The Mitochondrial Oxoglutarate Carrier: Cysteine-Scanning Mutagenesis of Transmembrane Domain IV and Sensitivity of Cys Mutants to Sulfhydryl Reagents†

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ABSTRACT: Using a functional mitochondrial oxoglutarate carrier mutant devoid of Cys residues (C-less carrier), each amino acid residue in transmembrane domain IV and flanking hydrophilic loops (from T179 to S205) was replaced individually with Cys. The great majority of the 27 mutants exhibited significant oxoglutarate transport in reconstituted liposomes as compared to the activity of the C-less carrier. In contrast, Cys substitution for G183, R190, Q198, and Y202, in either C-less or wild-type carriers, yielded molecules with complete loss of oxoglutarate transport activity. G183 and R190 could be partially replaced only by Ala and Lys, respectively, whereas Q198 and Y202 were irreplaceable with respect to oxoglutarate transport. Of the single-Cys mutants tested, only T187C, A191C, V194C, and N195C were strongly inactivated by *N*-ethylmaleimide and by low concentrations of methanethiosulfonate derivatives. Oxoglutarate protects Cys residues at positions 187, 191, and 194 against reaction with *N*-ethylmaleimide. These positions as well as the residues found to be essential for the carrier activity, except Y202 which is located in the extramembrane loop IV−V, reside on the same face of transmembrane helix IV, probably lining part of a water-accessible crevice or channel between helices of the oxoglutarate carrier.

The oxoglutarate transporter, also known as the oxoglutarate/malate carrier (OGC),[1] is a nuclear-encoded protein located in the inner mitochondrial membrane. This enzyme catalyzes the transport of 2-oxoglutarate in an electroneutral exchange for malate and plays an important role in several metabolic processes, including the malate−aspartate shuttle, the oxoglutarate−isocitrate shuttle, gluconeogenesis from lactate, and nitrogen metabolism (*1*).

Encoded by the OGC gene that in man maps to chromosome 17q25.3 (*2*), the carrier has been purified and kinetically characterized in reconstituted liposomes (for a review, see ref *3*). Like other functionally characterized members of the mitochondrial carrier family, the protein has a tripartite structure, made up of 3 tandemly repeated sequences of about 100 amino acids in length. On the basis of the hydropathy profile of the primary amino acid sequence (*4*), a secondary structure was proposed in which the carrier has six α-helical transmembrane domains connected by hydrophilic loops. Evidence supporting the general features of the model and demonstrating that both the N and C termini of OGC are on the cytoplasmic face of the membrane has been obtained from limited proteolysis and immunological studies (*5*). By using cross-linking reagents, it has been shown that the OGC exists as a homodimer (*6*). In addition, the OGC has been expressed in *E. coli* and refolded in reconstitutively active form (*7*). Previous site-directed mutagenesis studies showed that the three cysteinyl residues present in the primary structure of OGC are not involved in the transport mechanism since the C-less mutant is fully active (*8*).

In this study, Cys-scanning mutagenesis of the functional C-less mutant of OGC has been employed to examine the residues in transmembrane helix IV and in part of the flanking hydrophilic loops of OGC. The effects of individual Cys replacements for Thr179−Ser205 on the oxoglutarate transport activity are reported, as well as the sensitivity of the mutants to the alkylating agent *N*-ethylmaleimide (NEM). The results demonstrate that four of the residues (G183, R190, Q198, and Y202) are essential for the carrier activity, four Cys mutants (T187C, A191C, V194C, and N195C) are inactivated by NEM, and these mutants, except N195C, exhibit substrate protection against NEM inhibition. All these residues, except Y202 which is located in the extramembrane loop IV−V, appear to cluster on one face of helix IV, suggesting that this surface within the OGC protein may be important for substrate binding and/or translocation of oxoglutarate.

