Transmembrane Topography of the Mitochondrial Phosphate Carrier Explored by Peptide-Specific Antibodies and Enzymatic Digestion†

Loredana Capobianco,‡ Gérard Brandolin,§ and Ferdinando Palmieri*,‡

Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari and CNR Unit for the Study of Mitochondria and Bioenergetics, Bari, Italy, and Laboratoire de Biochimie, Département de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires, Grenoble Cedex, France

Received November 13, 1990; Revised Manuscript Received February 13, 1991

ABSTRACT: Two peptides corresponding to the amino acid sequences 1–10 (N-terminal peptide) and 303–313 (C-terminal peptide) of the bovine heart mitochondrial phosphate carrier have been synthesized. After being coupled to ovalbumin, they were injected into rabbits to raise polyclonal antibodies. The specificity of the generated antibodies was tested by enzyme-linked immunosorbent assay (ELISA) and/or Western blot. Anti-N-terminal antibodies and anti-C-terminal antibodies exclusively reacted with the corresponding terminal peptide; they also reacted with the isolated phosphate carrier as well as with the phosphate carrier protein in mitochondrial lysates. Both anti-N-terminal and anti-C-terminal antibodies bound to freeze–thawed mitochondria, indicating that both termini of the membrane-bound phosphate carrier are exposed to the cytoplasmic side of the inner mitochondrial membrane. These immunological data were complemented with results concerning enzymatic cleavage of the membrane-bound phosphate carrier by carboxypeptidase A and by an arginine-specific endoprotease. Carboxypeptidase A markedly decreased the binding of anti-C-terminal antibodies to phosphate carrier in freeze–thawed mitochondria. Arg-endoprotease cleaved the phosphate carrier in inside-out submitochondrial particles, but not in right-side-out particles, yielding two fragments of similar apparent molecular weight ($M_r \cong 14.5$K), which were immunodetected only by the anti-N-terminal antiserum, and a fragment of $M_r \cong 17$K which was detected only by the anti-C-terminal antiserum. It appears, therefore, that Arg-endoprotease cleavage sites of the phosphate carrier are present only at the matrix side of the inner mitochondrial membrane, at Arg-140 and/or Arg-152. It can be concluded that the N-terminal and the C-terminal regions of the phosphate carrier both protrude into the cytosol, giving an even number of transmembrane segments, and that the loop containing Arg-140 and Arg-152 protrudes into the matrix space.

The synthesis of ATP during oxidative phosphorylation requires uptake of ADP and phosphate into mitochondria. The translocation of phosphate across the inner mitochondrial membrane is catalyzed by a specific transport system known as the phosphate carrier. This carrier catalyzes the uptake of phosphate either by proton cotransport or in exchange for hydroxyl ions [for a review, see LaNoue and Schoolwerth (1984)]. The phosphate carrier has been purified and successfully reconstituted into phospholipid vesicles (Bisaccia & Palmieri, 1984; Kolbe et al., 1984; Kaplan et al., 1986). The complete amino acid sequence of the phosphate carrier from bovine heart and rat liver was deduced from the nucleotide sequence of cDNA clones (Runswick et al., 1987; Ferreira et al., 1989). The mature protein contains 3 highly conserved internal repeats of about 100 amino acids in length. Furthermore, it has been shown that the repetitive elements of the phosphate carrier are related to those of the ADP/ATP carrier, the uncoupling protein and the oxoglutarate carrier (Runswick et al., 1987, 1990), showing that the phosphate carrier is a member of a family of mitochondrial carrier proteins. It has been shown that residues of cysteine and lysine are essential for the activity of the carrier (Fonyo, 1979; Genchi et al., 1988). In addition, the phosphate carrier has been photolabeled with the competitive inhibitor 4-azido-2-nitrophenyl phosphate (Tommasino et al., 1987; Wohlrab et al., 1989). Whereas Kolbe and Wohlrab (1985) have shown that the site of *N*-ethylmaleimide binding is Cys-42, the essential lysine(s) and the site of photolabeling have not been localized.

Examination of the phosphate carrier sequence led Runswick et al. (1987) to propose a secondary structure model for the arrangement of the protein in the membrane in which the polypeptide consists of six transmembrane α helices connected by relatively hydrophilic loops [see also Ferreira et al. (1989)]. Aquila et al. (1987), on the other hand, have proposed that the phosphate carrier has seven transmembrane spans. It has been suggested that all the mitochondrial substrate carriers so far sequenced share a similar arrangement in the lipid bilayer, since the hydropathy analysis yields strikingly similar profiles (Runswick et al., 1987, 1990; Ferreira et al., 1989).

The precise transmembranous organization of the phosphate carrier as well as of any other mitochondrial carriers remains to be determined. Only in the case of the ADP/ATP carrier has the exposure of the N-terminal region of the polypeptide chain on the cytoplasmic side of the inner mitochondrial membrane been established by the use of site-specific antibodies (Brandolin et al., 1989). In the case of the phosphate carrier, it is not known whether the two termini are exposed to the same side of the membrane plane [as predicted from the model of Runswick et al. (1987)] or are located at opposite

† This work was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), the target project Biotechnology and Bioinstrumentation of Consiglio Nazionale delle Ricerche (CNR), and the Centre de la Recherche Scientifique (CNRS/UA 1130 allée à l'INSERM).

