Topography of the Membrane-Bound ADP/ATP Carrier Assessed by Enzymatic Proteolysis†

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ABSTRACT: The folding of the peptide chain of the beef heart ADP/ATP carrier in the inner mitochondrial membrane was investigated by enzymatic and immunochemical approaches, using specific proteases and polyclonal antibodies directed against the whole protein and specific regions of the carrier. The accessibility of the membrane-bound ADP/ATP carrier to proteases was followed by immunodetection of the cleavage products, using mitochondria devoid of outer membrane (mitoplasts) and inside-out submitochondrial particles (SMP) in the presence of either carboxyatractyloside (CATR) or bongkrekic acid (BA), two specific inhibitors which are able to bind to the outer face or the inner face of the carrier, respectively. Four types of particles were investigated, namely, mitoplasts–CATR, mitoplasts–BA, SMP–CATR, and SMP–BA. Only the ADP/ATP carrier in SMP–BA was cleaved by two specific proteases, namely, trypsin and lysine C endoprotease, at low doses for short periods of time. Two initial cleavage sites were found between Lys-42 and Glu-43, and between Lys-244 and Gly-245. After a longer period of incubation, an additional cleavage site between Lys-146 and Gly-147 could be demonstrated. Despite cleavage of the membrane-embedded carrier, the binding capacity and affinity of SMP for BA were not altered. A number of other proteases tested, including V8 protease, proline C endoprotease, thrombin, α-chymotrypsin, and thermolysin had virtually no effect. These results are explained by a dynamic model of the arrangement of the peptide chain of the ADP/ATP carrier. This model postulates that two pairs of short amphipathic sequences containing the amino acid sequences 140–151 and 153–167, on the one hand, and 237–249 and 253–267, on the other, which carry the binding sites for 2-azido-ADP [Dalbon et al. (1988) *Biochemistry 27*, 5141–5149] are juxtaposed and act as channel formers in the course of transport of ADP or ATP.

ATP generated by oxidative phosphorylation in mitochondria is exported to the cytosol, in exchange for ADP, by a specific carrier protein located in the inner mitochondrial membrane. Two species of specific inhibitors are known, namely, atractyloside (ATR)[1] and carboxyatractyloside (CATR), on the one hand, and bongkrekic acid (BA), on the other, which bind to the cytosolic (external) face and the matrix (internal) face of the carrier, respectively [for a review, see Vignais et al. (1985)]. During the course of transport, the ADP/ATP carrier adopts two different conformations, that can be trapped by CATR and by BA and are referred to as CATR and BA conformations, respectively. From the sequence of the bovine mitochondrial ADP/ATP carrier, which is 297 amino acids long (Aquila et al., 1982), a first model of the topography of the membrane-bound carrier, on the basis of hydropathy plots, was reported (Saraste & Walker, 1982). Since then, an number of experiments aimed at determining the topography of the membrane-bound carrier have been performed with bovine heart mitochondria and the derived inside-out submitochondrial particles (SMP) as biological material, using photolabels (Boulay et al., 1983; Dalbon et al., 1988), chemical modifiers (Boulay & Vignais, 1984; Bogner et al., 1986), antipeptide antibodies (Brandolin et al., 1989), and proteolytic enzymes (Brandolin et al., 1989). The finding that in mitochondria, but not in SMP, an azido derivative of the inhibitor ATR binds to residues 159–200 (Boulay et al., 1983) and that 2-azido-ADP binds to residues 153–200 and 250–281 (Dalbon et al., 1988) was interpreted by assuming that the above residues are exposed to the outside. The modification of Cys-56 by *N*-ethylmaleimide, a permeant SH reagent which inhibits ADP/ATP transport (Boulay & Vignais, 1984), and the enzymatic cleavage of the carrier protein at either Arg-30 or Arg-59 by an arginine-specific endoprotease, only in SMP (Brandolin et al., 1989), indicated that the region of the carrier containing Arg-30, Cys-56, and Arg-59 is exposed to the inside. Finally, antibodies directed against the N-terminal region of the carrier were found to react with mitochondria (Brandolin et al., 1989), leading to the conclusion that the N-terminal portion of the carrier is exposed to the outside. The present work combines immunochemical and enzymatic methods, in particular the use of antibodies directed against segments of the carrier chain, and proteolytic enzymes. The biological materials used were beef heart mitochondria devoid of outer membrane (mitoplasts), and the derived inside-out submitochondrial particles (SMP), with the

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[1] Abbreviations: ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; SMP, submitochondrial particle(s); TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; SPAGE, SDS–polyacrylamide gel electrophoresis; HTP, hydroxylapatite; PVDF, poly(vinylidene difluoride); PBS, phosphate-buffered saline; PBST; PBS supplemented with 0.05% Tween 20.

