Transmembrane Topography of the Mitochondrial Oxoglutarate Carrier Assessed by Peptide-Specific Antibodies and Enzymatic Cleavage†

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Received July 19, 1993; Revised Manuscript Received November 18, 1993⊛

ABSTRACT: The folding of the peptide chain of the bovine heart oxoglutarate carrier in the inner mitochondrial membrane and in the membrane of reconstituted proteoliposomes has been investigated by enzymatic and immunochemical approaches using proteinase K and polyclonal site-directed antibodies, respectively. Two peptides corresponding to the amino acid sequences 2–12 (N-terminal peptide) and 303–314 (C-terminal peptide) have been synthesized and coupled to ovalbumin before being used to immunize rabbits. The specificity of the generated antibodies was tested by enzyme-linked immunosorbent assay (ELISA) and by Western blot analysis. Both anti-N-terminal and anti-C-terminal antibodies reacted specifically with the corresponding peptides and with the isolated oxoglutarate carrier, whereas only anti-C-terminal antibodies immunodetected the carrier in mitochondrial lysates and reacted with the membrane-bound carrier in mitoplasts and in freeze-thawed mitochondria. This result indicated that the last 12 C-terminal amino acid residues of the oxoglutarate carrier protein are accessible from the cytosolic side of the inner mitochondrial membrane. Anti-C-terminal antibodies did not recognize the oxoglutarate carrier in reconstituted proteoliposomes unless the membrane was inverted, indicating that the carrier was inserted unidirectionally in proteoliposomes, with an orientation opposite that found in mitochondria. The immunological data were complemented by data from a limited proteolysis study performed on the membrane-bound oxoglutarate carrier in proteoliposomes, using proteinase K. Cleavage of the carrier caused a time-dependent inhibition of the oxoglutarate–oxoglutarate exchange activity of the reconstituted system. Four cleavage sites were identified, between Val-39 and Gln-40, between Tyr-61 and Lys-62, between Phe-169 and Arg-170, and between Arg-182 and Gly-183. These sites were assigned to the external side of the liposomal membrane and therefore to the matrix side of the inner mitochondrial membrane. The presence of three additional cleavage sites, located between Ala-5 and Ser-6, between Ser-22 and Val-23, and between Thr-103 and Val-104, was demonstrated in proteolysis experiments with inside-out proteoliposomes. It was concluded that the latter three sites are exposed to the internal side of the liposomal membrane and oriented toward the cytosol in intact mitochondria. These results are consistent with an arrangement of the peptide chain of the oxoglutarate carrier monomer into an even number of transmembrane segments, with the N- and C-terminal regions protruding into the cytosol.

The oxoglutarate carrier is a transport protein of the inner membrane of mitochondria which plays an important role in several metabolic processes, including the malate–aspartate shuttle, the oxoglutarate–isocitrate shuttle, gluconeogenesis from lactate, and nitrogen metabolism (Meijer & van Dam, 1981; Krämer & Palmieri, 1992). The oxoglutarate carrier is now a well-characterized protein. It has been purified and reconstituted into liposomes in an active form (Bisaccia et al., 1985; Indiveri et al., 1987). In the reconstituted system it has been shown that the oxoglutarate carrier catalyzes the transport of oxoglutarate in an electroneutral exchange for malate or other dicarboxylates and that the exchange reaction proceeds via a sequential mechanism (Indiveri et al., 1991). The amino acid sequence of the oxoglutarate carrier has been deduced from bovine heart cDNA sequences (Runswick et al., 1990), and there is only one gene for this protein in cow and man (Iacobazzi et al., 1992). Recently the oxoglutarate carrier protein has been expressed in abundant amounts in *Escherichia coli* and refolded in reconstitutively active form (Fiermonte et al., 1993). The oxoglutarate transport system belongs to a family of proteins of the mitochondrial inner membrane, such as the ADP/ATP carrier, the phosphate carrier, the uncoupling protein from brown adipose tissue, and proteins of unknown functions such as MRS3, MRS4 (Wiesenberger et al., 1991), and HML7 (Zarilli et al., 1989), which are thought to result from triplication and divergence of an ancestral sequence (Aquila et al., 1985; Runswick et al., 1987). The polypeptide chains of all these proteins consist of three tandemly repeated related sequences of approximately 100 amino acids. On the basis of the hydrophobic profile of the oxoglutarate carrier (Runswick et al., 1990) it has been proposed that each of the three repeated elements of this protein is folded into two transmembrane α-helices linked by an extensive polar region, forming a structure with six transmembrane α-helices. Apart from this proposal, the precise transmembrane organization of the oxoglutarate carrier, as well as that of any other mitochondrial carrier, remains to be established.

† This work was supported by the CNR Target Projects "Ingegneria Genetica" and "Biotechnology and Bioinstrumentation", the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), and the Centre de la Recherche Scientifique.

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⊛ Abstract published in *Advance ACS Abstracts,* March 1, 1994.

0006-2960/94/0433-3705$04.50/0

EXPERIMENTAL PROCEDURES

Materials. *N-t*-Boc[1] amino acids and Pam (phenylacetamidomethyl) resin were obtained from Neosystem Laboratories (Strasbourg, France). The sources of other chemicals were as follows: hydroxyapatite (Bio-Gel HTP), Bio-Rad; egg yolk phospholipids (lecithin from eggs), Fluka; cardiolipin, Avanti Polar Lipids; Triton X-114, Serva; ovalbumin and 3,3′,5,5′-tetramethylbenzidine, Miles Scientific; horseradish peroxidase-conjugated anti-rabbit Ig and 2-oxo-[5-^{14}C]glutarate from the radiochemical center, Amersham; proteinase K and phenylmethanesulfonyl fluoride, Boehringer; 3-maleimidobenzoic acid *N*-hydroxysuccinimidyl ester, Pierce; poly(vinylidene difluoride) membrane, Applied Biosystems. The antiserum directed against the F_1 part of the bovine heart F_1ATPase was a gift from Prof. F. Zanotti.

Biological Preparations. Bovine heart mitochondria were prepared according to the method of Smith (1967). Intact mitoplasts, corresponding to mitochondria from which the outer membrane and the intermembrane space components were removed, were prepared as described by Burnette & Batra (1985). Freshly prepared bovine heart mitochondria, suspended in a hypotonic medium consisting of 60 mM sucrose and 0.1 M EDTA, pH 7.2, were incubated with digitonin (1 mg/mg of protein) for 15 min at 25 °C. The integrity of the resulting mitoplasts was assessed by measuring the activity of the mitochondrial malate dehydrogenase, an enzyme specific for the matrix space, and the activity of the F_1ATPase, an enzyme of known orientation in the mitochondrial inner membrane. The malate dehydrogenase activity was measured as described by Hohorst (1962), and the ATPase activity was measured as described by Wehrle et al. (1978). In order to assess the integrity of the mitoplasts, we also investigated the orientation of F_1ATPase in our mitoplast preparation by ELISA performed with anti-F_1ATPase antibodies and intact and broken mitoplasts as described in this paper (see below). In addition to mitoplasts, freeze-thawed mitochondria, i.e., mitochondria stored in liquid nitrogen and thawed just before use, were employed in some experiments since it was shown that these particles have a damaged outer membrane but an intact inner membrane (Brandolin et al., 1989). To make the internal side of the membrane of mitoplasts accessible to antibodies, mitoplasts were suspended in a medium consisting of 12 mM KCl, 10 mM Tris, and 1 mM EDTA, final pH 7.2, and subjected to three cycles of freezing and thawing. Proteoliposomes were prepared from a mixture containing the purified oxoglutarate carrier, phospholipids, and Triton X-114, after removal of this detergent by hydrophobic chromatography (Indiveri et al., 1991). The composition of the initial mixture used for reconstitution was 300 μL of purified oxoglutarate carrier (0.2–0.4 μg of protein) prepared as described by Bisaccia et al. (1985) and Indiveri et al. (1987), 40 μL of 10% Triton X-114, 9 mg of phospholipids in the form of sonicated liposomes (Bisaccia et al., 1985), 20 mM oxoglutarate, and 10 mM PIPES (pH 7) in a final volume of 0.7 mL. After being vortexed, this mixture was passed 15 times through the same Amberlite XAD-2 column (0.5 × 4.5 cm) preequilibrated in a buffer containing 10 mM PIPES and 20 mM oxoglutarate (pH 7). All the operations were performed at 4 °C, except the passage through Amberlite, which was carried out at room temperature. The substrate outside the proteoliposomes and the carrier protein that had not been incorporated into the proteoliposomes were removed by passing 600 μL of the liposomal suspension through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated in a medium consisting of 50 mM NaCl/10 mM PIPES (pH 7.0). The first 750 μL of the slightly turbid eluate from the Sephadex column, containing the proteoliposomes, was collected. In order to scramble the orientation of the proteoliposomal membrane, the proteoliposomes were frozen at −80 °C and thawed three times and then pulse-sonicated for 20 s. These proteoliposomes were shown to be partially "inside out" by the fact that they exhibited two external K_m values for oxoglutarate/oxoglutarate exchange corresponding to opposite orientations of the reconstituted oxoglutarate carrier in the liposomal membrane (Indiveri et al.,1991).

