicles and the ability to catalyze carnitine/carnitine antiport were evaluated. The data reported above show that only a fraction of the protein added to the reconstitution mixture, ranging from 13% to 38%, was incorporated into liposomes. This may be due to incomplete refolding of the carrier after substitution of sarkosyl with Triton X-100 and treatment with cardiolipin (*8*) and/or to formation of a soluble form of the protein after interaction with the detergent. These hypotheses are in line with the following findings: (1) no loss of protein occurred during reconstitution, (2) after reconstitution a small amount (about 0.005%) of Triton X-100 was still present in the proteoliposomal suspension, and (3) the protein not incorporated in the membrane was lost after separation of the proteoliposomes by Sephadex chromatography and ultracentrifugation (results not shown). Incomplete reconstitution was previously found for other recombinant mitochondrial carriers (*12*, *38*−*42*). Our results further indicate that the group of mutants that share substitution C23S show a lower efficiency of reconstitution into liposomes. This observation suggests that the first transmembrane domain of CAC, in which Cys-23 is located, is important for the insertion of the carrier in the membrane. Thus a minimal alteration to this part of the protein may interfere with its insertion into the phospholipid bilayer.

None of the Native Cys Is Irreplaceable, and Cys-136 Is the Principal Target of the CAC by NEM, MTSES, p-CMBS, and Mersalyl. Since the transport activities of single, double, and quadruple replacement mutants are comparable to that of the wild-type CAC, none of the six Cys residues present in the CAC is essential to the translocation mechanism. In agreement with this conclusion, the V_{max} values of these mutants are not significantly different from that of the wild type, and the differences in the transport activities reported in Table 1 (at 0.1 mM carnitine) can be explained by the different half-saturation constants (K_m) for carnitine. However, the five replacement mutants and the Cys-less CAC exhibit significantly lower V_{max}. A possible explanation of this finding is that the substitution of more than four cysteines leads to a change in conformation of the protein. The inhibition of the CAC by SH reagents (*3*) is explained here as a consequence of their principal interaction with only one cysteine out of the six present in the carrier molecule, i.e., Cys-136. This conclusion is supported mainly by the NEM labeling assays and by the finding that the mutant C136S is the only single Cys replacement mutant to be almost unaffected by these reagents.

Cys-136 Is Localized in, or near, the Substrate Binding Site and Is Accessible from the External Medium. A possible interpretation for the protective effect of the substrate on the SH reagent inhibition of the wild-type CAC and the CAC mutants containing Cys-136 (but not of those in which Cys-136 was substituted) may be that Cys-136 is localized at, or

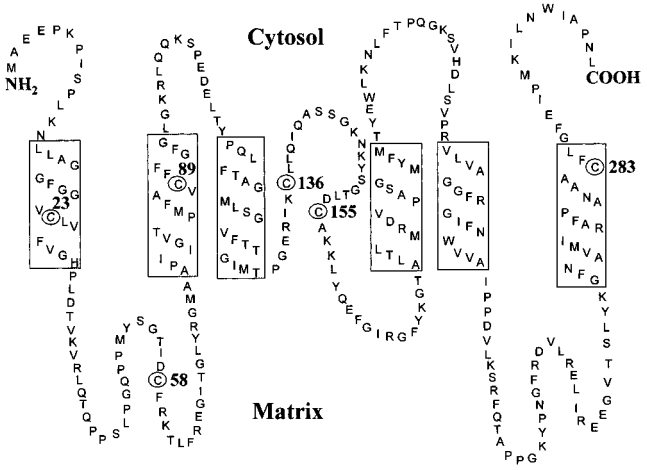

FIGURE 6: Secondary structure model of the rat CAC. The one-letter amino acid code is used. Putative transmembrane α-helices are shown in boxes. The Cys residues that were mutated are highlighted, and their number in the amino acid sequence is denoted.

near, the substrate binding site of the CAC. This hypothesis is strengthened by the protection exerted by the substrate against the NEM labeling of Cys-136. Since Cys-136 is not involved in transport function, it is possible that binding of Cys-136 of the CAC by SH reagents has an obstructing effect on the carnitine translocation path. However, the alternative explanation for the substrate protection, i.e., that substrate binding induces a change in the tertiary structure of the carrier protein, thereby decreasing the binding of the inhibitors to a site different from that of the substrate, is also feasible. As a result of the conformational change in the carrier, the reactivity or the accessibility of Cys-136 for SH reagents may be decreased. There exist several potential explanations for this effect. It may, for example, be caused by an increase in the p*K* of Cys-136, which may occur when the microenvironment surrounding the sulfydryl group becomes more hydrophobic (*22*). It may also be due to a relative decrease of positive charges close to the sulfydryl group.

In the primary structure of the CAC Cys-136 is present in the hydrophilic loop connecting transmembrane domains III and IV (see Figure 6). According to the folding model of the metabolite carrier proteins in the mitochondrial membrane (see ref *7* for references), which also applies to the CAC (*4*), this loop is located on the matrix side of the mitochondrial membrane. Since we have shown that both the purified (*29*) and the recombinant (this study) CAC proteins are oriented in proteoliposomes in the same direction as in mitochondria, loop III−IV containing Cys-136 is located inside the proteoliposomes. However, not only *N*-ethylmaleimide, which is a permeable reagent, but also MTSES and *p*-CMBS, which are hydrophilic impermeable reagents, react with Cys-136 when added externally to the proteoliposomes. This observation suggests that Cys-136 is accessible from the external side of the proteoliposomes and, therefore, loop III−IV protrudes into the membrane toward the outside, most likely between transmembrane domains (Figure 6). The proposal of intramembrane hydrophilic loops protruding from the matrix side was originally based on the observation that lysine groups, located in the hydrophilic matrix region of the ADP/ATP carrier, reacted with the membrane-impermeable reagent pyridoxal phosphate applied from the outside of the mitochondria (*43*). Furthermore, various covalently binding ATP derivatives were shown to be incorporated into the ADP/ATP carrier and the uncoupling protein in this matrix region, although they were not transported and were applied from the cytosolic side (*44*, *45*).

Finally, the experimental data obtained with $HgCl_2$ suggest that Cys-155 may perhaps be in close proximity to Cys-136, presumably within a distance of 4 Å. Thus, since Hg^{2+} is known to react with a single or two close SH groups (*22*), the lower extent of inhibition of mutant C155S by $HgCl_2$ as compared to the other SH reagents might suggest a close proximity of Cys-155 and Cys-136. Other arguments in favor of this interpretation come from the findings that the replacement of Cys-155 with Ser leads (a) to an increase in the transport affinity for carnitine and (b) to a lower extent of substrate protection against NEM or MTSES inhibition as compared to the wild-type CAC, suggesting a slight conformational change at or near the substrate binding site caused by the mutation in position 155. Cross-linking experiments between Cys-136 and Cys-155 are about to begin in our laboratory in order to prove or disprove the hypothesis of their proximity.

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