* Address correspondence to this author at the Dipartimento Farmaco-Biologico, Università di Bari, Trav. 200 Re David, 4, 70125 Bari, Italy.

‡ University of Bari and CNR Unit for the Study of Mitochondria and Bioenergetics.

§ Centre d'Etudes Nucléaires.

0006-2960/91/0430-4963$02.50/0

sides [as predicted from the model of Aquila et al. (1987)].

In order to answer these questions and to gain some insight into the transmembrane arrangement of the phosphate carrier in the inner mitochondrial membrane, we have raised antibodies to the N-terminal and C-terminal regions of the bovine heart phosphate carrier and have investigated their interaction with mitochondria and submitochondrial particles. In addition, enzymatic cleavage of the membrane-bound phosphate carrier was combined with the immunological approach, under the assumption that the cleavage sites are located in the exposed loops.

Experimental Procedures

Materials. Boc amino acid and Pam (phenylacetamidomethyl) resin were obtained from Neosystem Laboratories (Strasbourg, France). The sources of other chemicals were as follows: Bio-Gel P-2 minus 400-mesh gel and EIA-grade protein A–horseradish peroxidase conjugate, Bio-Rad; Ultrogel AcA 202, IBF; ovalbumin and 3,3′,5,5′-tetramethylbenzidine, Miles Scientific; ^{125}I-labeled protein A and anti-rabbit Ig horseradish peroxidase linked whole antibody, Amersham; Arg-C endoprotease[1] and carboxypeptidase A, Boehringer; 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester, Pierce.

Biological Preparations. Bovine heart mitochondria were prepared according to the method of Smith (1967). For antibody assays, we used bovine heart mitochondria stored in liquid nitrogen and thawed just before use. Freeze–thawed mitochondria have a damaged outer membrane but an intact inner membrane. They proved to be useful for testing the accessibility of antibodies to the inner membrane. Submitochondrial particles were obtained by differential centrifugation after ultrasonic irradiation of bovine heart mitochondria (Lauquin et al., 1977).

Proteolytic Digestion. Freeze–thawed mitochondria or submitochondrial particles (4 mg, 0.180 mL) in 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA, and 5 mM Tris, pH 7.4, were treated with Arg-C endoprotease for 1 h at 37 °C. For proteolysis with carboxypeptidase A, particles (4 mg, 0.180 mL) in 0.25 M sucrose, 10 mM Tris, and 1 mM EDTA, pH 8, were digested for different periods of time (0, 20, 40, and 60 min) at 37 °C. Proteolytic enzymes were used at a constant ratio of protein/exopeptidase (4/1 w/w). The reactions were stopped by the addition of 20 μL of the following mixture: 20% (w/v) $NaDodSO_4$, 50% (v/v) glycerol, 25% β-mercaptoethanol, and traces of bromophenol blue, and the samples were immediately heated for 2 min at 100 °C. Aliquots corresponding to about 30 μg of protein were loaded on slab acrylamide gels for SPAGE for characterization of the peptide fragments.

Synthesis of Peptides. The N-terminal sequence of the bovine heart phosphate carrier corresponding to residues 1–10 (Ala-Val-Glu-Glu-Gln-Tyr-Ser-Cys-Asp-Tyr) and the C-terminal sequence corresponding to residues 303–313 (Cys-Ser-Leu-Lys-Lys-Lys-Leu-Gly-Tyr-Thr-Gln) were synthesized by the Merrifield solid-phase method (Barany & Merrifield, 1980) using *t*-Boc as the temporary protecting group. A cysteine residue was substituted for glutamic acid at the N-terminal end of 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). If required, the coupling reaction was repeated until the test became negative. The Boc protecting groups were removed by treatment with trifluoroacetic acid as described by Stewart and 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 desalted over Bio-Gel- P-2 minus 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 composition and the concentration of the purified peptide were determined with a Waters (Pico-tag) apparatus after hydrolysis in 6 M HCl for 24 h at 110 °C. Fast atom bombardament mass spectrometry yielded signals corresponding to $(M + H)^+$ values of 1206 and 1268 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 1–10) was coupled with bisdiazotized benzidine (BDB) to ovalbumin through the hydroxyl group of Tyr-6 or Tyr-10. The coupling reaction was performed as described by Tamura and Bauer (1982). To allow specific coupling at the tyrosine residues, the synthetic peptide was first citraconylated at primary amino groups. Routinely, 0.1 μmol of ovalbumin was reacted with 3 μmol of N-terminal peptide. 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 303–313) was coupled with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) to ovalbumin through the SH group of Cys-303 (replacing Glu-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 μmol of ovalbumin was reacted with 3 μmol of C-terminal peptide. The ovalbumin–peptide conjugate was chromatographed over AcA202 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). For the generation of the anti-phosphate carrier protein antiserum, 0.2 mg of phosphate carrier purified from bovine heart dissolved in 0.5 mL of 0.9% NaCl was emulsified with an equal volume of complete Freund's adjuvant, and male New Zealand white rabbits were immunized as described by Zara et al. (1990). 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 (NUNC ref. 4-39454).