Proteolytic Digestion of the Oxoglutarate Carrier in Proteoliposomes. Cleavage of the oxoglutarate carrier by proteinase K on sites accessible on the external surface of the liposomal membrane was performed as follows: 200 mL of proteoliposomes, eluted from 270 Sephadex G-75 columns, was incubated with 30 μg of proteinase K in the presence of 5 mM $CaCl_2$ at 25 °C for 60 h. The generated proteolytic fragments were analyzed by SDS–PAGE and microsequencing as described in the following. In order to allow proteolysis of the carrier on regions exposed to the internal surface of the proteoliposomal membrane, a similar treatment was applied to "frozen/thawed/sonicated" proteoliposomes. However, under these conditions, the polypeptide pattern was not very different from that obtained with normal proteoliposomes, probably due to the relatively small amount of inside-out proteoliposomes present in the preparation. Proteolysis of the oxoglutarate carrier by proteinase K on regions exposed to the internal surface of proteoliposomes could be improved by internalization of the protease: 200 mL of proteoliposomes was subjected to the freeze/thaw/sonication treatment (see above) in the presence of 30 μg of proteinase K and 5 mM $CaCl_2$ and then incubated for 60 h at 25 °C. After proteinase K treatment, both normal and frozen/thawed/sonicated proteoliposomal preparations (200 mL of proteoliposomes, divided into aliquots of 600 μL) were subjected to Sephadex G-75 chromatography on 0.7 × 15-cm columns preequilibrated with 50 mM NaCl/10 mM PIPES (pH 7.0) to remove most of the protease and $CaCl_2$. Immediately after the Sephadex G-75 chromatography, the proteolytic reaction was blocked by the addition of a 10-fold excess of PMSF, relative to proteinase K, to the eluates. The proteoliposomes were precipitated with cold acetone (20 mL of acetone/1 mL of proteoliposomes) for 4 h at −20 °C. The sample was centrifuged at 44000*g* for 10 min at 0 °C. The lipids were removed essentially as described by Wessel and Flügge (1984). The pellet was suspended in 10 mL of cold distilled water containing 500 μg of PMSF. After the addition of 40 mL of methanol, the sample was vortexed and centrifuged (2 min at 6000*g*) to ensure total collection of the sample at the base of the tube. Twenty milliliters of chloroform and 30 mL of water were added and vortexed vigorously. The phases were separated by centrifugation at 9000*g* for 5 min. The upper phase was removed and discarded. A further 30 mL of methanol was added to the remaining lower and intermediate phases, which contained the precipitated protein. The mixture was vortexed, and the protein

[1] Abbreviations: BDB, bisdiazotized benzidine; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimidyl ester; *N-t*-Boc, *N-tert*-butoxycarbonyl; PBS, phosphate-buffered saline, consisting of 0.14 M NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4; PBS-T, PBS supplemented with 0.05% (w/v) Tween 20; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine.

was pelleted by centrifugation at 9000*g* for 4 min. The supernatant was removed, and the protein pellet was carefully dried in a stream of nitrogen. This procedure was repeated two times to ensure the complete removal of the lipids. The pellet was solubilized in 100 μL of sample buffer made up of 7% SDS (w/v), 45% glycerol (v/v), 50 mM DTE, 225 mM Tris-HCl (pH 6.8), and traces of bromophenol blue, in the presence of 500 μg of PMSF. The mixture was heated for 2 min at 100 °C. The sample was loaded on a slab 16.5% acrylamide gel in the presence of 6 M urea for characterization of the peptide fragments (Schägger & von Jagow, 1987).

Synthesis of Peptides. Peptides corresponding to the N-terminal region of the bovine heart oxoglutarate carrier (residues 2–12: Ala-Ala-Thr-Ala-Ser-Pro-Gly-Ala-Ser-Gly-Met) and to the C-terminal sequence (residues 303–314: Met-Asn-Lys-Ala-Tyr-Lys-Arg-Leu-Phe-Leu-Ser-Gly) were synthesized by the Merrifield solid-phase method (Barany & Merrifield, 1980) using *t*-Boc as the temporary protecting group. A tyrosine residue was substituted for Met-12 in the N-terminal peptide and a cysteine residue was substituted for Met-303 in the C-terminal peptide to facilitate coupling to the ovalbumin carrier. The completeness of all coupling reactions was monitored by the ninhydrin test (Kaiser et al., 1970). The Boc protecting groups were removed by treatment with trifluoroacetic acid as described by Stewart & Young (1984). The peptides were released from the resin with simultaneous removal of side-chain protecting groups by treatment with anhydrous hydrogen fluoride at 0 °C in the presence of 1% 2-mercaptopyridine, 10% anisole, 10% thioanisole, and 10% *p*-cresol as scavengers (Stewart & Young, 1984). The released peptide was chromatographed over Bio-Gel P2 (–400 mesh) in 10% acetic acid and ultimately purified by preparative HPLC using a C_{18} reversed-phase column (μBondapak C_{18}, 240 × 12 mm, Waters) eluted with a linear gradient (40–100%) of acetonitrile supplemented with 0.1% (v/v) trifluoroacetic acid. The amino acid compositions of the purified peptides were controlled on a Waters (Pico-tag) apparatus after hydrolysis in 6 M HCl for 24 h at 110 °C. Fast atom bombardment mass spectrometry yielded signals corresponding to $(M + H)^+$ values of 891 and 1398 for the N- and C-terminal peptides, respectively.