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carrier either in the CATR or in the BA conformation for each type of particles used. The results are discussed in terms of a dynamic model of the arrangement of the peptide chain of the carrier.

Experimental Procedures

Materials. The materials and their sources were as follows. Boc amino acids were obtained from Neo-System (Strasbourg), and the Merrifield resin (copolymer styrene–1% divinylbenzene chloromethylated) was from Peninsula Laboratories; CNBr-activated Sepharose 4B was from Pharmacia; ^{125}I-labeled A protein was from Amersham; PVDF membrane (Immobilon P) was from Millipore, trypsin was from Sigma, and endoprotease Lys-C was from Boehringer. Beef heart mitochondria were prepared according to the method of Smith (1967). Inside-out beef heart submitochondrial particles (SMP) were obtained by sonication of mitochondria as described by Lauquin et al. (1977). Contamination of SMP by mitochondrial membranes in the right-side-out conformation was assayed by [^{3}H]atractyloside binding (Lauquin et al., 1977). To make the inner mitochondrial membrane freely accessible to antibodies, mitochondria were frozen and thawed (Brandolin et al., 1989). These mitochondrial particles, devoid of a functional outer membrane, were referred to as mitoplasts. They were incubated at 20 mg of protein/mL for 2–3 min at 20 °C with 50 μM CATR or 50 μM BA and with 10 μM ADP to yield mito–CATR or mito–BA with the carrier in the CATR or BA conformation, respectively. As SMP bind BA, but not CATR, SMP–CATR were prepared by sonication of mito–CATR. SMP–BA were obtained by addition of 50 μM BA to SMP.

Anti-Peptide Antibodies. The peptide termed P40, corresponding to amino acid residues 40–50 of the beef heart ADP/ATP carrier, namely, Ala-Ser-Lys-Gln-Ile-Ser-Ala-Glu-Lys-Gln-Tyr, and the peptide corresponding to the C-terminal sequence, Cys-Val-Leu-Val-Tyr-Asp-Glu-Ile-Lys-Lys-Phe-Val (C-ter peptide), were synthesized by the Merrifield solid-phase method (Barany & Merrifield, 1980), using *t*-Boc as a temporary protecting group. A cysteine residue was substituted for phenylalanine at the N-terminal end of the C-ter peptide to permit coupling to the ovalbumin carrier, the coupling reagent being maleimidobenzoic acid *N*-hydroxysuccinimide ester (Lerner et al., 1981). Peptide P40 was coupled by its tyrosine residue 50 to ovalbumin with bis-diazotized benzidine (Brandolin et al., 1989). The binding stoichiometry of peptides with respect to ovalbumin (mol/mol) was between 15 and 20. The conjugates were used to immunize rabbits as previously described (Boulay et al., 1986). In addition to the P40 and C-ter peptide antisera, we used an antiserum raised against the ADP/ATP carrier protein denatured by sodium dodecyl sulfate (SDS-carrier) [cf. Boulay et al. (1986)]. Before use, the immune sera were decomplemented by treatment at 56 °C for 30 min. Antibodies directed against peptide P40 were purified by affinity chromatography on a Sepharose 4B–ADP/ATP carrier coupled column (Brandolin et al., 1989). The Western blot technique was used to test the reactivity of the immune sera against the ADP/ATP carrier protein and the derived fragments (Towbin et al., 1979). The data in Figure 1 illustrate the immunoreactivity of the three antisera with respect (1) to the ADP/ATP carrier protein in SDS-lysed SMP, (2) to the purified carrier, and (3) to the 20- and 10-kDa peptide fragments arising from acidolytic cleavage of the purified carrier between Asp-203 and Pro-204. All three antisera were immunoreactive to the whole carrier protein. In addition, the SDS-carrier antiserum reacted with the 20- and 10-kDa fragments. As expected from the localization of the site of acidolytic cleavage, the P40 antiserum reacted against the 20-kDa fragment and the C-terminal peptide antiserum against the 10-kDa fragment.

