Identification by Site-directed Mutagenesis and Chemical Modification of Three Vicinal Cysteine Residues in Rat Mitochondrial Carnitine/Acylcarnitine Transporter*

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The proximity of the Cys residues present in the mitochondrial rat carnitine/acylcarnitine carrier (CAC) primary structure was studied by using site-directed mutagenesis in combination with chemical modification. CAC mutants, in which one or more Cys residues had been replaced with Ser, were overexpressed in *Escherichia coli* and reconstituted into liposomes. The effect of SH oxidizing, cross-linking, and coordinating reagents was evaluated on the carnitine/carnitine exchange catalyzed by the recombinant reconstituted CAC proteins. All the tested reagents efficiently inhibited the wild-type CAC. The inhibitory effect of diamide, Cu^{2+}-phenanthroline, or phenylarsine oxide was largely reduced or abolished by the double substitutions C136S/C155S, C58S/C136S, and C58S/C155S. The decrease in sensitivity to these reagents was much lower in double mutants in which Cys^{23} was substituted with Cys^{136} or Cys^{155}. No decrease in inhibition was found when Cys^{89} and/or Cys^{283} were replaced with Ser. Sb^{3+}, which coordinates three cysteines, inhibited only the Cys replacement mutants containing cysteines 58, 136, and 155 of the six native cysteines. In addition, the mutant C23S/C89S/C155S/C283S, in which double tandem fXa recognition sites were inserted in positions 65–72, *i.e.* between Cys^{58} and Cys^{136}, was not cleaved into two fragments by fXa protease after treatment with diamide. These results are interpreted in light of the homology model of CAC based on the available x-ray structure of the ADP/ATP carrier. They indicate that Cys^{58}, Cys^{136}, and Cys^{155} become close in the tertiary structure of the CAC during its catalytic cycle.

The mitochondrial carnitine/acylcarnitine carrier (CAC)[1] plays a central role in the translocation of fatty acids as acyl-carnitines into the mitochondrial matrix, where the acyl groups are used for fatty acid oxidation (1, 2). After some pioneer studies in intact mitochondria, the CAC was purified (3) and characterized in reconstituted liposomes (see Ref. 4 and references therein). In particular, the CAC was found to be very sensitive to cysteine-specific reagents such as *N*-ethylmaleimide, mercurials, and diamide (4). Later, the amino acid sequence of the carrier was determined by cDNA sequencing (5), showing that it belongs to the mitochondrial carrier protein family (reviewed in Ref. 2). On the basis of the hydrophobic profile of the CAC and its sequence similarity with the other mitochondrial carriers, a model for its arrangement in the inner mitochondrial membrane has been proposed (5). According to this model, the CAC polypeptide chain has six transmembrane α-helices (I–VI) traversing the membrane, connected by hydrophilic loops, and both the N and C termini protruding toward the cytosol. This asymmetric orientation of the membrane-embedded CAC is also supported by functional studies indicating different substrate-binding sites on the inner and outer faces in both intact mitochondria (6) and reconstituted liposomes (7). The CAC is encoded in man by the gene *SLC25A20* (2) that maps to chromosome 3p21.31 (8), in *Saccharomyces cerevisiae* by the gene *CRC* (9), and in *Aspergillus nidulans* by the gene *acuH* (10). The rat *CAC* gene was expressed in *Escherichia coli* and refolded in an active form (11), which opened the way to using site-directed mutagenesis to elucidate structure-function relationships of this metabolically important transporter. Recently, it was found that Cys^{136}, located in loop III-IV, is accessible to membrane-impermeable reagents from the external (cytosolic) side of the proteoliposomes (12).

The primary sequence of the rat CAC contains six cysteines (Cys^{23}, Cys^{58}, Cys^{89}, Cys^{136}, Cys^{155}, and Cys^{283}). In this work, we aimed to determine the relationships among the six Cys residues of CAC. By functional analysis of Cys-mutants treated with SH oxidizing, cross-linking, and coordinating reagents, we have identified three cysteine residues that become close in the tertiary structure of the CAC during its catalytic cycle. They are Cys^{58}, Cys^{136}, and Cys^{155}.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-50, G-75, and G-200 were purchased from Amersham Biosciences, L-[*methyl*-³H]carnitine from Amersham Biosciences, and egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), Pipes, Triton X-100, cardiolipin, L-carnitine, and *N*-dodecanoyl sarcosine (sarcosyl) and diamide 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 from total rat liver cDNA as described previously (11). The Cys/Ser replacements were constructed with complementary mutagenic primers using the

[1] The abbreviations used are: CAC, carnitine/acylcarnitine carrier; fXa, factor 10 of blood coagulation; Pipes, 1,4-piperazinediethanesulfonic acid.