MATERIALS AND METHODS

Materials. 2-Keto[1-^{14}C]glutaric acid and [^{14}C]NEM were purchased from Dupont De Nemours; Pipes, Triton X-114, Amberlite XAD-4, and egg yolk phospholipids (lecithin from

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[1] Abbreviations: DTE, dithioerythritol; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; NEM, *N*-ethylmaleimide; OGC, oxoglutarate carrier; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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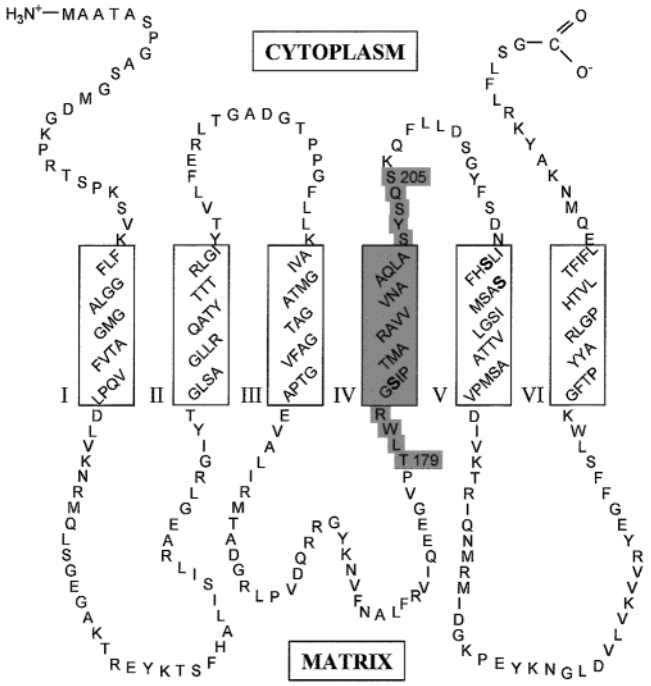

FIGURE 1: Secondary structure model of the C-less bovine OGC. The one-letter amino acid code is used. Putative transmembrane α-helices are shown in boxes, and the Ser residues replacing the three native Cys residues are enlarged and emboldened. The shaded area highlights the region of the carrier subjected to Cys-scanning mutagenesis.

eggs) from Fluka; *N*-dodecanoylsarcosine (sarkosyl) from Sigma; MTSEA, MTSES, and MTSET from Fluorescent Dyes Inc.; and Sephadex G-75 from Pharmacia. All other reagents were of analytical grade.

Construction of Plasmids and Site-Directed Mutagenesis. The coding region for the bovine OGC was amplified by PCR from bovine heart cDNA essentially as described previously (*7*). The forward and reverse oligonucleotide primers corresponded to the extremities of the coding sequence for OGC [nucleotides 48−64 and 976−992 of the cDNA for OGC (*4*) followed by the stop codon and with *Nde*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively]. The C-less OGC was obtained by replacing the three native cysteines (C184S, C221S, and C224S) with serine residues using the OGC cDNA fragment as template (*8*). The C-less OGC was then employed as template to generate the single-Cys-replacement mutants. Each amino acid residue from T179 to S205, a region containing the putative transmembrane helix IV and part of the flanking hydrophilic loops (Figure 1), was replaced individually with cysteine in C-less OGC. Mutant S184C restores a single native Cys to C-less OGC. G183, R190, Q198, and Y202 were also mutated to various other residues in C-less OGC. In addition, mutants G183C, R190C, Q198C, or Y202C were also cloned into the wild-type OGC gene. All mutations were introduced in the wild-type OGC cDNA or in the C-less OGC cDNA by the overlap extension PCR method (*9*) using oligonucleotides carrying appropriate mutations in their sequences. The PCR products were cloned into the expression vector pMW7, which was transformed into *E. coli* DH5α cells. Transformants selected on 2 × TY plates containing ampicillin (100 μg/mL) were screened by direct-colony PCR, and by restriction digestion of the purified plasmid DNA. All mutations were verified by DNA sequencing, and, except for the desired base changes, all the sequences were identical to those of OGC or C-less OGC.