Coupling of Peptides to Ovalbumin and Generation of Rabbit Anti-Peptide Antibodies. The N-terminal peptide (residues 2–12) was coupled with bisdiazotized benzidine (BDB) to ovalbumin through the hydroxyl group of Tyr-12. The coupling reaction was performed as described by Tamura & Bauer (1982). To allow specific coupling at the tyrosine residues, the synthetic peptide was first citraconylated at the primary amino groups. The ovalbumin–peptide conjugate was first dialyzed for 2 h against 5% acetic acid for deprotection of the primary amino groups, and a further dialysis step against PBS was performed to remove excess BDB and free peptide. The C-terminal peptide (residues 303–314) was coupled with 3-maleimidobenzoic acid *N*-hydroxysuccinimidyl ester (MBS) to ovalbumin through the SH group of Cys-303 (replacing Met-303). To allow specific coupling of MBS at amino groups of ovalbumin, the ovalbumin was reacted with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Lerner et al., 1981). Routinely, 0.1 mmol of ovalbumin was reacted with 3 mmol of C-terminal peptide. The ovalbumin–peptide conjugate was chromatographed over AcA 202 in PBS. The conjugates corresponding to 100 μg of coupled peptide in 0.5 mL of PBS were supplemented with 0.5 mL of complete Freund's adjuvant, and male New Zealand white rabbits were immunized as previously described (Boulay et al., 1986). Sera were decomplemented by treatment at 56 °C for 30 min.

Antibody Assays by ELISA. The ability of antisera to react with the synthetic peptides, the isolated carrier, and the membrane-bound carrier was tested by ELISA, using microtitration polystyrene plates. Two hundred microliters of the synthetic peptides diluted in PBS; of the isolated oxoglutarate carrier protein precipitated in acetone and suspended in a 0.05 M sodium carbonate buffer, pH 9.6; of mitoplasts suspended in 0.12 M KCl, 10 mM Tris, and 1 mM EDTA (pH 7.3); or of proteoliposomes in 50 mM NaCl and 10 mM PIPES (pH 7) was added to each well of the plate. After an overnight incubation at 4 °C, the solution was removed, and the wells were washed with PBS. They were then filled with 200 μL of a 1% BSA solution in PBS, and the plate was allowed to stand for 1 h at room temperature for saturation of binding sites. After the plate was washed with PBS-T, 200 μL of antiserum diluted in PBS-T was added to the wells, and the plate was incubated at room temperature for 2 h. The plate was washed with PBS-T, and 200 μL of a solution of horseradish peroxidase-conjugated anti-rabbit Ig diluted 2000-fold with PBS-T was added to each well. After another 2-h incubation period at room temperature followed by washings, each well was filled with 200 μL of a solution of 3,3′,5,5′-tetramethylbenzidine (TMB) in DMSO (1%, w/v) diluted 100-fold with 0.1 M sodium acetate/citric acid, pH 6, and supplemented with 3 μL of 30% H_2O_2. The peroxidase reaction was left to develop for 1 h in the dark at room temperature; it was stopped by addition of 50 μL of 2 M H_2SO_4. The absorbance of the reaction medium was determined at 450 nm with an automatic reader. In back-titration experiments, mitoplasts suspended in 0.12 M KCl, 10 mM Tris, and 1 mM EDTA (pH 7.3) or inside-out proteoliposomes in 50 mM NaCl and 10 mM PIPES (pH 7) were incubated with the anti-C-terminal antiserum for 2 h at room temperature (mitoplasts) and 12 h at 4 °C (inside-out proteoliposomes). After centrifugation at 100000*g* for 5 min at room temperature (mitoplasts) or at 150000*g* for 1 h at 4 °C (inside-out proteoliposomes), the supernatants were collected. The unreacted antibodies present in the supernatants were assayed by ELISA against the purified oxoglutarate protein coated onto microtiter plates as described above.

Western Blot and Sequence Analysis. Besides ELISA, the Western blot technique (Towbin et al., 1979) was used to test the reactivity of the anti-peptide antisera against the oxoglutarate carrier protein and the derived fragments. Proteins from mitochondrial lysates (500 μg) or resulting from the proteinase K cleavage of the oxoglutarate carrier in proteoliposomes (12 μg) were separated by polyacrylamide gel electrophoresis according to Schägger and von Jagow (1987). After electroblotting according to Boulay et al. (1986), the nitrocellulose sheets were treated with the antiserum indicated in the captions to Figures 2 and 6 and then incubated with horseradish peroxidase-conjugated anti-rabbit Ig. The immunoreaction proteins were detected by the peroxidase reaction performed with 20 mL of a mixture of 4-chloro-1-naphthol (0.05%, w/v), methanol (16%, v/v), and BSA (0.5%, w/v) in a medium containing 0.14 M NaCl and 0.01 M phosphate (pH 7.0) with the final addition of 12 μL of 30% H_2O_2.

Peptide Sequencing. The peptides generated from 200 mL of proteoliposomes after treatment with proteinase K were separated by the SDS–PAGE system described by Schägger and von Jagow (1987) and then transferred to a poly(vinylidene difluoride) (PVDF) membrane (Matsudaira, 1987; Fearnley et al., 1989). Single bands detected by Coomassie blue staining were excised and subjected to Edman degradation in a pulsed-liquid protein Sequencer (Applied Biosystems 477A) equipped with an on-line PTH-amino acids analyzer.

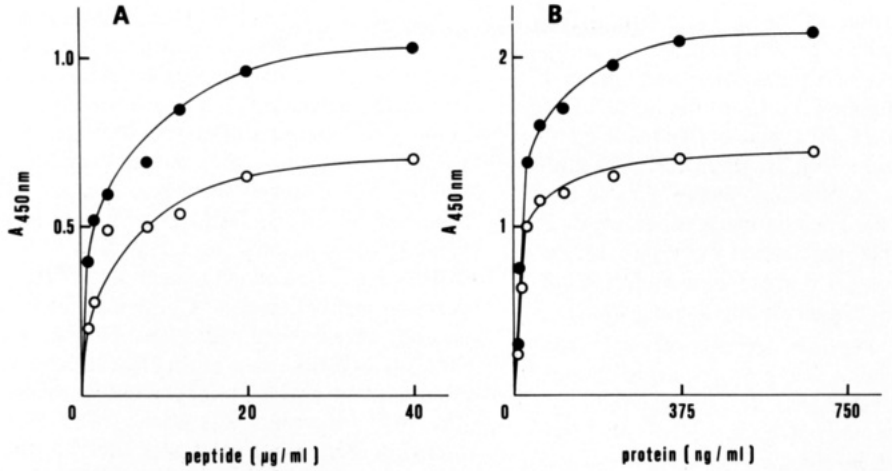

FIGURE 1: (A) Reactivity of anti-N-terminal peptide antiserum and anti-C-terminal peptide antiserum with the N-terminal and the C-terminal peptides assessed by ELISA. Microtiter plates were coated with the N-terminal peptide or the C-terminal peptide used in the indicated amounts. The immobilized peptides were incubated with the corresponding antisera, anti-N-terminal antiserum (○) or anti-C-terminal antiserum (●), used at a dilution of $3:10^3$ in PBS–Tween. The immunological reaction was detected by a chromogenic reaction as described under Experimental Procedures. (B) Reactivity of anti-N-terminal and anti-C-terminal antisera to oxoglutarate carrier protein assessed by ELISA. Microtiter plates, coated with the indicated amounts of purified oxoglutarate carrier, were incubated either with the anti-N-terminal antiserum (○) or with the anti-C-terminal antiserum (●), used at a dilution of $3:10^3$ in PBS-Tween. Immunoreactivities were revealed as described under Experimental Procedures.

Measurements of Transport Activity. The proteoliposomes were distributed in reaction vessels (150 µL) and used to measure the oxoglutarate–[^{14}C]oxoglutarate exchange activity at 25 °C by the inhibitor stop method (Palmieri & Klingenberg, 1979). Transport was started by the addition of [^{14}C]-oxoglutarate (10 µL, 0.1 mM) and stopped after 5 min by addition of 10 µL of a solution containing 480 mM pyridoxal 5′-phosphate and 160 mM bathophenanthroline (Indiveri et al., 1991). In control assays, the inhibitors were added together with the labeled substrate. External [^{14}C]oxoglutarate was removed from each sample of proteoliposomes (150 µL) by chromatography on an anion-exchange column (Dowex AG1-X8, acetate form; 0.5 × 5 cm). The proteoliposomes were eluted with 1 mL of 50 mM NaCl, and their radioactivity was determined by scintillation counting. The transport activity was determined after correction of the measurements by subtraction of the control values.