Two hundred microliters of the synthetic peptides diluted in PBS or the isolated phosphate carrier protein in a carbonate buffer was added to each well of the plate. The carrier protein, purified as described previously (Bisaccia & Palmieri, 1984)

[1] Abbreviations: 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; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; BDB, bisdiazotized benzidine; TMB, 3,3′,5,5′-tetramethylbenzidine; SPAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *N-t*-Boc, *N-tert*-butoxycarbonyl; TFA, trifluoroacetic acid; Arg-endoprotease, arginine-specific endoprotease; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; EMA, eosinyl-5-maleimide.

and chromatographed on Sephacryl S 200 in 70% formic acid, was lyophilized and diluted in 0.05 M $NaHCO_3/Na_2CO_3$, pH 9.6, before being coated. 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 protein A–horseradish peroxidase conjugate 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 (Titertek Multiskan PLUS, Flow Laboratories). For assays of the reactivity of antibodies against the membrane-bound carrier, the wells of the microtiter plates were coated overnight, at 4 °C, with freeze–thawed mitochondria or submitochondrial particles and then diluted to the appropriate concentration with a medium consisting of 0.12 M KCl, 10 mM Tris, and 1 mM EDTA, final pH 7.3. The subsequent steps were as described above.

In back-titration experiments, freeze–thawed bovine heart mitochondria or submitochondrial particles diluted in a medium consisting of 0.12 M KCl, 10 mM Tris, and 1 mM EDTA, pH 7.4, were incubated with the antisera for 2 h at room temperature. After a 5-min centrifugation at 100000*g* (Airfuge, Beckman) at room temperature, supernatants were collected. The unreacted antibodies present in the supernatant were assayed by ELISA against the corresponding peptides coated onto microtiter plates as described above.

Western Blot Analysis. Besides ELISA, the Western blot technique (Towbin et al., 1979) was used to test the reactivity of the anti-peptide antisera against the phosphate carrier protein and the derived fragments. Thirty microgram of proteins of lysed mitochondria or submitochondrial particles were separated by polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$. After being electroblotted (Boulay et al., 1986), the nitrocellulose sheets were treated with the antiserum indicated in the legends to the figures and then incubated either with ^{125}I-labeled protein A or with anti-rabbit Ig horseradish peroxidase linked whole antibody. The immunoreaction proteins were detected by autoradiography or by the peroxidase reaction, respectively. The peroxidase reaction was performed by 20 mL of a mixture of 0.05% 4-chloro-1-naphthol, 16% methanol, and 0.5% bovine serum albumin in 0.14 M NaCl and 0.01 M phosphate (pH 7.0) with the final addition of 12 μL of 30% H_2O_2.

Results

Characterization of Peptide Antibodies by ELISA. The reactivity of the antibodies raised against the ovalbumin-conjugated N- or C-terminal peptides was investigated by ELISA using microtiter plates with the peptides coated on the wells. Figure 1A,B shows that the anti-N-terminal antiserum and the anti-C-terminal antiserum reacted strongly with the corresponding terminal peptides. In order to check the specificity of the antibodies, controls were performed in which the anti-N-terminal and the anti-C-terminal antisera were assayed against the coated C-terminal peptide and N-terminal peptide, respectively. As shown in Figure 1A,B, no cross-reaction was detected. Similarly, no reaction was found with the preimmune sera or with an antiserum directed to ovalbumin (not shown).

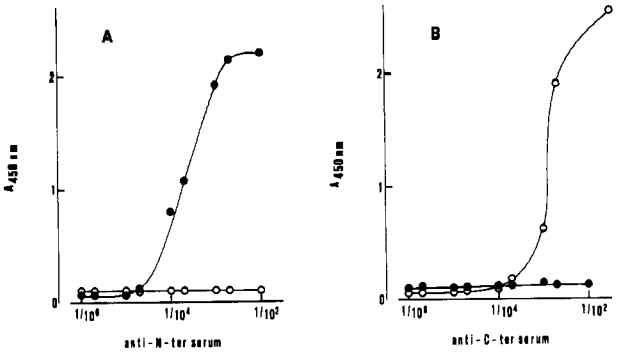

FIGURE 1: Reactivity of anti-N-terminal peptide antiserum and anti-C-terminal peptide antiserum to N-terminal and C-terminal peptides assessed by ELISA. Microtiter plates were coated with the N-terminal peptide or the C-terminal peptide (800 ng/mL in PBS), and the immobilized peptides were incubated with the anti-N-terminal antiserum (A) or with the anti-C-terminal antiserum (B) used at different dilutions. (●) N-Terminal peptide coated; (○) C-terminal peptide coated. After peroxidase-conjugated protein A incubation, reactivities of the antisera were detected by a chromogenic reaction as described under Experimental Procedures.

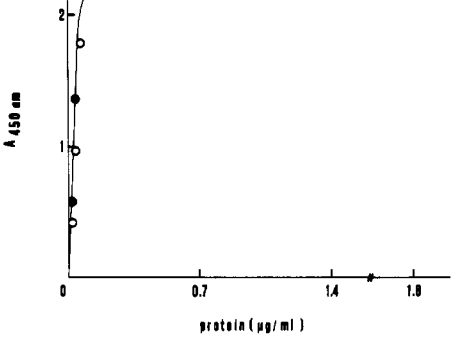

FIGURE 2: Reactivity of anti-N-terminal and anti-C-terminal peptide antisera to phosphate carrier protein assessed by ELISA. Microtiter plates coated with the indicated amounts of purified phosphate carrier were incubated either with the anti-N-terminal antiserum (●) or with the anti-C-terminal antiserum (○) at a dilution of $3/10^3$ in PBS–Tween. Immunoreactivities were revealed as described in Figure 1.