Bacterial Expression of OGC and OGC Mutants. The overproduction of OGC mutants as inclusion bodies in the bacterial cytosol was accomplished as described before for the wild-type bovine oxoglutarate carrier (*7*), except that the host cells were *E. coli* CO214(DE3) (*10*). The inclusion bodies, purified as described previously (*7*), were washed at 4 °C first with TE buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA), then twice with a buffer containing 10 mM Pipes, pH 7.0, Triton X-100 (3%, w/v) and 0.1 mM EDTA, and then once again with TE buffer. OGC and OGC mutants were solubilized in a buffer containing 2.5% (w/v) sarkosyl, 10 mM Tris-HCl, pH 7.0, and 0.1 mM EDTA. Insoluble material was removed by centrifugation (258000*g* for 1 h at 4 °C). Each mutant of about 31.5 kDa molecular mass, identified by its reaction with a rabbit antiserum raised against the OGC isolated from bovine heart mitochondria, was more than 90% pure.

Reconstitution of OGC and OGC Mutants into Liposomes and Transport Measurements. The recombinant proteins in sarkosyl were diluted 10-fold with a buffer containing 2 mM Pipes, pH 7.0, 0.6% (w/v) Triton X-114, and 0.2 mM EDTA and reconstituted into liposomes in the presence of 20 mM oxoglutarate, as described previously (*11*). The external substrate was removed from proteoliposomes on Sephadex G-75 columns. Transport at 25 °C was started by adding [^{14}C]oxoglutarate to eluted proteoliposomes and terminated by the addition of 20 mM pyridoxal 5′-phosphate and 16 mM bathophenanthroline (*11*). In controls, the inhibitors were added at the beginning together with the labeled substrate. Finally, the external substrate was removed from quenched samples on Dowex AG1-X8, and the radioactivity in the liposomes was measured (*11*). The experimental values were corrected by subtracting control values, and the transport activities were calculated by taking into account the efficiency of reconstitution (i.e., the share of successfully incorporated protein).

[^{14}C]NEM Labeling of OGC Mutants. Proteoliposomes were incubated with 0.25 mM [^{14}C]NEM for 10 min at 25 °C, and the reaction was stopped by adding 20 mM DTE. Then the proteoliposomes were passed through Sephadex G-75 columns preequilibrated with 50 mM NaCl, 10 mM Pipes, pH 7.0, and 20 mM DTE. The turbid eluates from Sephadex were pooled and precipitated with a 20-fold excess of cold acetone for 2 h at −20 °C. The sample was centrifuged at 44000*g* for 10 min at 0 °C. The lipids were removed as described previously (*12*). After delipidation, the pellet was solubilized in the sample buffer for SDS−PAGE. For fluorographic detection of radioactivity, the gels stained with Coomassie Blue were treated with AMPLIFY for 30 min and dried on filter paper in vacuo (70 °C, 90 min), and exposed (Kodak X-OMAT films) 4−6 weeks at −80 °C.

Other Methods. SDS−PAGE was performed according to Laemmli (*13*) as described previously (*12*). The amount of recombinant protein was estimated from Coomassie Blue-stained SDS−PAGE gels with an LKB 2202 Ultroscan laser densitometer, with carbonic anhydrase as standard. Western blotting was carried out as described (*5*) with a rabbit antiserum raised against the OGC isolated from bovine heart mitochondria. The extent of incorporation of the recombinant protein into liposomes was determined as described in Phelps