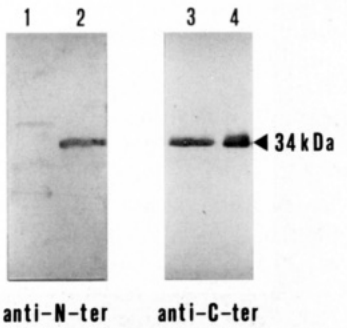

FIGURE 2: Binding of antibodies to the oxoglutarate carrier explored by Western blot analysis. Aliquots of 500 µg of lysed mitochondria (lanes 1 and 3) and 12 µg of purified oxoglutarate carrier (lanes 2 and 4) were electrophoresed on a 16.5% polyacrylamide gel in the presence of SDS. After being electroblotted, the nitrocellulose sheets were treated with the anti-N-terminal antiserum (1:500 dilution) (lanes 1 and 2) and with the anti-C-terminal antiserum (1:500 dilution) (lanes 3 and 4). Immunodetections were performed as described under Experimental Procedures.

RESULTS

Characterization of Peptide-Specific Antibodies. The reactivity of anti-peptide antibodies directed to the N- and C-terminal regions of the bovine heart oxoglutarate carrier was investigated by ELISA with either the peptides or the isolated carrier protein coated onto the wells of microtiter plates. Figure 1A shows that both the anti-N-terminal antiserum and the anti-C-terminal antiserum reacted with the corresponding terminal peptides. No cross reaction was detectable (data not shown). The reactivity of the anti-C-terminal antibody, however, was higher than that of the anti-N-terminal antibody. The same results were obtained when the anti-N- and the anti-C-terminal antisera were assayed against the isolated protein (Figure 1B). These observations suggested a higher immunogenicity of the C-terminal peptide–ovalbumin conjugate compared to that of the N-terminal peptide–ovalbumin conjugate. Before the anti-C- and anti-N-terminal antibodies were reacted with the membrane-bound oxoglutarate carrier in mitoplasts, it was important to check their reactivity toward the isolated carrier and toward the proteins of SDS mitochondrial lysates by Western blot analysis. As shown in Figure 2, both the anti-N- and the anti-C-terminal antisera immunodecorated the purified oxoglutarate carrier protein (lanes 2 and 4), although the anti-N-terminal antiserum reacted much less strongly than the anti-C-terminal antiserum. In the mitochondrial lysates, on the other hand, only the anti-C-terminal antiserum recognized a band of M_r 34 kDa corresponding to the oxoglutarate carrier protein (lane 3), whereas the anti-N-terminal antiserum gave no detectable reaction (lane 1), thus confirming its lower efficiency.

Reactivity of the Anti-C-terminal and Anti-N-terminal Antisera with the Membrane-Bound Oxoglutarate Carrier in Mitoplasts. Investigation of the orientation of the C- and N-terminal regions in the membrane-bound bovine heart oxoglutarate carrier was performed by ELISA with mitoplasts coated onto microtiter plates. The data presented in Figure 3A show that the binding of the anti-C-terminal antibodies to the oxoglutarate carrier increased with the amount of coated mitoplasts. This result indicates that the C-terminal region of the membrane-bound oxoglutarate carrier is accessible to specific antibodies from the cytosolic side of the inner mitochondrial membrane. In order to check whether the C-terminal region of the oxoglutarate carrier is exposed, besides to the cytosolic side, to the matrix side of the mitochondrial membrane, i.e., whether the oxoglutarate carrier is randomly inserted in the mitochondrial membrane, immunotitrations were performed with anti-C-terminal anti-

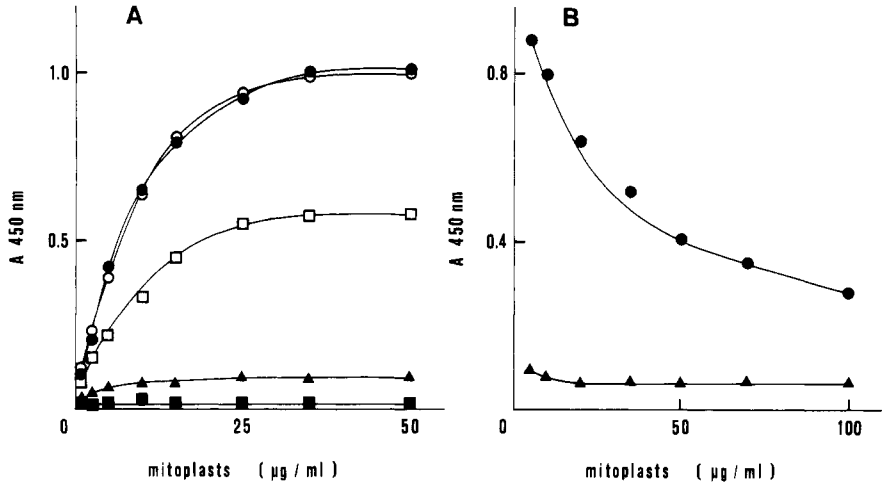

FIGURE 3: (A) Reactivity of the anti-C-terminal peptide antiserum to the membrane-bound oxoglutarate carrier in intact and permeabilized mitoplasts, assessed by ELISA. Microtiter plates were coated with the indicated amounts of intact (●, ▲, ■) or permeabilized (○, □) mitoplasts. The immobilized particles were incubated with the following antisera: anti-C-terminal peptide antiserum (●, ▲, ○) used at a dilution of $3:10^3$ or anti-F_1ATPase antiserum (■, □) used at a dilution of $1:10^3$. In (▲) the antiserum was used in the presence of the competing C-terminal peptide at 20 µg/mL. Binding of antibodies was detected by a chromogenic reaction as described under Experimental Procedures. (B) Back-titration by ELISA of the anti-C-terminal antibodies after reaction with the membrane-bound oxoglutarate carrier in intact mitoplasts. The anti-C-terminal antiserum ($3:10^3$ dilution), in the presence (▲) or in the absence (●) of the competing C-terminal peptide at 20 µg/mL, was incubated with intact mitoplasts used at increasing concentrations. The mitoplasts were sedimented by centrifugation, and unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the purified oxoglutarate carrier at 20 µg/mL. Binding of antibodies was detected by a chromogenic reaction as described under Experimental Procedures.

bodies allowed to react with mitoplasts permeabilized by osmotic shock and freeze-thawing, i.e., in which the matrix side of the inner membrane is accessible to antibodies. The data presented in Figure 3A show that the reactivity of the anti-C-terminal antibodies with the oxoglutarate carrier in intact mitoplasts was not enhanced by permeabilization of the mitoplasts, indicating that the C-terminal region of the oxoglutarate carrier is located exclusively at the external side of the inner mitochondrial membrane. The specificity of the immunoreaction was demonstrated by carrying out a similar assay in the presence of the C-terminal peptide, which drastically reduced the reaction (Figure 3A). The anti-N-terminal antiserum, used at 1:100 dilution, gave no detectable reaction with either intact or disrupted mitoplasts used at concentrations to 100 µg protein/mL. These observations suggest that the anti-N-terminal antibodies do not react with the corresponding epitope on the membrane-bound oxoglutarate carrier protein because of improper conformation or, alternatively, that the N-terminal region of the oxoglutarate carrier is not accessible on either face of the mitochondrial membrane. Similar results with both anti-C-terminal and anti-N-terminal antibodies were obtained using coated freeze-thawed mitochondria instead of coated mitoplasts. As a control, Figure 3A also shows that an antiserum directed against the F_1 part of the F_1ATPase did not react with intact mitoplasts, whereas it reacted strongly with broken mitoplasts, i.e., when the matrix side of the inner membrane was accessible to the anti-F_1ATPase antibodies.