The reactivity of anti-N-terminal and anti-C-terminal antisera toward the isolated phosphate carrier was investigated by ELISA. The data presented in Figure 2 demonstrate that both the anti-N-terminal and the anti-C-terminal antisera reacted with the phosphate carrier protein purified from bovine heart mitochondria. These results were corroborated by immunoblotting of $NaDodSO_4$ lysates of bovine heart mitochondria (Figure 3). In these experiments, both antisera immunodecorated a band of M_r 33K, corresponding to the phosphate carrier protein (lanes 2 and 5). The specificity of labeling was assessed by preincubating the antisera with the respective peptides for 10 min at 25 °C: the immunoreaction was markedly decreased (lanes 3 and 6). Preimmune sera were ineffective (lanes 4 and 7). The ADP/ATP carrier (M_r about 30K) was used as a control (lane 8) since its N-terminal region is known to be immunodetected by antibodies (Brandolin et al., 1989).

Reactivity of the Anti-N-Terminal and the Anti-C-Terminal Antibodies with the Membrane-Bound Phosphate Carrier. To

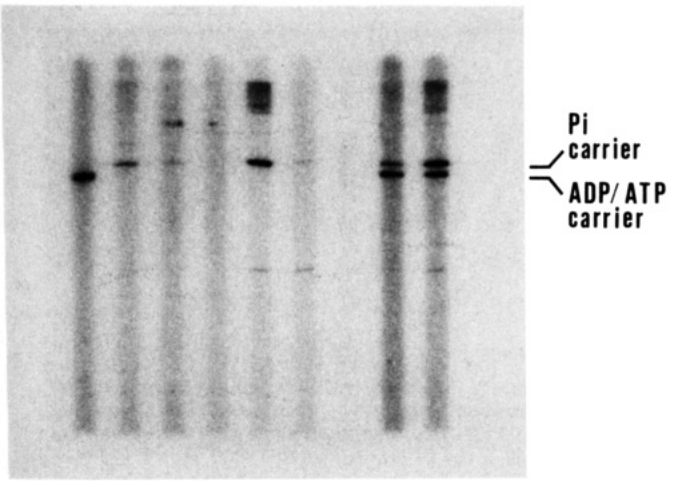

FIGURE 3: Binding of antibodies to the phosphate carrier explored by Western blot analysis. Samples of lysed mitochondria (lanes 1–9) were electrophoresed on a 20% polyacrylamide gel in the presence of $NaDodSO_4$. After being electroblotted, the nitrocellulose sheets were treated with the anti-ADP/ATP carrier antiserum (1/100 dilution) (lane 1), the anti-N-terminal antiserum (1/50 dilution) in the absence or in the presence of competing N-terminal peptide (6 μg/mL) (lanes 2 and 3, respectively), preimmune sera (1/50 dilution) (lanes 4 and 7), the anti-C-terminal antiserum (1/50 dilution) in the absence or in the presence of competing C-terminal peptide (6 μg/mL) (lanes 5 and 6, respectively), both the anti-ADP/ATP carrier (1/100 dilution) and anti-N-terminal antiserum (1/50 dilution) (lane 8), and both the anti-ADP/ATP carrier (1/100 dilution) and anti-C-terminal antiserum (1/50 dilution) (lane 9). Immunodetections were performed as described under Experimental Procedures.

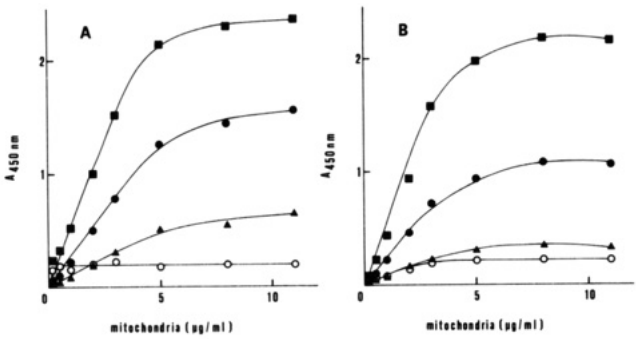

FIGURE 4: Reactivity of the anti-N-terminal peptide and the anti-C-terminal peptide antisera to the membrane-bound phosphate carrier in mitochondria assessed by ELISA. Microtiter plates were coated with the indicated amounts of freeze–thawed bovine heart mitochondria. Immobilized particles were incubated with either the anti-N-terminal peptide antiserum (A) or the anti-C-terminal peptide antiserum (B) added at three different dilutions: $3/10^3$ (■), $1/10^3$ (●), and $3/10^4$ (▲). Antisera were used at a dilution of $3/10^3$ in the presence of the corresponding N-terminal (○) or C-terminal (○) peptide, at a concentration of 5 μg/mL. Binding of antibodies was detected by a chromogenic reaction as described in Figure 1.