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overlap extension method (13) and the High Fidelity PCR system (Roche Applied Science). The PCR products were purified by the Gene Clean Kit (La Jolla Pharmaceutical Company), digested with NdeI and HindIII (restriction sites added at the 5′ end of forward and reverse primers, respectively), and ligated into the pMW7 expression vector. Single or double tandem fXa recognition sites were inserted in positions 65–72 of the four-Cys replacement mutant C23S/C89S/C155S/C283S by replacing the sequence REGITGLY with IEGRIEGR using the overlap extension method (13). 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* C0214 (11). Bacterial overexpression, isolation of the inclusion body fraction, and solubilization and purification of the wild-type CAC and mutant CAC proteins were performed as described previously (11).

Reconstitution of CAC and CAC Mutants into Liposomes—The recombinant proteins were reconstituted into liposomes in the presence of 13 mM carnitine, as described previously (11). The external substrate was removed from proteoliposomes on Sephadex G-75 columns.

Transport Measurements and Effect of SH Oxidizing, Cross-linking, and Coordinating Reagents—Transport at 25 °C was started by adding 0.1 mM [³H]carnitine to proteoliposomes and terminated by the addition of 1.5 mM *N*-ethylmaleimide (11). In controls, the inhibitor was added together with the labeled substrate, according to the inhibitor stop method (14). Finally, the external substrate was removed by chromatography on Sephadex G-50 columns, and the radioactivity in the liposomes was measured (14). The experimental values were corrected by subtracting control values. All of the transport activities were determined by taking into account the efficiency of reconstitution (*i.e.* the share of successfully incorporated protein). To study the effect of diamide, Cu²⁺-phenanthroline, or phenylarsine oxide on the transport activity of wild-type CAC and CAC mutants, the proteoliposomes were preincubated with each reagent (under the conditions indicated in the legends to figures 1, 2, 3, 4, and 6) before transport was started. In the case of Sb³⁺, this reagent was added to the reconstitution mixture as potassium antimony tartrate.

Digestion with fXa Protease and Electrophoresis Analyses—The mutant containing fXa recognition sites was reconstituted into liposomes, as described above, and incubated in the presence or absence of 2 mM diamide for 30 min at room temperature. Next, the proteoliposomes were passed through Sephadex G-200, ultracentrifuged at 110,000 × *g* for 90 min at 4 °C, resuspended in fXa digestion buffer (50 mM Tris-HCl, pH 8.0, and 1 mM CaCl₂) and 2% Triton X-100, and digested with fXa protease (4 µg/ml) for 24 h at 4 °C. Finally, the samples were analyzed by SDS-PAGE in the presence or absence of dithioerythritol.

Other Methods—SDS-PAGE was performed according to Laemmli (15) as described previously (12). The amount of recombinant protein was estimated on Coomassie Blue-stained SDS-polyacrylamide gels by a 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 by Phelps *et al.* (16), with the modifications reported in Ref. 12. N-terminal sequencing was carried out as described previously (17). The amino acid sequences were aligned with ClustalW (version 1.7). The homology model of the CAC was built by the Swiss-Model protein modeling server (18–20) using the x-ray structure of the carboxyatractyloside-ADP/ATP carrier complex as a template (21).

RESULTS

Transport Activity of CAC Mutants—As reported previously, all of the single Cys mutants exhibited transport activities similar to the wild-type protein (12). Table I shows the transport activities of the mutants containing more than one substitution. Most of these mutants had transport activities ranging from ~20 to 110% of the wild-type CAC. The mutants C23S/C58S/C136S, C58S/C136S/C155S, C23S/C58S/C136S/C155S, and C23S/C58S/C89S/C155S were more or less inactive (transport activity <5% of the wild type). A comparable loss of activity was observed previously for the C-less CAC and for the five-replacement mutants C23S/C58S/C89S/C136S/C283S and C23S/C58S/C89S/C155S/C283S (12). All of these virtually inactive mutants are characterized by substitution of at least three Cys residues among the four residues Cys²³, Cys⁵⁸, Cys¹³⁶, and Cys¹⁵⁵, independently of the presence or absence of Cys⁸⁹ and Cys²⁸³ (Table I).

TABLE I
Transport activity of CAC mutants

CAC mutants, in which two or more Cys residues were substituted with Ser, were overexpressed in *E. coli,* reconstituted into liposomes, and tested for their transport activity. The activity was measured as [³H]carnitine/carnitine exchange (see "Experimental procedures"). C23S/C89S/C155S/C283S (2 fXa) represents the four-Cys replacement mutant in which double tandem fXa recognition sites were inserted. Similar results were obtained in at least three independent experiments in duplicate.