It might be argued that coating the mitoplasts on the microtiter plates could disorganize the membrane structure, allowing access of anti-C-terminal antibodies to initially unexposed epitopes. To test this possibility, a back-titration assay was carried out as described by Brandolin et al. (1989). Intact mitoplasts used at increasing concentrations were incubated with a fixed concentration of anti-C-terminal antiserum. After centrifugation the unreacted antibodies remaining in the supernatants were back-titrated by ELISA, using microtiter plates coated with the purified carrier protein. The results of back-titration ELISA are shown in Figure 3B. The reaction of the anti-C-terminal antibodies against the coated purified protein markedly decreased on increasing the amount of mitoplasts, demonstrating that uncoated mitoplasts are able to bind the anti-C-terminal antibodies. As a control of the specificy of the immunoreaction, it was shown that when the anti-C-terminal antibodies were allowed to react with the respective peptide in solution prior to being added to the mitochondrial suspension, virtually no further reaction was detected (Figure 3B).

Reactivity of the Anti-C-terminal and Anti-N-terminal Antisera with the Membrane-Bound Oxoglutarate Carrier in Proteoliposomes. Additional ELISAs were perfomed using coated normal and frozen-thawed-sonicated proteoliposomes. In the latter particles, freeze/thaw/sonication was used to invert the membrane of part of the proteoliposomal population. It was shown that the anti-C-terminal antibodies did not react with normal proteoliposomes (Figure 4A). Instead, they reacted strongly with frozen/thawed/sonicated proteoliposomes (Figure 4A). This reaction was drastically reduced by the addition of the C-terminal peptide, demonstrating the specificity of the immunological reaction (Figure 4A). Back-titration ELISA confirmed that uncoated frozen/thawed/sonicated proteoliposomes are able to bind the C-terminal peptide-specific antibodies (Figure 4B). In this case, too, the addition of the C-terminal peptide virtually abolished the immunological reaction (Figure 4B). These results indicate that the C-terminal region of the oxoglutarate carrier in the membrane of proteoliposomes protrudes toward the inside, in contrast with the situation found in mitochondria.

Localization of Proteinase K Cleavage Sites of the Membrane-Bound Oxoglutarate Carrier. Further investigation of the trasmembrane arrangement of the oxoglutarate carrier was carried out by means of proteinase K digestion of the carrier protein inserted in the membrane of normal and of frozen/thawed/sonicated proteoliposomes. Liposomes reconstituted with the purified oxoglutarate carrier were chosen for these studies, instead of mitoplasts, because the sequence of the peptides generated by proteolysis could be determined without purification. Obviously, mitoplasts are not suitable for such studies because the cleavage fragments of the oxoglutarate carrier, which is a minor component of the

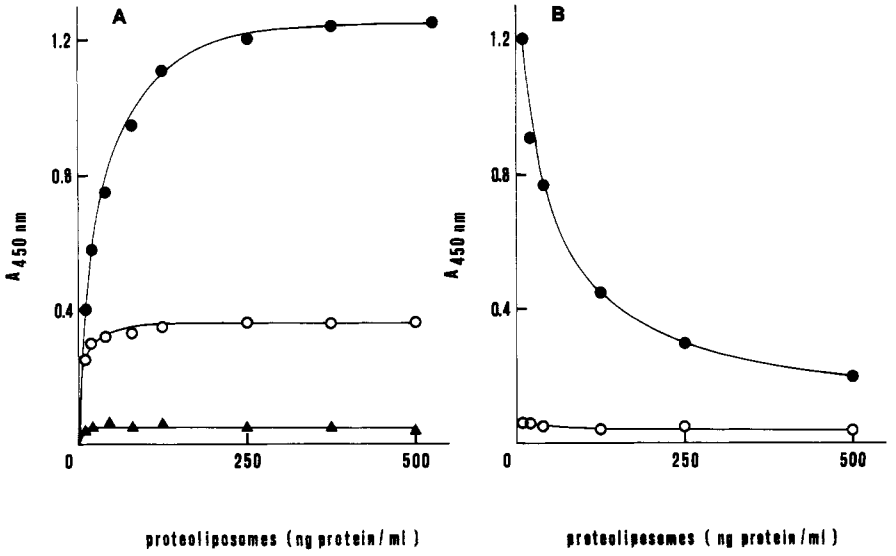

FIGURE 4: (A) Reactivity of the anti-C-terminal peptide antiserum to the membrane-bound oxoglutarate carrier in proteoliposomes assessed by ELISA. Microtiter plates were coated with increasing concentrations of normal proteoliposomes (▲) or frozen/thawed/sonicated proteoliposomes (● and ○). The immobilized particles were incubated with the anti-C-terminal peptide antiserum ($3:10^3$ dilution). In (○) the antiserum was used in the presence of the competing C-terminal peptide (25 μg/mL). Binding of antibodies was detected by a chromogenic reaction as described under Experimental Procedures. (B) Back-titration by ELISA of anti-C-terminal antibodies after reaction with the membrane-bound oxoglutarate carrier in frozen/thawed/sonicated proteoliposomes. The anti-C-terminal antiserum ($3:10^3$ dilution), in the presence (○) or in the absence (●) of the competing C-terminal peptide at 20 μg/mL, was incubated with increasing concentrations of frozen/thawed/sonicated proteoliposomes. The proteoliposomes were spun down by centrifugation, and unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the purified oxoglutarate carrier at 20 μg/mL. Binding of antibodies was detected by a chromogenic reaction as described under Experimental Procedures.

mitochondrial membrane, would be contaminated by other proteins (or by peptides of other mitochondrial proteins) and therefore could not be sequenced without being isolated.

Proteinase K digestion of the purified oxoglutarate carrier reconstituted into liposomes requires high protease/protein ratios and long times of incubation. Similar experimental conditions have been described for the enzymatic digestion of halorhodopsin incorporated into liposomes (Schobert et al., 1988). To check the stability of the oxoglutarate carrier in the liposomal membrane, the oxoglutarate–oxoglutarate exchange activity was followed during aging of proteoliposomes, in the presence and in the absence of proteinase K. Control experiments illustrated in Figure 5 show that the activity of the oxoglutarate carrier remains absolutely constant for 60 h at 25 °C. However, incubation of the proteoliposomes with proteinase K caused a time-dependent inhibition of the oxoglutarate carrier activity (to 35% of the control value after 60 h), suggesting an action of the protease on the carrier protein. *Staphylococcus aureus* V8 protease used under the same conditions did not affect significantly the oxoglutarate–oxoglutarate exchange activity.

Figure 6A shows SDS–PAGE of the oxoglutarate carrier after it has been digested with proteinase K in proteoliposomes. In the case of normal proteoliposomes (lane 1), many bands were visible corresponding to the uncleaved material and to several peptides of apparent molecular mass ranging between 33 and 8 kDa. When proteolysis of the carrier was performed in frozen/thawed/sonicated proteoliposomes, a smaller number of peptides, ranging between 32 and 15 kDa, were detected by silver nitrate staining, in addition to the large band corresponding to the uncleaved protein (lane 2). The paucity of visible peptides in lane 2 is probably due to the fact that proteinase K was present on both sides of the membrane of frozen/thawed/sonicated proteoliposomes, thus causing a broad fragmentation of the oxoglutarate carrier protein with formation of many small peptides ($M_r \leq 2000$–3000), which were not detected on the gel.