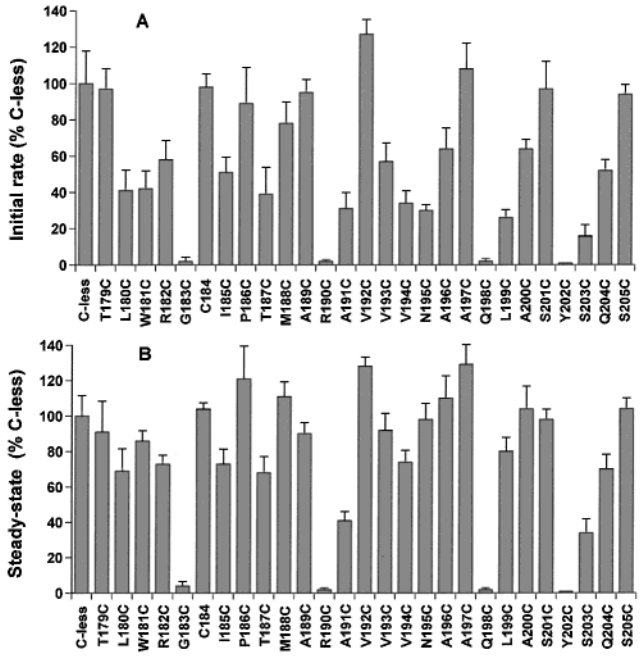

FIGURE 2: Oxoglutarate transport by individual Cys-replacement mutants or C-less OGC. The single-letter amino acid code along the horizontal axis denotes the original residues replaced with Cys in increasing order from Thr179 to Ser205. Proteoliposomes were preloaded internally with 20 mM oxoglutarate, and transport was started by the addition of 3 mM [^{14}C]oxoglutarate. (A) Rates of oxoglutarate/oxoglutarate exchange measured at 30 s. The rate for C-less OGC averaged 2552 ± 458 nmol min^{-1} (mg of protein)$^{-1}$ in 118 experiments. Results are expressed as a percentage of this value. (B) Steady-state levels of oxoglutarate/oxoglutarate exchange measured at 120 min. Results are expressed as a percentage of the C-less value, which was 12762 ± 1489 nmol (mg of protein)$^{-1}$ in 95 experiments. The data represent means ± SD of at least three independent experiments in duplicate.

et al. (*14*). Protein was determined by the modified Lowry method after precipitation with trichloroacetic acid and extraction of detergent and lipid by organic solvents (*15*).

RESULTS

Reconstitution of OGC Mutants into Liposomes. To measure the ability of the OGC mutants to catalyze oxoglutarate transport, the recombinant proteins were reconstituted into liposomes. The amount of each mutant recovered in the proteoliposomes after reconstitution (i.e., the efficiency of reconstitution) varied between 14 and 27% of the protein added to the reconstitution mixture. These values, which are similar to those found for other recombinant reconstituted mitochondrial carrier proteins (*10*, *14*, *16*−*18*), were used to calculate the transport activities reported in this work.

Transport Activity of the Reconstituted OGC Mutants. Both the initial rate and the steady-state of oxoglutarate transport are presented in Figure 2 for each OGC mutant as a percentage of the C-less values. The majority of the 27 mutants transported oxoglutarate at very significant rates (Figure 2A). A total of 13 mutants exhibited rates that were between 60% and 100% or more of C-less OGC, whereas 9 mutants transported oxoglutarate at lower but significant rates (>20% of C-less). Only G183C, R190C, Q198C, Y202C, and S203C exhibited a markedly diminished rate of oxoglutarate exchange (<20% of C-less). Steady-state levels of oxoglutarate transport for the great majority of mutants also

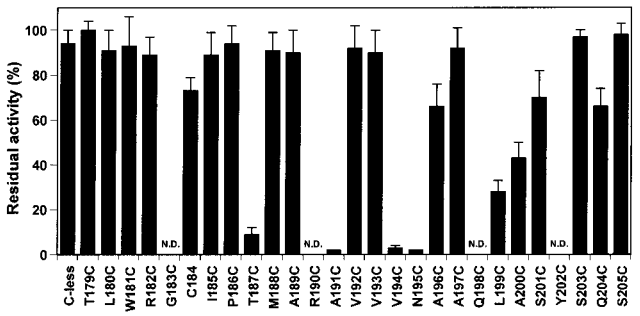

FIGURE 3: Effect of NEM on the rate of oxoglutarate transport in proteoliposomes reconstituted with C-less OGC or single Cys-replacement mutants of C-less OGC. Proteoliposomes were preincubated with and without 2 mM NEM for 10 min at 25 °C. Transport was initiated by adding 0.3 mM [^{14}C]oxoglutarate and 20 mM DTE, and terminated after 30 s. Rates are presented as a percentage of the rate measured in the absence of NEM. The data represent means ± SD of at least three independent experiments in duplicate. NEM inhibition of mutants G183C, R190C, Q198C, and Y202C which displayed negligible transport could not be assayed (N.D., not determined).