Mutant proteins	Cys present	Transport activity
		µmol/10 min/g protein
Wild type	All	1580
C23S/C58S	89/136/155/283	1336
C23S/C136S	58/89/155/283	1321
C23S/C155S	58/89/136/283	983
C23S/C283S	58/89/136/155	912
C58S/C136S	23/89/155/283	863
C58S/C155S	23/89/136/283	313
C89S/C136S	23/58/155/283	1152
C136S/C155S	23/58/89/283	415
C136S/C283S	23/58/89/155	867
C23S/C58S/C136S	89/155/283	58
C23S/C58S/C89S	136/155/283	1311
C23S/C58S/C283S	89/136/155	1484
C58S/C136S/C155S	23/89/283	46
C23S/C58S/C89S/C283S	136/155	1288
C23S/C58S/C136S/C155S	89/283	63
C23S/C58S/C89S/C155S	136/283	56
C23S/C89S/C136S/C283S	58/155	1573
C23S/C89S/C155S/C283S	58/136	986
C23S/C89S/C155S/C283S (2 fXa)	58/136	358
C58S/C89S/C136S/C283S	23/155	747
C58S/C89S/C155S/C283S	23/136	595
C89S/C136S/C155S/C283S	23/58	1718

Effect of Diamide on CAC Cys Mutants—Fig. 1 shows the effect of the SH-oxidizing reagent diamide (22), at concentrations from 0.1 to 0.5 mM, on single CAC Cys mutants. Diamide strongly inactivated the wild-type protein, reaching nearly complete inhibition of transport activity at 0.5 mM. The mutants C23S, C58S, C89S, C155S, and C283S were inactivated similarly to the wild-type CAC. Only C136S was much less sensitive to the reagent. Because protein inactivation caused by diamide is used to monitor the formation of S-S bridge(s) (4), these results indicate that Cys¹³⁶ is involved in the formation of S-S bridge(s). To gain information about the second Cys residue involved in the disulfide bridge(s), double mutants were tested for their sensitivity to diamide concentrations up to 2 mM. In a first set of double mutants, Cys¹³⁶ was substituted together with a second Cys residue (Fig. 2*A*). The double mutants C89S/C136S and C136S/C283S showed a sensitivity to diamide that was virtually indistinguishable from that exhibited by the single mutant C136S, indicating that Cys⁸⁹ and Cys²⁸³ are not involved in S-S formation. The sensitivity to diamide of the double mutant C23S/C136S and in particular of C58S/C136S was lower than that of the single mutant C136S; a complete loss of sensitivity to diamide was observed with the mutant C136S/C155S, indicating that the presence of Cys¹³⁶ and/or Cys¹⁵⁵ is essential for the formation of S-S bridges either with each other or in combination with other Cys residues. The dependence on diamide of the transport activity exhibited by the mutants C23S/C58S, C23S/C155S, and C58S/C155S is shown in Fig. 2*B*. C23S/C58S and C23S/C155S showed a sensitivity to diamide similar to or only slightly lower than that of the wild type, whereas C58S/C155S had a much lower sensitivity to diamide than the wild type, indicating that an S-S bridge is formed between Cys⁵⁸ (but not Cys²³) and Cys¹⁵⁵. We then analyzed the effect of diamide on CAC mutants in which only two of the four Cys residues 23, 58, 136, and 155 were present (Fig. 3). C23S/C58S/C89S/C283S showed a sensitivity
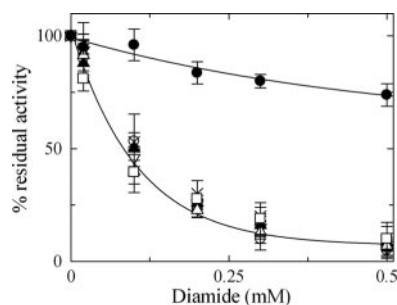
FIG. 1. **Dependence on diamide of carnitine transport catalyzed by reconstituted CAC single Cys mutants.** Proteoliposomes containing 13 mM carnitine were preincubated for 1 min with 0–0.5 mM diamide. Transport was started by the addition of 0.1 mM [³H]carnitine and stopped after 5 min. Results are expressed as percent of residual activity, *i.e.* percent of the activity in the presence of diamide with respect to the control value without inhibitor. The data represent means ± S.D. of at least three independent experiments in duplicate. ○, wild type; ×, C23S; ▲, C58S; △, C89S; ●, C136S; □, C155S; ▽, C283S.
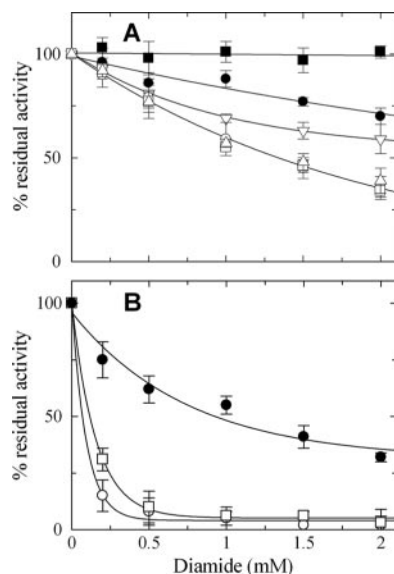

FIG. 2. **Dependence on diamide of carnitine transport catalyzed by reconstituted CAC double Cys-mutants.** Proteoliposomes were preincubated with 0–2 mM diamide. Other conditions were the same as in Fig. 1. *A*, the results with the mutants C136S (□), C23S/C136S (▽), C58S/C136S (●), C89S/C136S (○), C136S/C155S (■), and C136S/C283S (△). *B*, the results with the mutants C23S/C58S (○), C23S/C155S (□), and C58S/C155S (●).