investigate whether the anti-N-terminal and the anti-C-terminal antibodies were able to react with the membrane-bound phosphate carrier, ELISA tests were performed with coated freeze–thawed mitochondria. It has been shown that, in these particles, the inner mitochondrial membrane is made accessible to antibodies as a result of damage to the outer membrane (Brandolin et al., 1989). The data presented in Figure 4A,B show that the binding of the anti-N-terminal antibodies as well as the binding of the anti-C-terminal antibodies to freeze–thawed mitochondria increased with the concentration of the antibodies and with the amount of mitochondria to a plateau value. These results indicate that both the N-terminal and the C-terminal regions of the membrane-bound phosphate carrier are accessible to specific antibodies from the outer side of the inner mitocondrial membrane. The specificity of the

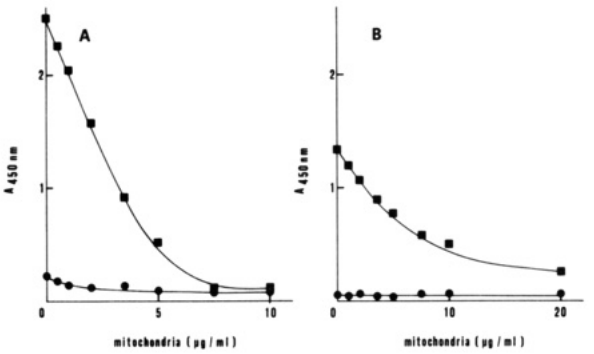

FIGURE 5: Back-titration by ELISA of anti-N-terminal antibodies or anti-C-terminal antibodies after reaction with the membrane-bound phosphate carrier in mitochondria. The anti-N-terminal antiserum (A) or the anti-C-terminal antiserum (B) diluted 1000-fold was incubated for 2 h at room temperature with freeze–thawed bovine heart mitochondria at the indicated concentrations in the absence (■) or in the presence (●) of competing corresponding N-terminal or C-terminal peptide at 4 μg/mL. The mitochondria were spun down by centrifugation, and unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the N-terminal peptide at 2 μg/mL (A) or the purified phosphate carrier (B) at 0.2 μg/mL. Binding of antibodies was detected by a chromogenic reaction as described in the legend of Figure 1.

immunoreaction was demonstrated by adding N-terminal and C-terminal peptides which drastically reduced the reaction (Figure 4A,B).

In order to exclude the possibility that coating the freeze–thawed mitochondria might disorganize the membrane structure, making initially nonexposed epitopes of the membrane-bound carrier accessible to externally added antibodies, back-titration ELISA tests were performed. In these experiments, increasing concentrations of freeze–thawed mitochondria were incubated with a fixed concentration of anti-N-terminal antiserum or anti-C-terminal antiserum. After centrifugation, the unreacted antibodies remaining in the supernatant were back-titrated by ELISA, using microtiter plates coated with the N-terminal peptide or with the phosphate carrier protein. The results of back-titration ELISA are shown in Figure 5. The reaction of the anti-N-terminal antibodies against the coated N-terminal peptide (Figure 5A) as well as the reaction of the anti-C-terminal antibodies against the coated C-terminal peptide or against the coated phosphate carrier (Figure 5B) markedly decreased on increasing the amount of mitochondria, proving that "non coated" mitochondria are able to bind the two terminal peptide-specific antibodies. As a control of the specificity of the immunological reactions, it was shown that when the two types of antibodies were allowed to react with the respective peptides in solution prior to being added to the mitochondrial suspension, virtually no further reaction was detected (Figure 5).

With submitochondrial particles, in which the membrane is thought to be predominantly inverted, the intensity of the reaction of the two antisera was about 40% lower than that observed with mitochondria. However, as will be discussed later, no definite conclusion can be drawn about the orientation of the phosphate carrier embedded in the membrane of submitochondrial particles, because of the possible contamination of the inverted particles with right-side-out particles.

Localization of Carboxypeptidase A and Arg-Endopeptidase Cleavage Sites of the Membrane-Bound Phosphate Carrier. To further investigate the transmembrane arrangement of the phosphate carrier in the inner mitocondrial membrane, enzymatic digestion of the carrier protein in the membrane of freeze–thawed mitochondria and of submitochondrial particles was combined with the immunological

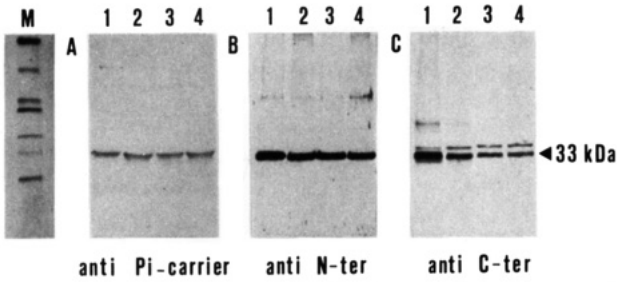

FIGURE 6: Reactivity of the anti-phosphate carrier antiserum, the anti-N-terminal antiserum, and the anti-C-terminal antiserum to the phosphate carrier protein treated by carboxypeptidase A in freeze–thawed mitochondria. Freeze–thawed mitochondria (32 μg) were incubated with 8 μg of carboxypeptidase A for 20, 40, and 60 min at 37 °C (lanes 2, 3, and 4, respectively). Lane 1 is control. Mitochondria were lysed by 2% SDS and proteins separated by SPAGE. After being electroblotted on nitrocellulose, immunoreactive peptides were detected by anti-phosphate carrier, anti-N-terminal peptide, and anti-C-terminal peptide antibodies used at 1/500, 1/1300, and 1/500 dilutions, respectively, followed by reaction with anti-rabbit Ig horseradish peroxidase linked whole antibody as described under Experimental Procedures.