approximated those of C-less OGC (Figure 2B). A total of 21 mutants exhibited steady-state levels that were between 60% and 100% or more of C-less OGC, 2 levels about 40%, and 4 (G183C, R190C, Q198C, Y202C) negligible levels as compared to that of C-less OGC. Despite a diminished initial rate, mutants V194C, N195C, L199C, and S203C showed an appreciable oxoglutarate transport at steady-state (between 35% and 115% of C-less). Since the OGC gene encoding C-less carrier contains three mutations, it was important to test mutations in the wild-type background before concluding that a residue is essential for the function of the OGC. Therefore, the mutations G183C, R190C, Q198C, and Y202C were cloned into the wild-type OGC gene and each mutant was expressed and tested for oxoglutarate transport in proteoliposomes. The resulting mutants were still unable to catalyze oxoglutarate transport to any significant extent (data not presented). In other experiments (not shown), Gly183 was further mutated to Ala, Thr, or Val, Arg190 to Lys, His, Gln, or Leu, Gln198 to Ala, Asn, Phe, Lys, or Glu, and Tyr202 to Ala, Val, Thr, or Phe, and the mutants were examined for transport activity. For all these mutants, except for G183A and R190K, the reconstituted transport of oxoglutarate measured both at initial rate and at the steady-state was negligible with respect to the C-less OGC (<5%). G183A exhibited a lower but significant initial rate and steady-state level of oxoglutarate transport as compared to those of C-less OGC (32% and 63%, respectively). R190K displayed a very low transport rate (5% of C-less) and a markedly reduced, but significant steady-state level of oxoglutarate transport (20% of the C-less OGC).

Effect of Sulfhydryl Reagents on the Activity of OGC Mutants. The effect of NEM on the initial rate of oxoglutarate transport is shown in Figure 3 for all the mutants from T179C to S205C, with the exception of G183C, R190C, Q198C, and Y202C which had negligible activity. Although the activity of the majority of the Cys-replacement mutants was not affected significantly by NEM, four mutants (T187C, A191C, V194C, and N195C) were inhibited by the alkylating reagent by more than 90% and two mutants (L199C and A200C) by 72% and 57%, respectively. The four Cys-replacement mutants, that were nearly completely inactivated

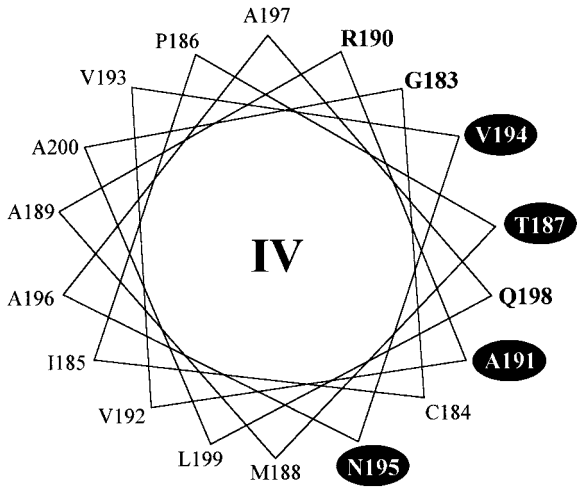

FIGURE 4: Helical wheel plot of putative transmembrane helix IV (from Gly183 to Ala200). Transmembrane helix IV is viewed from the matrix surface of the membrane. Positions sensitive to NEM, MTSEA, MTSES, and MTSET treatment (>90% inhibition of initial rate) are highlighted against a dark background. Also shown (boldface letters) are the positions where Cys replacement results in loss of activity.