to diamide similar to the wild type, indicating that Cys[136] and Cys[155] are at or can come to an optimal distance for forming an S-S bridge. In contrast, C89S/C136S/C155S/C283S was completely insensitive to diamide, indicating that Cys[23] and Cys[58] cannot form an internal S-S bridge. C23S/C89S/C155S/C283S and C23S/C89S/C136S/C283S were strongly sensitive to diamide, indicating that Cys[58] is involved in S-S formation with Cys[136] or Cys[155]. A much lower sensitivity was observed with C58S/C89S/C155S/C283S and especially with C58S/C89S/C136S/C283S, suggesting a minor contribution of Cys[23] to S-S formation with Cys[136] or Cys[155]. It should be noted that the same high sensitivity to diamide observed with C23S/C58S (Fig. 2*B*) was found for the triple mutants C23S/C58S/C89S and C23S/C58S/C283S and that the addition of 15 mM dithioerythritol after treatment with 2 mM diamide led to a complete recovery of the activity of all the mutants described (not shown). In addition, the effect of diamide on mutants C23S/C58S/C136S, C58S/C136S/C155S, C23S/C58S/C136S/C155S, and C23S/C58S/C89S/C155S was not tested because of their very low transport activity.
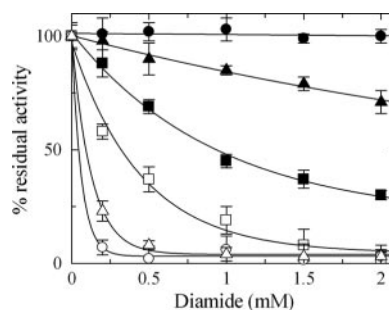

FIG. 3. **Dependence on diamide of carnitine transport catalyzed by reconstituted CAC four-Cys replacement mutants.** Proteoliposomes were preincubated with 0–2 mM diamide. Other conditions were as in Fig. 1. ○, C23S/C58S/C89S/C283S; □, C23S/C89S/C136S/C283S; △, C23S/C89S/C155S/C283S; ▲, C58S/C89S/C136S/C283S; ■, C58S/C89S/C155S/C283S; ●, C89S/C136S/C155S/C283S.

Effect of Cu²⁺-phenanthroline and Phenylarsine Oxide on CAC Four-Cys Replacement Mutants—We also tested the effect of Cu^{2+}-phenanthroline, another reagent that, like diamide, oxidizes vicinal SH residues to S-S bridges (23), on the activity of the wild-type CAC and of the four-replacement mutants C23S/C58S/C89S/C283S, C23S/C89S/C136S/C283S, C23S/C89S/C155S/C283S, C58S/C89S/C136S/C283S, C58S/C89S/C155S/C283S, and C89S/C136S/C155S/C283S (Fig. 4*A*). The wild-type protein and the C23S/C58S/C89S/C283S mutant were totally inhibited by 2 μM Cu^{2+} phenanthroline, confirming that Cys[136] and Cys[155] are at or can come to an optimal distance for S-S formation. C23S/C89S/C155S/C283S was also efficiently inhibited, showing maximal effect at about 5 μM Cu^{2+}-phenanthroline. C23S/C89S/C136S/C283S and C58S/C89S/C155S/C283S were about 70% inactivated at 10 μM Cu^{2+}-phenanthroline. C58S/C89S/C136S/C283S was about 30% inactivated at 10 μM Cu^{2+}-phenanthroline, and C89S/C136S/C155S/C283S was nearly unaffected at the same concentration.

In another set of experiments, the effect of phenylarsine oxide was investigated. This reagent does not induce the formation of S-S bridges but reacts with two Cys residues close enough to be cross-linked with the reagent (24). Fig. 4*B* shows that the wild-type CAC, C23S/C58S/C89S/C283S, and C23S/C89S/C155S/C283S were strongly inhibited by phenylarsine oxide. C23S/C89S/C136S/C283S and C58S/C89S/C155S/C283S were inhibited to a lesser extent, and C58S/C89S/C136S/C283S and especially C89S/C136S/C155S/C283S were affected poorly by phenylarsine oxide up to 1 mM. Taken together, these results indicate that Cys[136] and Cys[155] can form an S-S bridge not only with each other but also with Cys[58] and perhaps with Cys[23].