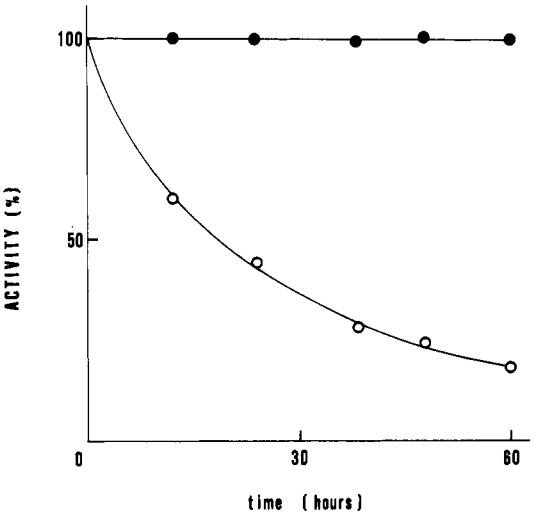

FIGURE 5: Effect of proteinase K on the activity of the oxoglutarate carrier reconstituted into liposomes. Seven milliliters of normal proteoliposomes in the presence of 5 mM $CaCl_2$ was incubated in the presence (○) or in the absence (●) of proteinase K (1.05 μg) for the indicated periods of time at 25 °C. The proteolytic reaction was blocked by addition of 10 μg of PMSF followed by filtration of the proteoliposomes through a Sephadex G-75 column. Then the oxoglutarate–[^{14}C]oxoglutarate exchange activity was measured as described under Experimental Procedures. The control value of uninhibited oxoglutarate transport, determined in the presence of 0.1 mM external substrate, was 2.7 mmol/5 min per gram of protein.

In parallel experiments, the protein bands corresponding to those shown in Figure 6A were transferred onto a nitrocellulose membrane to test their immunoreactivity with the anti-C-terminal antibodies (Figure 6B) and onto a PVDF membrane for protein sequencing. The results of immunodecoration and of sequencing of each fragment are shown in Table 1. The protein of band 1 was found to be N-terminal blocked and corresponded to the uncleaved oxoglutarate carrier (cf. lane 3). The fragment migrating as band 2, which could not be sequenced and did not react with anti-C-terminal antibodies,

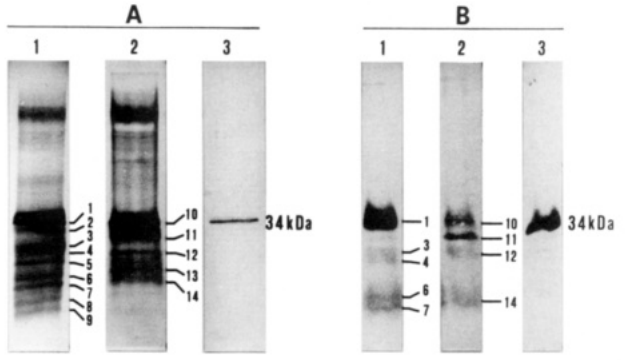

FIGURE 6: Cleavage of the membrane-bound oxoglutarate carrier in normal and frozen/thawed/sonicated proteoliposomes by proteinase K. Normal or frozen/thawed/sonicated proteoliposomes were incubated with proteinase K at 25 °C for 60 h. The fragments resulting from the cleavage of the oxoglutarate carrier were separated by SDS–PAGE according to the method described by Schägger and von Jagow (1987). Immunoreactive peptides were detected by anti-C-terminal antibodies (antiserum used at 1:500 dilution) after electroblotting on nitrocellulose as described under Experimental Procedures. (A) Silver nitrate staining of the protein bands: lane 1, cleavage fragments of the oxoglutarate carrier obtained from normal proteoliposomes (5 μg); lane 2, cleavage fragments of the carrier inserted in the membrane of frozen/thawed/sonicated proteoliposomes (5 μg); lane 3, purified oxoglutarate carrier (0.5 μg). (B) Immunodetection of proteolytic fragments of the oxoglutarate carrier with the anti-C-terminal antiserum: lane 1, cleavage fragments from normal proteoliposomes (10 μg); lane 2, cleavage fragments from frozen/thawed/sonicated proteoliposomes (10 μg); lane 3, purified oxoglutarate carrier (10 μg).

Table 1

sample (band no.)	M_r (kDa)	reactivity with anti-C-terminal antiserum	determined sequence
	Normal Proteoliposomes		
1	34	+	none
2	33	–	none
3	27	+	Q_{40} PLDLVKNRM
4	26	+	K_{62} TSFHALISI
5	20	–	none
6	15	+	R_{170} IVQEEGVPT
7	13	+	G_{183} CIPTMARAV
8	10	–	K_{62} TSFHALISI
9	8	–	none
	Frozen/Thawed/Sonicated Proteoliposomes		
10	34	±	S_6 PGASGMDGK
11	32	+	V_{23} KFLFGGLAG
12	25	±	V_{104} LFERLTGAD
13	20	–	none
14	15	±	R_{170}IVQEEGVPT

resulted most probably from cleavage of the oxoglutarate carrier by proteinase K at a site close to the C-terminal extremity of the peptide chain. Analysis of the amino acid sequences of five proteolytic peptides (Table 1) demonstrated that proteinase K cleaved the reconstituted oxoglutarate carrier in normal proteoliposomes at the level of the following sites: V_{39}–Q_{40}, Y_{61}–K_{62}, F_{169}–R_{170}, and R_{182}–G_{183}. Since normal proteoliposomes were incubated with externally added proteinase K, these sites could be assigned to the external side of the liposomal membrane. Only one out of the five peptides which could be sequenced (i.e., peptide 8, starting at position K_{62}) was not recognized by anti-C-terminal antibodies, indicating that it lacked the C-terminal end of the carrier protein. Considering its M_r of 10 kDa, it may be proposed that peptide 8 was generated by a second cleavage at the level of the F_{169}–R_{170} bond, which is a proteinase K cleavage site identified from sequencing of band 6. The fragments corresponding to bands 5 and 9 could not be sequenced or reacted with the anti-C-terminal antibodies; therefore, they probably start with the N-terminal end of the carrier protein, and on the basis of their M_r's , they can be proposed to be generated by cleavage at the level of the R_{182}–G_{183} and Y_{61}–K_{62} bonds, respectively.

The results obtained from limited proteolysis of the oxoglutarate carrier in frozen/thawed/sonicated proteoliposomes, i.e., incubated with proteinase K at both membrane sides, are very interesting (Figure 6, lane 2; Table 1). We were surprised to find that band 10, which corresponds to the N-terminal blocked uncleaved protein (cf. Figure 6, band 1 of lane 1 and the purified oxoglutarate carrier of lane 3), displayed the sequence starting as follows: S_6PGASGMDGK. This result shows that band 10 comprises, in addition to the uncleaved protein, another polypeptide of very similar M_r, corresponding to a proteinase K cleavage product of the oxoglutarate carrier lacking the first five amino acids at the N-terminal end. Cleavage of the oxoglutarate carrier at the A_5–S_6 bond was not obtained in normal proteoliposomes, demonstrating that the N-terminal region of the carrier protrudes into the intraliposomal space and is accessible to proteinase K only in frozen/thawed/sonicated proteoliposomes. Band 11 contains a very distinct peptide of 32 kDa which was not present in the proteinase K cleavage pattern of the oxoglutarate carrier in normal proteoliposomes (cf. Figure 6, lane 1). This peptide reacted with the anti-C-terminal antibodies and was shown to start with the sequence V_{23}KFLFGGLAG (Table 1). Peptide corresponding to band 12 reacted with the anti-C-terminal antibodies, although weakly, either because it was present in too little amount or possibly due to a further cleavage in its C-terminal extremity. It displayed also a characteristic sequence starting at position V_{104} (Table 1). The peculiar sequences found by sequencing the peptides generated by proteinase K digestion of the oxoglutarate carrier in frozen/thawed/sonicated proteoliposomes show that the cleavage sites A_5–S_6, S_{22}–V_{23}, and T_{103}–V_{104} are exposed to the water phase at the internal side of the liposomal membrane. The peptide migrating as band 13 displays a M_r of 20 kDa, is N-terminal blocked, and does not react with anti-C-terminal antibodies: it corresponds most probably to the proteolytic fragment of band 5. The fragment of the oxoglutarate carrier corresponding to band 14 displays the same sequence as that of band 6. The generation of some identical peptides (e.g., in bands 5 and 13 and in bands 6 and 14) during the proteinase K digestion of the oxoglutarate carrier in both frozen/thawed/sonicated proteoliposomes and normal proteoliposomes is not surprising since both membrane sides were made accessible to proteinase K in frozen/thawed/sonicated proteoliposomes.