by NEM, display helical periodicity, and, moreover, they reside on the same face of helix IV as positions G183, R190, and Q198 when modeled on a helical wheel plot (Figure 4). In other experiments, we studied the dependence of OGC mutant inhibition on NEM concentration. After 1 min preincubation, half-maximal inhibition of reconstituted V194C, A191C, N195C, and T187C was obtained with approximately 0.05, 0.1, 0.7, and 1.5 mM NEM, respectively (results not shown). Under the same conditions (preincubation for 1 min), the oxoglutarate/oxoglutarate transport rate of reconstituted L199C and A200C was virtually unaffected by 2 mM NEM. To investigate whether the substrate has any protective effect on NEM inhibition of the mutants T187C, A191C, V194C, and N195C, proteoliposomes were incubated with NEM in the presence and absence of oxoglutarate. In these experiments, the NEM concentration and the incubation time were kept sufficiently low in order to prevent the inhibition from being total. Under the conditions shown in Figure 5, all the mutants tested, except N195C, exhibit substantial substrate protection, indicating that the reaction of NEM with cysteines in these positions is prevented by the presence of oxoglutarate. It is noteworthy that the protective effect of oxoglutarate is specific, since other chemically related anions, i.e., α-ketoadipate, glutamate, and glutarate, which are not substrates of the OGC, had no effect (data not shown).

In an attempt to understand why most of the Cys-replacement mutants were not inhibited by NEM, we investigated whether [^{14}C]NEM binds to these mutants. The results reported in Figure 6 demonstrate that V192C, I185C, A196C, and A189C and (not shown) M188C, P186C, and A197C were labeled by [^{14}C]NEM. Therefore, the lack of sensitivity of these mutants to NEM is not caused by the inability of NEM to react with cysteinyl residues in these positions. As a control, Figure 6 also shows that the mutants V194C, A191C, and N195C, which were inhibited by NEM, as well as the mutants R190C and G183C, that reside on the same face of helix IV, were all labeled by [^{14}C]NEM.

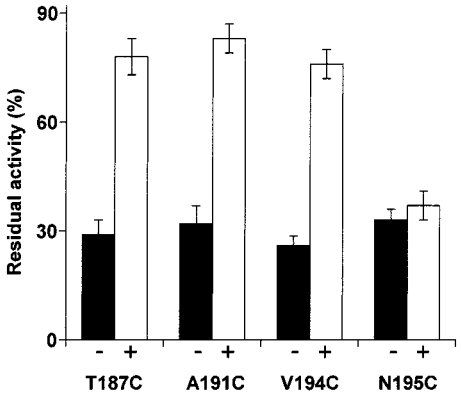

FIGURE 5: Influence of substrate on the inhibition of OGC mutants by NEM. Proteoliposomes were preincubated for 1 min (A191C, V194C, and N195C) or 3 min (T187C) with 0.1 mM (V194C), 0.2 mM (A191C), or 1.2 mM (T187C and N195C) NEM, in the presence (+) or absence (−) of 5 mM oxoglutarate. Transport was initiated by adding 3 mM [^{14}C]oxoglutarate and 20 mM DTE and terminated after 30 s. Results are expressed as percent residual activity, i.e., percent of the activity in the presence of NEM with respect to the control value without inhibitor. The data represent means ± SD of at least three independent experiments in duplicate.