Effect of Sb³⁺ on CAC Cys Mutants—To obtain further support for the existence of vicinal SH groups in the CAC molecule, the effect of Sb^{3+} was investigated. Sb^{3+} is known to form a tricoordinate complex with a cluster of three Cys residues in the tertiary structure of proteins (25, 26). We first found that 0.8 mM Sb^{3+} completely inhibited the activity of the wild-type CAC, indicating that Sb^{3+} can form a complex with three of the Cys residues present in the carrier protein. Then we compared the effects of the reagent, at a concentration giving half-maximal inhibition of the wild-type protein (0.2 mM), on CAC mutants in which one or more of the four Cys residues 23, 58, 136, and 155 were substituted (Fig. 5). All of the four-replacement mutants tested (containing only two Cys residues) were insensitive to Sb^{3+}. The double mutant C23S/C283S (containing Cys[58], Cys[89], Cys[136], and Cys[155]) was strongly inhibited by Sb^{3+}, similarly to the wild-type protein. In contrast, little inhibition by Sb^{3+} was observed with the double mutants in which at least one of the three cysteines 58, 136, and 155 was substituted. Among the single mutants, C23S, C89S, and
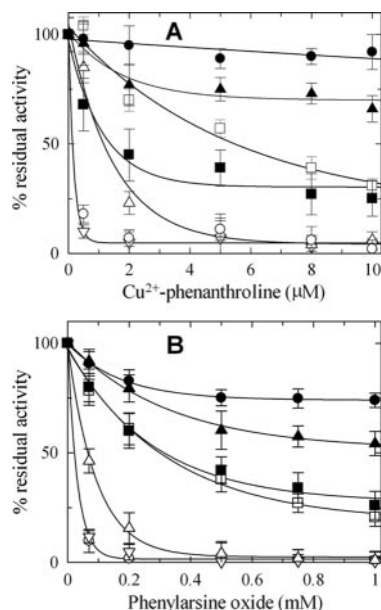
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FIG. 4. **Dependence on Cu^{2+}-phenanthroline (*A*) or phenylarsine oxide (*B*) of carnitine transport catalyzed by reconstituted CAC four-Cys replacement mutants.** Proteoliposomes, reconstituted with wild type (▽), C23S/C58S/C89S/C283S (○), C23S/C89S/C136S/C283S (□), C23S/C89S/C155S/C283S (△), C58S/C89S/C136S/C283S (▲), C58S/C89S/C155S/C283S (■), and C89S/C136S/C155S/C283S (●), were preincubated with 0–10 μM Cu^{2+}-phenanthroline for 5 min (*A*) or with 0–1 mM phenylarsine oxide for 1 min (*B*). Other conditions were the same as in Fig. 1.
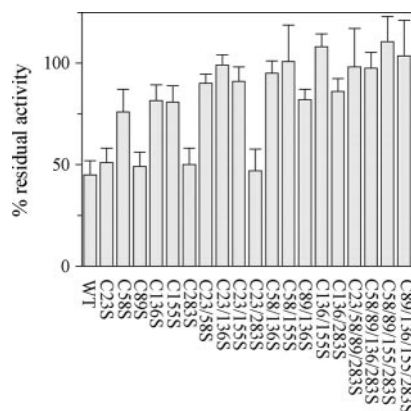

FIG. 5. **Effect of Sb^{3+} on CAC mutants.** The reconstitution mixture was preincubated for 2 min with 0.2 mM Sb^{3+}, which was added as potassium antimony tartrate. The resulting proteoliposomes were assayed for transport as described under "Experimental Procedures" and as described in the Fig. 1 legend, except that the incubation time was 10 min. *WT*, wild type.

C283S were markedly inhibited similarly to the wild-type CAC, whereas C58S, C136S, and C155S were only slightly inhibited by Sb^{3+}. These results point to the importance of each of the three cysteines 58, 136, and 155 in the formation of the tricoordinate complex with Sb^{3+}.

The Chemical Modifications Involve Only Cysteines Belonging to a Single Polypeptide Chain—It is important to know whether the cysteine pairs of the CAC oxidized by diamide and Cu^{2+}-phenanthroline and cross-linked by phenylarsine oxide, as well as the three cysteines coordinated by Sb^{3+}, are located on the same polypeptide or on different polypeptide subunits. This question was investigated by subjecting the wild-type CAC and its mutants to SDS-PAGE, in the absence of dithioerythritol, after treatment with the above mentioned reagents. The results obtained showed no significant variation in the
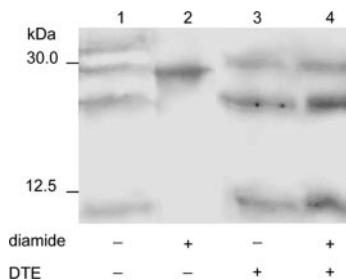

FIG. 6. **Site-specific proteolysis of the CAC mutant C23S/C89S/C155S/C283S containing double tandem fXa recognition sites and the effect of diamide.** Proteoliposomes, reconstituted with C23S/C89S/C155S/C283S containing double tandem fXa recognition sites, were incubated with (*lanes 2 and 4*) or without (*lanes 1 and 3*) diamide and then with fXa protease. Subsequently, the samples were subjected to SDS-PAGE in the presence (*lanes 3 and 4*) or absence (*lanes 1 and 2*) of dithioerythritol (*DTE*). Other conditions were as described under "Experimental Procedures."

apparent molecular mass of any protein tested with or without preincubation with the chemical modifiers (not shown). This result clearly indicates that diamide, Cu^{2+}-phenanthroline, phenylarsine oxide, and Sb^{3+} do not cause dimerization of CAC and its mutants, and therefore all the cysteines involved in the modifications described above are located on a single polypeptide subunit.