DISCUSSION

Due to severe difficulties encountered in the crystallization of membrane transport proteins, the need of understanding their structure–function relationships has led to the development of methods able to investigate the topography of the carriers within the membrane. Such approaches include the use of structure-prediction algorithms and site-directed mutagenesis, as well as specific or nonspecific chemical reagents and endo- or exoproteases. To overcome possible drawbacks inherent to uncontrolled membrane permeability of some reagents, antibodies and proteases which are nonpermeant have been widely used to determine the sidedness of emerging segments of membrane proteins (for reviews, see Ovchinnikov (1987), Jennings (1989), and Traxler et al. (1993)). These studies include the *Escherichia coli* lactose/ H^+ carrier

(Seckler et al., 1983, 1986; Carrasco et al., 1984), the human erythrocyte glucose carrier (Davies et al., 1987), the LDL receptor in the plasma membrane of fibroblasts (Schneider et al., 1983), the quinone-binding protein of the thylakoid membrane (Sayre et al., 1986), the acetylcholine receptor of the postsynaptic membrane (Young et al., 1985; Ratnam et al., 1986), and the chloride pump of halobacteria (Schobert et al., 1988). More recently, anti-peptide antibodies have been used to localize the N- and C-terminal regions of the (Ca^{2+}–Mg^{2+})-ATPase on the cytoplasmic side of sarcoplasmic reticulum membranes (Matthews et al., 1989). A similar approach has been used in combination with controlled proteolysis to investigate the topography of two mitochondrial carriers, namely, the ADP/ATP carrier and the phosphate carrier. These studies allowed the proposal of possible arrangements of the polypeptide chains of both carriers, demonstrating in particular the conformation-sensitive cytosolic exposure of the N-terminal segment of the ADP/ATP carrier (Brandolin et al., 1989; Marty et al., 1992) and the orientation toward the cytosol of both the N- and C-terminus of the phosphate carrier (Capobianco et al., 1991). In the present work, we have used antipeptide antibodies to probe the orientation of the N- and C-terminal regions of the membrane-bound oxoglutarate carrier either in mitoplasts or in proteoliposomes. In addition, the access of the peptide chain of the membrane-embedded carrier to proteinase K was studied.

Antibodies were raised in rabbits against two synthetic peptides corresponding to the N- and C-terminal regions of the oxoglutarate carrier. Both antisera were shown to recognize the corresponding peptides with no detectable cross reaction. However, when assayed for immunodetection of the oxoglutarate carrier on Western blots, they displayed different immunoreactivities. The anti-C-terminal antibodies were highly reactive toward the carrier both in the isolated state and among the proteins of a mitochondrial lysate, whereas the anti-N-terminal antibodies reacted weakly with the isolated carrier, giving no detectable response with mitochondrial lysate. Lack of reactivity of the anti-N-terminal antibodies toward the mitochondrial lysate may be explained by the very low amount of oxoglutarate carrier present in the lysate and by the relatively low efficiency of the anti-N-terminal antibodies, as shown by the fact that the anti-C-terminal antibodies give a higher response under similar experimental conditions. In addition, in contrast to anti-C-terminal antibodies, anti-N-terminal antibodies did not react toward the membrane-bound oxoglutarate carrier and therefore could not be used to probe the orientation of its N-terminal region.

ELISA performed with mitoplasts and freeze-thawed mitochondria, either coated onto microtiter plates or in back-titration assays, using anti-C-terminal antibodies demonstrated clearly the cytosolic exposure of the C-terminal region of the oxoglutarate carrier. As disruption of mitoplasts, which allowed the reaction of anti-C-terminal antibodies with regions of the peptide chain of the carrier exposed to the mitochondrial matrix, did not enhance the immunoreaction, it was concluded that the oxoglutarate carrier is asymmetrically inserted in the membrane. This result excluded the possibility of antiparallel orientation of oxoglutarate subunits in carrier dimers (Palmieri et al., 1992).

For further insight into the transmembrane arrangement of the oxoglutarate carrier, limited proteolysis experiments were carried out with proteinase K on the carrier incorporated in the membrane of proteoliposomes. This reconstituted system has been previously characterized in terms of oxoglutarate transport activity (Indiveri et al., 1991). Two types of proteoliposomes were used: normal and inside-out proteoliposomes. In the latter particles, the membrane of part of the proteoliposomal population has been inverted by a freeze/thaw/sonication procedure, and the reactivity of the oxoglutarate carrier peptide chain has been probed on both faces of the membrane. The efficiency of both preparations in terms of oxoglutarate–oxoglutarate exchange activity was checked (data not shown; see also Indiveri et al. (1991)).

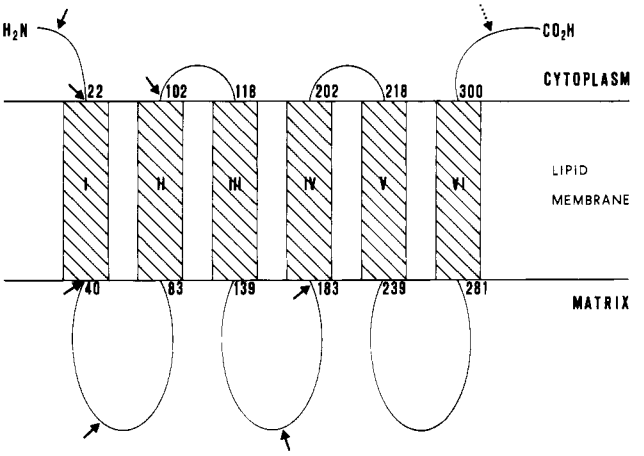

FIGURE 7: Scheme illustrating the postulated transmembrane arrangement of the oxoglutarate carrier monomer in the mitochondrial membrane. This model is based on the hydrophobic profile predicted from the sequence of the carrier protein (Runswick et al., 1990) and on immunochemical and enzymatic experimental results presented in this study. The N-terminal and C-terminal regions of the carrier are proposed to protrude into the cytosol. Proteolytic cleavage sites assigned to either the cytosolic or the matrix face of the membrane are indicated by arrows.

From preliminary experiments carried out by ELISA using anti-C-terminal antibodies it was demonstrated that the oxoglutarate carrier was inserted asymmetrically in proteoliposomes, with the C-terminus exposed to the inside, i.e., oriented inside out compared to intact mitochondria. The asymmetric inside-out orientation of the oxoglutarate carrier in proteoliposomes might result from favored interactions of the carrier with one or the other of the leaflets of the membrane of liposomes, possibly due to the asymmetrical distribution of the lipids within this membrane and/or because of intrinsic features of the carrier. Along this line of argument, neither the nature of the detergent used during the isolation process nor the lipid composition of the liposomes can be excluded as influencing the asymmetrical insertion of the isolated carrier into the membrane of liposomes. This interesting behavior of the oxoglutarate carrier deserves further investigation.