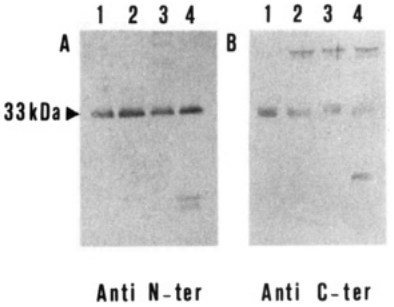

FIGURE 7: Reactivity of anti-N-terminal and anti-C-terminal antibodies to the phosphate carrier and the Arg-endoprotease cleavage products in freeze–thawed mitochondria and submitochondrial particles, analyzed by immunoblotting. Freeze–thawed mitochondria (32 μg) or submitochondrial particles (32 μg) were incubated for 1 h at 37 °C in the absence (lanes 1 and 3, respectively) and in the presence of 8 μg of Arg-endoprotease (lanes 2 and 4, respectively). Immunodecoration with anti-N-terminal antiserum (A) and anti-C-terminal antiserum (B). Samples were then processed as described in the legend of Figure 6, and blotted peptides were immunodetected either with anti-N-terminal antiserum (A) or with anti-C-terminal antiserum (B). After incubation with anti-rabbit Ig horseradish peroxidase linked whole antibody, bound antibodies were assayed by a chromogenic reaction (cf. Experimental Procedures).

approach; i.e., the anti-N-terminal and anti-C-terminal antisera were used to detect the fragments of the phosphate carrier generated upon proteolysis.

In the experiment reported in Figure 6, freeze–thawed mitochondria were incubated with carboxypeptidase A for 20, 40, and 60 min (lanes 2, 3, and 4, respectively) at 37 °C. After this incubation period, the mitochondria were solubilized in $NaDodSO_4$; the proteins were separated by gel electrophoresis and immunodecorated with the anti-phosphate carrier in panel A, the anti-N-terminal antiserum in panel B, and the anti-C-terminal antiserum in panel C. It is clear that the mitochondrial phosphate carrier reacted with the anti-phosphate carrier antiserum and with the anti-N-terminal antiserum independently of the treatment by carboxypeptidase A. In contrast, the reaction of the mitochondrial phosphate carrier with the anti-C-terminal antiserum was substantially decreased by the treatment with carboxypeptidase A (cf. lanes 2–4 with lane 1 of panel C). However, binding of the anti-C-terminal antibodies was not abolished even after 60 min of treatment. This result might be explained by the fact that carboxypeptidase A has low reactivity in the presence of lysine, arginine, aspartic acid, glutamic acid, and proline residues (Petra, 1970). At the C-terminal end of the phosphate carrier, the lysine residue at position 308 might inhibit digestion by carboxypeptidase A.

Further investigation of the topography of the membrane-bound phosphate carrier was carried out by means of enzymatic digestion of the carrier with an arginine-specific endoprotease. Figure 7 shows immunoblots of mitochondria or submitochondrial particles lysed and electrophoresed after treatment with Arg-endoprotease. The resulting fragments are detected either by anti-N-terminal peptide or by anti-C-terminal peptide antisera. Lanes 2 and 4 correspond to mitochondria and submitochondrial particles treated with Arg-endoprotease, respectively. It can be seen that the phosphate carrier was not cleaved when mitochondria were treated with this enzyme, as evidenced by the absence of fragments detected with either the anti-N-terminal antiserum or the anti-C-terminal antiserum. In contrast, the phosphate carrier in submitochondrial particles was efficiently cleaved by Arg-endoprotease that generated several fragments containing the N- or C-terminal regions of the phosphate carrier. This is demonstrated by the observation that the anti-N-terminal antiserum revealed the appearance of two peptides with a similar apparent molecular weight of about 14.5K, whereas the anti-C-terminal antiserum identified a peptide with an apparent molecular weight of 17K. Therefore, it appears that two Arg-endoprotease cleavage sites are located close together on the matrix surface of the inner mitochondrial membrane, approximately halfway along the polypeptide chain of the phosphate carrier. Two obvious candidates for the cleavage sites in this matrix-exposed region of the carrier are Arg-140 and Arg-152.

Discussion

Immunological and enzymatic approaches have been widely used to study the orientation and topography of membrane proteins since they involve the use of nonpermeant reagents able to interact with amino acids at the surface of the membrane [for a review, see Ovchinnikov (1987)]. 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), and the acetylcholine receptor of the postsynaptic membrane (Young et al., 1985; Ratnam et al., 1986). More recently, Matthews et al. (1989) have used anti-peptide antibodies to localize the N- and the C-terminus of the (Ca^{2+}–Mg^{2+})-ATPase on the cytoplasmic side of the sarcoplasmic reticulum membranes. In the present work, a similar approach was used to investigate the topology of the membrane-embedded mitochondrial phosphate carrier in mitochondria and in inside-out particles. In addition, we have studied the access of the peptide chain of the membrane-bound carrier to carboxypeptidase A and an Arg-specific endoprotease.