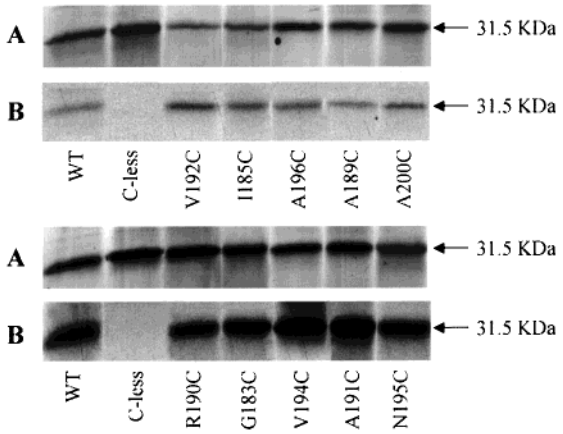

FIGURE 6: Labeling of Cys-replacement mutants of C-less OGC in proteoliposomes by [^{14}C]NEM. Proteoliposomes were incubated with 0.25 mM [^{14}C]NEM for 10 min at 25 °C. After removal of unbound NEM, the OGC mutant proteins were delipidated and separated by SDS−PAGE. (A) Coomassie Blue staining; (B) fluorography. Similar results were obtained in at least two separate experiments.

Since NEM is a known membrane-permeable thiol reagent, we extended our investigation to the hydrophilic, relatively membrane-impermeable sulfhydryl-specific reagents MTSEA, MTSES, and MTSET (*19*−*23*). All these reagents, at very low concentrations, inhibited the rate of oxoglutarate transport of reconstituted A191C, V194C, and N195C nearly completely, whereas only MTSEA, but not MTSES and MTSET, decreased the activity of T187C by 87% (Table 1). The other two mutants, which were partially inhibited by NEM and are close to the surface of the membrane, i.e., L199C and A200C, were inhibited by MTSEA by 70% and 26%, respectively, but not by MTSES and MTSET. All the other Cys-replacement mutants were not affected significantly by the MTS reagents (data not shown), in agreement with the results obtained with NEM.

DISCUSSION

In this study, Cys-scanning mutagenesis has been employed to identify functionally important residues in putative

Table 1: Effect of MTS Reagents on Cys-Replacement Mutants of C-less OGC[a]

	inhibition of oxoglutarate transport (%)		
OGC mutants	MTSEA	MTSES	MTSET
C-less	6 ± 3	9 ± 4	8 ± 4
T187C	87 ± 4	12 ± 5	10 ± 5
A191C	100 ± 2	98 ± 5	95 ± 1
V194C	98 ± 3	97 ± 7	95 ± 6
N195C	98 ± 4	92 ± 5	96 ± 5

[a] Proteoliposomes, reconstituted with C-less OGC or single Cys-replacement mutants of C-less OGC, were preincubated with or without 5 μM MTSEA, 5 μM MTSES, or 5 μM MTSET for 10 min at 25 °C. Transport was initiated by adding 0.3 mM [^{14}C]oxoglutarate and stopped after 30 s. Results are expressed as percent inhibition of the rate measured in the absence of the MTS reagents. The values are averages ± SD from four experiments.

transmembrane helix IV and in part of the flanking hydrophilic loops of the mitochondrial OGC. This approach has been extensively applied to the lactose permease of *E. coli* (reviewed in refs *24* and *25*) and subsequently extended to other transport proteins (*23*, *26*−*29*). It is in fact assumed that any residue that can be replaced by Cys in either C-less or the wild-type background without leading to complete loss of transport is unlikely to be essential for the transport mechanism (*30*). The results reported above show that the great majority of the residues between Thr179 and Ser205 tolerate replacement with Cys with relatively little or no significant effect on oxoglutarate transport activity and can therefore be excluded for playing a specific role in the mechanism of oxoglutarate transport. Among the 27 Cys-replacement mutants in the region studied here, only mutants G183C, R190C, Q198C, and Y202C appear to be completely defective. Their importance is confirmed by the findings that these amino acids also do not tolerate substitution with other residues (except for G183A) and that when the same mutations (G183C, R190C, Q198C, and Y202C) are transferred to the wild-type background, the mutants remain totally inactive. Furthermore, the virtually complete loss of activity displayed by these four mutants is not due to a defective insertion in the liposomal membrane since the transport activities were corrected for small differences in the efficiency of reconstitution.