From a methodological point of view, the reconstituted system is of considerable interest for a proteolytic fragmentation study of the oxoglutarate carrier since it allows the identification of the sites of cleavage without requiring the purification of the generated fragments. Obviously, mitoplasts are not suitable for such an investigation, due to their low content in oxoglutarate carrier protein. Cleavage of the oxoglutarate carrier inserted in normal liposomes by proteinase K occurred at the V_{39}–Q_{40}, Y_{61}–K_{62}, F_{169}–R_{170}, and R_{182}–G_{183} bonds, which indicated their access to the protease on the external surface of the liposomal membrane. Under the assumption that the oxoglutarate carrier is oriented inside out compared to mitochondria, these bonds can be assigned to the matrix side of the inner mitochondrial membrane. Further proteolysis experiments were carried out on the oxoglutarate carrier inserted in proteoliposomes whose membrane had been

inverted, resulting in the additional cleavage of the carrier at bonds A_5–S_6, S_{22}–V_{23}, and T_{103}–V_{104}. Therefore, it was concluded that these bonds are accessible to the protease on the internal side of the liposomal membrane and consequently are exposed on the cytosolic site of the inner mitochondrial membrane.

A possible arrangement of the peptide chain of the oxoglutarate carrier monomer in the mitochondrial membrane is shown in Figure 7, based on the hydropathy profile previously reported by Runswick et al. (1990) and on the data obtained by immunochemical and enzymatic approaches presented in this work. This model, in which both the N-terminal and the C-terminal region of the carrier are proposed to protrude into the cytosol, includes the presence of six transmembrane α-helices connected by hydrophilic loops. It would be consistent with the threefold symmetry proposed for the members of the mitochondrial family (Walker, 1992).

The results reported in this paper provide preliminary information on the transmembrane topography of the oxoglutarate carrier. Further investigations are obviously required to refine the proposed folding model.

ACKNOWLEDGMENT

We are indebted to Dr. J. Willison for critical reading of the manuscript.

REFERENCES

Aquila, H., Link, T. A., & Klingenberg, M. (1985) *EMBO J. 4*, 2369–2376.

Barany, G., & Merrifield, R. B. (1980) in *The Peptides* (Gross, E., & Meienhoffer, J., Eds.) Vol. 2, pp 1–284, Academic, New York.

Bisaccia, F., Indiveri, C., & Palmieri, F. (1985) *Biochim. Biophys. Acta 810*, 362–369.

Boulay, F., Lauquin, G. J. M., & Vignais, P. V. (1986) *Biochemistry 25*, 7567–7571.

Brandolin, G., Boulay, F., Dalbon, P., & Vignais, P. V. (1989) *Biochemistry 28*, 1093–1100.

Burnette, B., & Batra, P. P. (1985) *Anal. Biochem. 145*, 80–86.

Capobianco, L., Brandolin, G., & Palmieri, F. (1991) *Biochemistry 30*, 4963–4969.

Carrasco, N., Herzlinger, D., Mitchell, R., De Chiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672–4676.

Davies, S., Meeran, K., Cairns, M. T., & Baldwin, S. A. (1987) *J. Biol. Chem. 262*, 9347–9352.

Fearnley, I. M., Runswick, M. J., & Walker, J. E. (1989) *EMBO J. 8*, 665–672.

Fiermonte, G., Walker, J. E., & Palmieri, F. (1993) *Biochem. J. 294*, 293–299.

Hohorst, H. (1962) in *Methods of Enzymatic Analysis* (Bergmeyer, H., Ed.) p 328, Verlag Chemie, Weinheim.

Iacobazzi, V., Palmieri, F., Runswick, M. J., & Walker, J. E. (1992) *DNA Sequence 3*, 79–88.

Indiveri, C., Palmieri, F., Bisaccia, F., & Krämer, R. (1987) *Biochim. Biophys. Acta 809*, 310–318.

Indiveri, C., Dierks, T., Krämer, R., & Palmieri, F. (1991) *Eur. J. Biochem. 198*, 339–347.

Jennings, M. L. (1989) *Annu. Rev. Biochem. 58*, 999–1027.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.

Krämer, R., & Palmieri, F. (1992) in *Molecular mechanisms in bioenergetics* (Ernster, L., Ed.) pp 359–384, Elsevier Science Publishers, Amsterdam.

Lerner, R. A., Green, N., Alexander, H., Lin, F. T., & Sutcliffe, J. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3403–3407.

Marty, I., Brandolin, G., Gagnon, J., Brasseur, R., & Vignais, P. V. (1992) *Biochemistry 31*, 4058–4065.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Matthews, I., Colyer, J., Mata, A. M., Green, N. M., Sharma, R. P., Lee, A. G., & East, J. M. (1989) *Biochem. Biophys. Res. Commun. 161*, 683–688.

Meijer, A. J., & van Dam, K. (1981) in *Membrane transport* (Bonting, S., & De Pont, J., Eds.) pp 235–256, Elsevier, Amsterdam.

Ovchinnikov, Y. A. (1987) *Trends Biochem. Sci. 12*, 434–438.

Palmieri, F., & Klingenberg, M. (1979) *Methods Enzymol. 56*, 222–232.

Palmieri, F., Bisaccia, F., Iacobazzi, V., Indiveri, C., & Zara, V. (1992) *Biochim. Biophys. Acta 1101*, 223–227.

Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986) *Biochemistry 25*, 2633–2643.

Runswick, M. J., Powell, S. J., Nyren, P., & Walker, J. E. (1987) *EMBO J. 6*, 1367–1373.

Runswick, M. J., Walker, J. E., Bisaccia, F., Iacobazzi, V., & Palmieri, F. (1990) *Biochemistry 29*, 11033–11040.

Sayre, R. T., Andersson, B., & Bogorad, L. (1986) *Cell (Cambridge, Mass.) 47*, 601–608.

Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Schneider, W. J., Slaughter, C. J., Goldstein, J. L., Anderson, R. G. W., Capra, J. D., & Brown, M. S. (1983) *J. Cell Biol. 97*, 1635–1640.

Schobert, B., Lanyi, J. K., & Oesterhelt, D. (1988) *EMBO J. 7*, 905–911.

Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817–10820.

Seckler, R., Moroy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403–2409.

Smith, A. L. (1967) *Methods Enzymol. 10*, 81–86.

Stewart, J. M., & Young, J. D. (1984) in *Solid phase peptide synthesis*, Pierce Chemical Co., Rockford, IL.

Tamura, T., & Bauer, H. (1982) *EMBO J. 1*, 1479–1485.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Traxler, B., Boyd, D., & Beckwith, J. (1993) *J. Membr. Biol. 132*, 1–11.

Walker, J. E. (1992) *Curr. Opin. Struct. Biol. 2*, 519–526.

Wehrle, J. P., Cintron, N. M., & Pedersen, P. L. (1978) *J. Biol. Chem. 253*, 8598–8603.

Wessel, D., & Flügge, U. I. (1984) *Anal. Biochem. 138*, 141–143.

Wiesenberger, G., Link, T. A., von Ahsen, U., & Schweyen, R. J. (1991) *J. Mol. Biol. 217*, 23–38.

Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. W., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626–630.

Zarilli, R., Oates, E. L., McBride, W., Lerman, M. I., Chan, J. Y., Santisteban, P., Ursini, M. V., Notkins, A. L., & Kohn, L. D. (1989) *Mol. Endocrinol. 3*, 1498–1508.