Disulfuric Linkage in the C23S/C89S/C155S/C283S CAC Mutant with Inserted fXa Recognition Sites—In other experiments, double tandem fXa recognition sites were inserted in positions 65–72 of the C23S/C89S/C155S/C283S mutant. After incubation with fXa protease, this mutant was proteolyzed to an appreciable extent, giving rise to two fragments (Fig. 6, *lane 1*). As deduced from their N-terminal sequencing, the smaller fragment, with a molecular mass of ~7.5 kDa, extended from the N terminus to the cleavage site, and the larger fragment, with a molecular mass of ~25 kDa, extended from the cleavage site to the C terminus. Interestingly, if the mutant was treated with diamide before the incubation with fXa protease, no cleavage was observed (Fig. 6, *lane 2*), indicating that under these conditions, the two peptides are linked by a diamide-induced S-S bridge. To support this conclusion, aliquots of the proteins that had been treated with fXa protease without (Fig. 6, *lane 1*) and with (*lane 2*) diamide were supplemented with dithioerythritol before being subjected to SDS-PAGE (*lanes 3 and 4*). After the dithioerythritol addition, the two peptides also appeared in the diamide-treated sample (Fig. 6, *lane 4*), *i.e.* the S-S bridge formed by diamide was reduced. It is noteworthy that the smear of the protein bands shown in Fig. 6 was caused by the presence of phospholipids derived from the proteoliposomes from which the CAC proteins were extracted. Furthermore, it is possible that internal disulfide bridges contributed slightly to the apparent molecular mass in the absence of dithioerythritol (Fig. 6, *lane 2*). This also explains why the mutant C23S/C89S/C155S/C283S often appears as two close bands after gel electrophoresis, due to either spontaneous oxidation of a fraction of the protein or incomplete reaction with diamide. It should be mentioned that after reconstitution into liposomes, the mutant containing double tandem fXa recognition sites showed much lower activity than the wild-type CAC (Table I) and that the same mutant containing only one fXa recognition site showed higher transport activity but was poorly cleaved by fXa protease (not shown, see also Ref. 27).

Homology Model of CAC Based on the X-ray Structure of the ADP/ATP Carrier—The rat mitochondrial CAC is homologous with the bovine mitochondrial ADP/ATP carrier for which a crystal structure is available (21). In fact, the sequences of the two carriers can be aligned unambiguously (Fig. 7); they share

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CAC  MAEEPKPISPLKNLLAGGFGGVCLVFVGHPLDTVKVRLQTQPPS-LPGQPPMYSGTIDCF  60
AAC  ---SDQALSFLKDFLAGGVAAAISKTAVAPIERVKLLLQVQHASKQISAEKQYKGIIDCV
     . .* **..**** .        *.. **. ** * *        * * ***

CAC  RKTLFREGITGLYRGMAAPIIGVTPMFAVCFFGFGLGKRLQQKSPEDELTYPQLFTAGML  120
AAC  VRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDRHKQFWRYFAGNLA
      .    .*.  ..** * .*  *  *. *       *..  .     . .*. .

CAC  SG----VFTTGIMTPGERIKCLLQIQASSGKNK--YSGTLDCAKKLYQEFGIRGFYKGTA  180
AAC  SGGAAGATSLCFVYPLDFARTRLAADVGKGAAQREFTGLGNCITKIFKSDGLRGLYQGFN
     **      .   .   .* *      *    *  .*  .*   *... *.**.*.*

CAC  LTLMRDVPASGMYFMTYEWLKNLFTPQGKSVHDLSVPRVLVAGGFRGIFNWVVAIPPDVL  240
AAC  VSVQGIIIYRAAYFGVYDTAKGMLPDPKNVHIIVSWMIAQTVTAVAGLVSYPFDTVRRRM
     ...      ** *. .*     .     .*         *. .     . .

CAC  KSRFQTAPPGKYPNGFRDVLRELIREEGVTSLYKGFNAVMIRAFPANAACFLGFEIPMKI  300
AAC  MMQSGRKGADIMYTGTVDCWRKIAKDEGPKAFFKGAWSNVLRGMGGAFVLVLYDEIKKFV
      .         * * *.. ..** ...** ...*. .      *  ** .

CAC  LNWIAPNL
AAC  --------
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19.6% identical amino acids and 39.6% highly conserved residues. Interestingly, Cys^{58} and Cys^{155} of the rat CAC are conserved in the bovine ADP/ATP carrier corresponding to Cys^{56} and Cys^{159}, respectively. We therefore built a homology model of CAC (Fig. 8) based on the three-dimensional structure of the carboxyatractyloside-ADP/ATP carrier complex. Fig. 8 highlights the positions of the six cysteine residues of CAC, which are grouped at the matrix side (Cys^{58}, Cys^{136}, and Cys^{155}) and at the cytoplasmic side (Cys^{23}, Cys^{89}, and Cys^{283}) of the membrane-embedded CAC protein.