Antibodies were raised against two synthetic peptides corresponding to the N-terminal and to the C-terminal regions of the phosphate carrier. These peptides were chosen on the basis of their expected flexibility and the accessibility to aqueous medium of the N- and C-terminal regions of proteins (Chavez & Scheraga, 1979; Thornton & Sibanda, 1983). Immunological assays by ELISA showed that N- and C-terminal peptide-specific antibodies were elicited in rabbits immunized with the appropriate peptide–ovalbumin conjugates; no cross-reaction was detected. Both of the anti-peptide sera

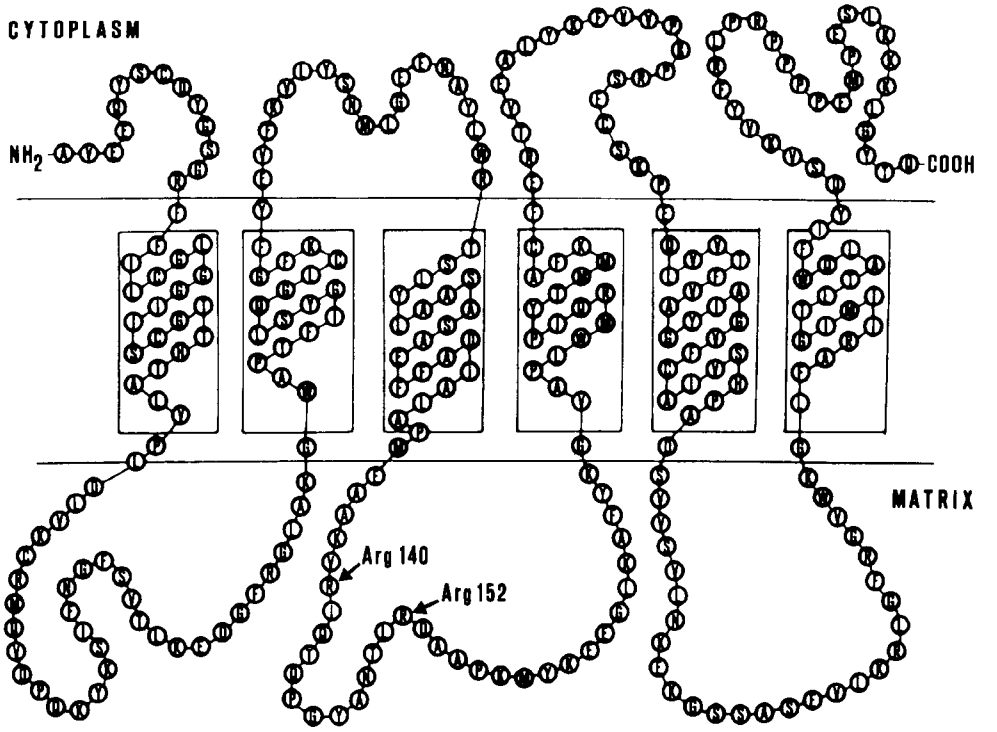

FIGURE 8: Possible model of the transmembrane arrangement of the phosphate carrier as predicted from the primary sequence and the hydrophobic profile (Runswick et al., 1987). The N-terminal and C-terminal regions of the polypeptide chain are exposed to cytosol, and the segment containing Arg-140 and Arg-152 protrudes into the matrix.

recognized specifically the isolated phosphate carrier bound to microtiter plates and also recognized the carrier among the proteins of a mitochondrial lysate after Western blotting. Thus, it was possible to use these antibodies to investigate the locations of the N-terminal and C-terminal regions of the carrier with respect to the plane of the membrane.

ELISA performed with freeze–thawed mitochondria bound to microtiter plates demonstrated clearly the cytoplasmic exposure of the N- and C-terminal regions of the phosphate carrier. These results were confirmed by back-titration ELISA experiments. In addition, the stepwise proteolysis of the membrane-bound phosphate carrier in freeze–thawed mitochondria with carboxypeptidase A provided further evidence that the C-terminus of the protein is exposed at the cytoplasmic side of the inner mitochondrial membrane.

Similar immunological assays were carried out with inverted submitochondrial particles obtained by sonication of mitochondria. In contrast to the clear-cut results obtained when mitochondria were used, no definite conclusion could be drawn about the orientation of the phosphate carrier in the membrane of submitochondrial particles. This might be due to the fact that inside-out particles are heterogeneous and contaminated to some extent by noninverted material (Lauquin et al., 1977).

The uncertainty concerning the orientation of the phosphate carrier in the membrane of inside-out particles was overcome by the use of an arginine-specific endoprotease that was shown to cleave the carrier in inside-out particles but not in freeze–thawed mitochondria. This result demonstrated the asymmetry of the membrane-bound phosphate carrier with respect to the plane of the membrane, and, if the carrier is dimeric (or multimeric), as suggested from *N*-ethylmaleimide binding data [see Wohlrab (1986)], an antiparallel orientation of the monomers can be excluded.