It is interesting that the three residues essential in helix IV, namely, G183, R190, and Q198, cluster on one face where all the hydrophilic residues of this transmembrane segment are located. In this respect, it is remarkable that the four NEM-sensitive positions described here all reside on the same relatively polar face of helix IV. It is also interesting that replacement of Ala191, located on the same face, with the bulkier Cys residue partially compromises activity (Figure 2) and NEM treatment causes additional loss of activity (Figure 3), indicating further that one face of helix IV is important. The possibility that Cys-replacements at other positions are inaccessible to the alkylating reagent and/or do not react with NEM has been ruled out by the finding that single-Cys residues at positions that are not inhibited by NEM are in fact alkylated with [^{14}C]NEM, demonstrating that modification at these positions fails to affect activity. The bulky maleimide at transport-sensitive positions might hinder conformational flexibility in this region of the protein, or alternatively it may protrude into the translocation path and hinder transport for the substrate. The virtually complete inhibition of reconstituted A191C, V194C, and N195C by low concentrations of the hydrophilic, membrane-impermeable MTSEA, MTSES, and MTSET supports the conclusion that the face of helix IV containing the strongly NEM-inhibited positions forms part of a hydrophilic interhelical crevice or channel, which is accessible to the aqueous medium, and that this may be the substrate translocation pathway. This conclusion is strengthened by the protective effect of the substrate on the NEM-inhibition of reconstituted A191C, V194C, and T187C. The opposite face of helix IV, which is hydrophobic and contains positions that are insensitive to either Cys-replacement alone or Cys-replacement and NEM-treatment, may be in contact with either the hydrophobic face of another helix or the lipid phase of the membrane.

Some comments are in order regarding the functionally important residues identified in helix IV and its flanking loops. Replacement of G183 with the bulkier residues of Cys, Thr, or Val severely compromises the activity, whereas its substitution with Ala is relatively well tolerated. Since G183 does not carry functional groups likely to be involved in oxoglutarate translocation or binding, the defects in G183C, G183T, and G183V may result from structural perturbation caused by introducing bulkier groups in this position. It is also possible that, although Gly183 is not obligatory for activity, the presence of the bulkier residues of Cys, Thr, or Val at position 183 interferes with the conformational changes that occur during turnover of the carrier. In agreement with the latter explanation, it was previously shown that the substrate-induced conformational change of OGC leads to increased sulfhydryl reagents binding to Cys184 (*12*), i.e., to a region very close to Gly183. In addition, G183 of the bovine OGC is very highly conserved at the beginning of putative helix IV in the members of the mitochondrial carrier family (*3*, *31*). Therefore, though G183 is not essential for oxoglutarate transport, it is a key location in the structure, and possibly in the overall turnover cycle, of the mitochondrial carriers. R190 is essential for oxoglutarate transport since it does not tolerate substitution with other residues, with the partial exception of Lys. The most likely explanation is that R190 plays an important role in substrate binding either by direct interaction with the highly charged substrate or by stabilizing local conformation in the vicinity of the substrate-binding site. It is also possible that R190 and other positively charged residues of OGC, including the intramembrane R90, R98, and R288, act within a charge network rather than in single ion bonds. This hypothesis was previously put forward by Klingenberg et al. to explain the importance of the intrahelical arginines of the adenine nucleotide carrier and the uncoupling protein (*32*, *33*). The other Cys-replacement mutant in helix IV with completely impaired activity, i.e., Q198, might participate in hydrogen bonding within the carrier molecule and with the carbonyl group of the substrate. Since Asn-replacement for Gln completely inactivates the carrier, both the presence of a H^+ donor group and the length of the side chain of Q198 are crucial for the role played by this residue. In the cytoplasmic loop IV−V, the mutant Y202C is virtually inactive, and the substitutions of Phe, Thr, Ala, or Val for Tyr 202 are not tolerated. Therefore, the Tyr hydroxyl at position 202 is essential, probably being required for

H-bonding, and cannot be replaced in its function by the thiol group of Cys and the hydroxyl group of Thr.

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