DISCUSSION

The resolution of the tertiary structure of mitochondrial carriers by x-ray crystallography is still in its infancy. So far, only the structure of the ADP/ATP carrier-carboxyatractyloside inhibited complex has been determined after crystallization of the purified carrier from bovine mitochondria (21). Therefore, especially for a protein like CAC, which is present in a very minute amount in mitochondria, the site-directed mutagenesis and chemical modification approach still represents a useful tool to obtain structural and dynamic information and to define structure-function relationships.

We found previously that formation of disulfide(s) in the CAC protein, purified from mitochondria, leads to transport inhibition, and we therefore concluded that at least two of the six Cys residues present in the CAC amino acid sequence are in close proximity (4). Identifying the specific residues that can form disulfides or can be cross-linked has important implications on the structure and dynamic properties of the CAC, because the protein segments that contain these Cys residues have to be sufficiently close or become close to each other during the conformational changes accompanying the translocation of the substrate through the protein. The functional analysis described above of one-, two-, and four-Cys replacements mutants of CAC, as well as of the wild-type recombinant protein, clearly shows that Cys^{58}, Cys^{136}, and Cys^{155} can be oxidized by diamide and Cu^{2+}-phenanthroline or cross-linked by phenylarsine oxide. Therefore the residues 58, 136, and 155 of CAC are close or become close in the tertiary structure of the protein during its catalytic cycle. This conclusion is supported by the observation that Sb^{3+}, a reagent forming a tricoordinate complex with three cysteines (25, 26), inhibits only the CAC mutants containing the above mentioned cysteines. It is also supported by the observation that the CAC mutant C23S/C89S/C155S/C283S, containing the cysteines 58 and 136 and two tandem fXa recognition sites in positions 65–72, is not cleaved by fXa protease into two fragments if pretreated with diamide.

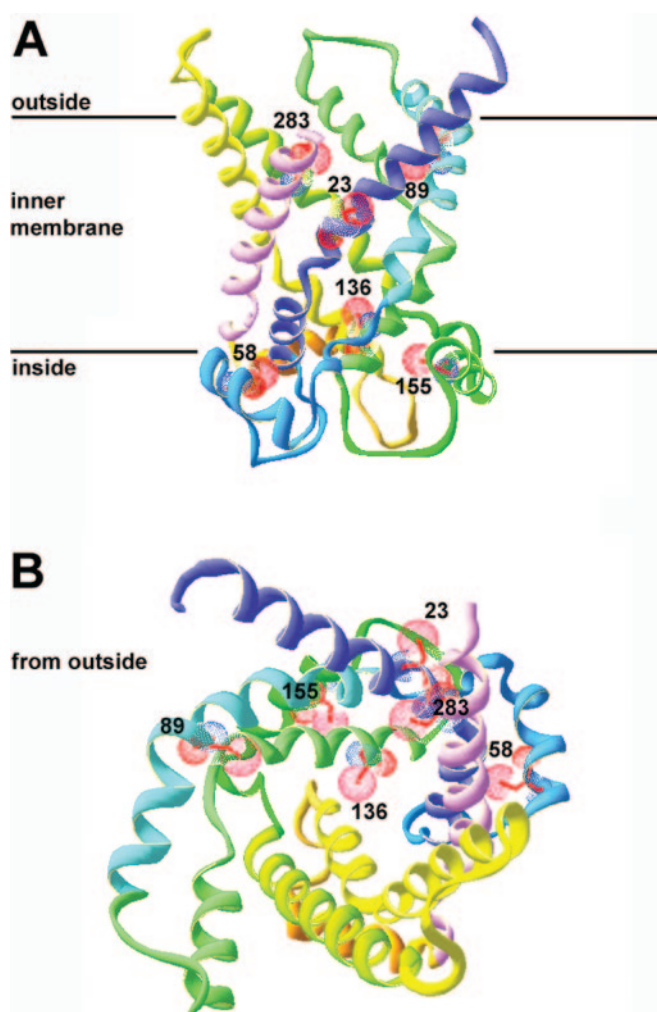The CAC and the ADP/ATP carrier are homologous and must

FIG. 8. **Structural model of the CAC.** Ribbon diagrams viewing the carrier from the side (*A*) or from the outside (*B*). The transmembrane α-helices are colored as follows: TM I, *blue*; TM II, *light blue*; TM III, *light green*; TM IV, *yellow-green*; TM V, *yellow*; TM VI, *pink*. Red dot surfaces highlight the SH groups of the Cys residues indicated by their number in the CAC sequence. The distances for Cys^{58}–Cys^{136} and Cys^{136}–Cys^{155}, calculated by the Swiss Protein Database Viewer software, are 14.8 and 9.4 Å, respectively.

have the same overall structure. Therefore, we discuss our results in light of the homology model of CAC (Fig. 8) based on the available x-ray structure of the ADP/ATP carrier (21). It is particularly interesting to compare the three-dimensional structure

with our Cys mutagenesis and chemical modification data because the former can only provide static information, whereas the latter can give a more dynamic view of the structure.