The molecular weight estimation of the fragments generated upon Arg-endoprotease digestion of the carrier allowed the localization of the cleavage site at Arg-140 and/or Arg-152 on the matricial face of the mitochondrial membrane.

The combined use of site-directed anti-peptide antibodies and site-specific proteases presented in this paper provided direct information about the topography of the phosphate carrier in the mitocondrial membrane. A possible arrangement of the polypeptide chain of the carrier, based on the hydropathy plots reported by Runswick et al. (1987) and our experimental results, is shown in Figure 8. This model includes 6 transmembrane α helices, made up of about 20 amino acid residues each, connected by 5 extramembrane hydrophilic loops. The N-terminal and C-terminal extremities of the peptide chain are oriented toward the cytosol, and the segment containing Arg-140 and Arg-152 faces the matrix compartment. This model disagrees with that proposed by Aquila et al. (1987), which predicts an odd number of transmembrane segments and a location of the two termini at opposite sides of the membrane. Our model also disagrees with that reported by Ferreira et al. (1989) for the phosphate carrier protein from rat liver mitochondria. These authors proposed a secondary structure model of the phosphate transporter containing six membrane-spanning helices and in which the amino and carboxyl termini are both located at the matrix side of the membrane. Such an orientation was deduced from the postulated cytoplasmic exposure of cysteine at position 41 (that corresponds to cysteine-42 in bovine heart phosphate carrier). However, this assumption deserves some comments. Cys-42 was identified as the NEM binding site in the phosphate carrier of bovine heart mitochondria (Kolbe & Wohlrab, 1985). From competitive binding experiments performed on mitochondria with *N*-ethylmaleimide (NEM) and eosinyl-5-maleimide (EMA), Houstek and Pedersen (1985) concluded that the same SH group was involved in binding of both reagents. As EMA is considered to be a membrane-impermeable reagent, cysteine-41 was assigned to the cytoplasmic face. However, in the experiments carried out by Houstek and Pedersen, the following sequence was followed: (1) binding of mersalyl; (2) reaction with cold NEM; (3) removal of mersalyl by DTT; and (4) labeling with [^{3}H]NEM (prevented

by EMA). In this way, the EMA-sensitive NEM binding to the phosphate carrier was directed to mersalyl-sensitive SH group(s) that may be distinct from Cys-42 due to the very different membrane permeabilities exhibited by mersalyl and NEM.

The results reported in this paper provide the first direct information on the arrangement of the phosphate carrier in the mitochondrial inner membrane. Further studies are obviously required to delineate a more precise folding model.

ACKNOWLEDGMENTS

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

REFERENCES

Aquila, H., Link, T. A., & Klingenberg, M. (1987) *FEBS Lett. 212*, 1–9.

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

Bisaccia, F., & Palmieri, F. (1984) *Biochim. Biophys. Acta 766*, 386–394.

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.

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.

Chavez, L. G., & Scheraga, H. A. (1979) *Biochemistry 18*, 4386–4395.

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

Ferreira, G. C., Pratt, R. D., & Pedersen, P. L. (1989) *J. Biol. Chem. 264*, 15628–15633.

Fonyo, A. (1979) *Pharmacol. Ther. 7*, 627–645.

Genchi, G., Petrone, G., De Palma, A., Cambria, A., & Palmieri, F. (1988) *Biochim. Biophys. Acta 936*, 413–420.

Houstek, J., & Pedersen, P. L. (1985) *J. Biol. Chem. 260*, 6288–6295.

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

Kaplan, R. S., Pratt, R. D., & Pedersen, P. L. (1986) *J. Biol. Chem. 261*, 12767–12773.

Kolbe, H. V. J., & Wohlrab, H. (1985) *J. Biol. Chem. 260*, 15899–15906.

Kolbe, H. V. J., Costello, D., Wong, A., Lu, R. C., & Wohlrab, H. (1984) *J. Biol. Chem. 259*, 9115–9120.

LaNoue, K. F., & Schoolwerth, A. C. (1984) in *Bioenergetics* (Ernster, L., Ed.) pp 221–268, Elsevier, Amsterdam.

Lauquin, G. J. M., Villiers, C., Michejda, J. W., Hryniewiecka, L. V., & Vignais, P. V. (1977) *Biochim. Biophys. Acta 460*, 331–345.

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

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.

Ovchinnikov, Y. A. (1987) *Trends Biol. Sci. (Pers. Ed.) 12*, 434–438.

Petra, P. H. (1970) *Methods Enzymol. 19*, 460–503.

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.

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.

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.

Thornton, J. M., & Sibanda, B. L. (1983) *J. Mol. Biol. 167*, 443–460.

Tommasino, M., Prezioso, G., & Palmieri, F. (1987) *Biochim. Biophys. Acta 890*, 39–46.

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

Wohlrab, H. (1986) *Biochim. Biophys. Acta 853*, 115–134.

Wohlrab, H., Bukusoglu, C., & DeFoe, H. (1989) in *Anion carriers of mitochondrial membranes* (Azzi, A., et al., Eds.) pp 113–121, Springer-Verlag, Berlin.

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.

Zara, V., De Benedittis, R., Ragan, C. I., & Palmieri, F. (1990) *FEBS Lett. 263*, 295–298.