As shown in the homology-modeled structure of the CAC, the three important cysteines, Cys^{58}, Cys^{136}, and Cys^{155}, are clustered at approximately identical distances from the membrane/aqueous interface near the mitochondrial matrix side. In the three-dimensional structure, the distances between the side chains of these cysteines (for instance, 9.4 Å between Cys^{136} and Cys^{155}) are greater than those required for the chemical modifications described in this study. However, it is possible that Cys^{58}, Cys^{136}, and Cys^{155} become closer during some stage of the catalytic cycle. The presence of the substrate carnitine, inside and outside of the proteoliposomes, probably facilitates the cross-linking of the CAC cysteine pairs observed here. It has indeed been shown that on binding the substrate, the carrier-substrate complex undergoes a conformational change, causing the binding center to switch between the external and internal states (reviewed in Ref. 28). Therefore, substrates may confer on the above mentioned cysteines the mobility necessary for gaining sufficient proximity to be cross-linked. We demonstrated previously that Cys^{136} is accessible from the extraliposomal (cytosolic) side to the membrane-impermeant reagents sodium(2-sulfonatoethyl)-methane-thiosulfonate and *p*-(chloromercuri)benzene-sulfonate and that this interaction is prevented by the presence of the CAC substrate (12). Interestingly, the homology model of CAC shows that Cys^{136} (corresponding to Thr-138 of the ADP/ATP carrier) protrudes into the large water-accessible cavity that, in the crystallographic structure of the ADP/ATP carrier-carboxyatractyloside complex, is exposed toward the cytosolic side of the mitochondrial membrane and is occupied by the inhibitor or possibly by the cytosolic ADP (21).

The effects of the various modification reagents on Cys^{23}-containing mutants are relatively minor, and the kinetics of the cross-linking reactions are also clearly different for Cys^{23}-containing mutants than for the mutants containing at least two of the three cysteines 58, 136, and 155 (Figs. 2 and 4). These results alone make it unlikely that Cys^{23} can be oxidized or cross-linked with Cys^{58}, Cys^{136}, or Cys^{155}. This possibility is definitively ruled out by the homology model of CAC. Cys^{23} is pointing into the lipid phase at the cytoplasmic side of the carrier protein, and its distance from Cys^{58}, Cys^{136}, and Cys^{155} (24.2, 21.3, and 24.2 Å, respectively) is too great to be cross-linked with one of these cysteines, unless drastic structural changes take place. In this study, some reconstituted mutants containing Cys^{23}, but with various mutations in the other native cysteines, have considerably lower activity than the wild-type protein (for example 19.8% of the wild-type activity for C58S/C155S). Because none of the native cysteines (including Cys^{23}) are important for the transport activity *per se* (12), the decreased activity of these mutants may be caused by structural/folding instability. A plausible interpretation of the results, which seemed to suggest involvement of Cys^{23} in disulfide bridge(s) with Cys^{136} or Cys^{155}, is that in certain recombinant mutants containing Cys^{23} and mutations in other native cysteines, there may be a portion of misfolded molecules that give anomalous cross-linking. This would in fact agree with previous results showing that C23S mutants have low yields of reconstitution into liposomes, which is likely because of structural/folding instability (12).

The CAC contains two other Cys residues, Cys^{89} and Cys^{283}. Our mutagenesis and chemical modification data, which show

that neither Cys^{89} nor Cys^{283} can be oxidized or cross-linked with Cys^{58}, Cys^{136}, or Cys^{155}, can be explained easily by the CAC homology model, because they are located at the cytoplasmic side of the carrier and are >20 Å from the three important cysteines Cys^{58}, Cys^{136}, and Cys^{155}. However, we cannot exclude the possibility that Cys^{89} and Cys^{283} may form a disulfide bridge with each other or with Cys^{23} without loss of transport activity.

Another result of this work is that at least two of the three important Cys residues of the rat CAC (Cys^{58}, Cys^{136}, and Cys^{155}) have to be present in reduced form for efficient activity of transport. It can be excluded, however, that these cysteines have a catalytic function because they are not conserved in evolutionarily distant organisms such as *S. cerevisiae* and *A. nidulans*. It is likely that their oxidation, cross-linking, and coordination by Sb^{3+} rigidifies the protein structure, causing reduction in transport activity. SH groups interact both with hydrophilic residues (SH, OH, NH_2) via hydrogen bond (29) and with hydrophobic residues (30). It is possible that in the rat CAC, two thiol groups of the three cysteines 58, 136, and 155 dynamically switch from hydrophilic to hydrophobic interactions, being structurally and dynamically important, probably because they are involved in one or more conformational changes during the translocation process.

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