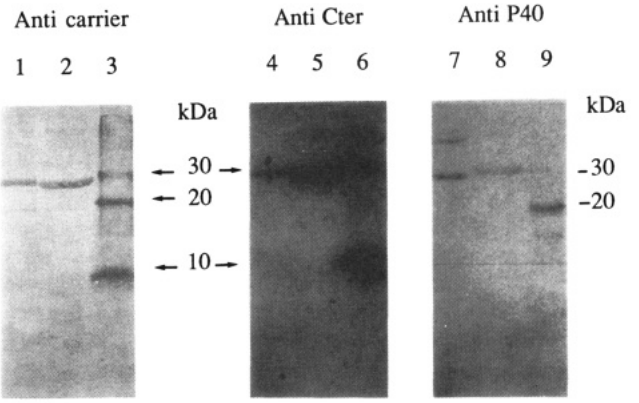

FIGURE 1: Reactivity of anti-P40 antibodies against the ADP/ATP carrier protein and its acidolytic fragments explored by Western blot analysis. Samples of lysed SMP, purified ADP/ATP carrier, or ADP/ATP carrier after acidolytic cleavage were electrophoresed on a 20% acrylamide gel in the presence of 0.5% $NaDodSO_4$ and then electroblotted onto nitrocellulose. The nitrocellulose sheets were treated either with anti-SDS-denatured carrier antiserum (1/100 dilution), with anti-C-terminal peptide antiserum (1/500 dilution), or with affinity-purified anti-peptide 40–50 antibodies (1/10 dilution). After incubation with ^{125}I-labeled protein A, the immunoreactive proteins were detected by autoradiography. Lanes 1, 4, and 7, SMP lysate (10 μg/lane); lanes 2, 5, and 8, purified ADP/ATP carrier protein (2 μg/lane); lanes 3, 6, and 9, ADP/ATP carrier after acidolytic cleavage (2 μg/lane). As expected, in addition to the ADP/ATP carrier itself, only the 20-kDa acidolytic fragment of the ADP/ATP carrier (Ser^1–Asp^{203}) was found to react with the P40 antibodies, whereas only the 10-kDa acidolytic fragment (Pro^{204}–Val^{297}) was immunodetected by the anti-C-ter peptide antibodies.

Proteolysis of the Membrane-Bound Carrier. Mito–CATR, mito–BA, SMP–CATR, and SMP–BA prepared as described above were diluted at 4 mg/mL in 0.27 M sucrose, 10 mM borate, 1 mM EDTA, and 20 mM ammonium bicarbonate, pH 9. Proteolytic digestion of the ADP/ATP carrier in mitochondria and SMP was carried out at 30 °C for lysine C endoprotease and at 10 °C for trypsin. Trypsin digestion was stopped by addition of soybean trypsin inhibitor (twice as much in weight as trypsin) and lysine C endoprotease digestion by addition of 1 mM TLCK (final concentration). For the other proteases, 1 mM DFP was used to stop proteolysis. In kinetic studies, an aliquot of the digest was added to a tube containing an equal volume of the dissociation medium, consisting of 250 mM Tris, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, and bromophenol blue, pH 6.8, that had just been heated at 100 °C for 2 min, and the mixture was further heated for 2 more min at 100 °C. The samples were electrophoresed on a minislab sodium dodecyl sulfate–polyacrylamide gel (SPAGE) with about 10 μg of protein/lane for further characterization of the proteolytic fragments.

Antibody Assays by ELISA. The ability of antisera to react with the membrane-bound ADP/ATP carrier was tested by ELISA using microtitration polystyrene plates (NUNC ref 4-S9454) as previously described (Brandolin et al., 1989). The wells of microtiter plates were coated overnight at 4 °C with freeze–thawed mitochondria or with SMP and diluted to appropriate concentrations (up to 100 μg/mL) with a medium consisting of 0.12 M KCl/10 mM MOPS, pH 6.7, in a final volume of 200 μL.

After the wells were washed with PBS and unspecific sites on the microtiter plates were saturated with 1% BSA in PBST, 200 μL of diluted anti-peptide antiserum was added to the wells, and the plates were incubated at room temperature for

2 h. Bound antibodies were allowed to react with peroxidase-conjugated protein A (Bio-Rad) diluted 2000-fold in PBST, and the peroxidase activity was measured by a chromogenic reaction using 3,3′,5,5′-tetramethylbenzidine as an indicator. The reactivity of antibodies to the membrane-bound ADP/ATP carrier in freeze–thawed mitochondria or in SMP was alternatively measured by back-titration ELISA. Freeze–thawed mitochondria or SMP were diluted at an appropriate concentration in a medium consisting of 0.12 M KCl and 10 mM MOPS, pH 6.7. Anti-peptide antisera were added, and incubation was allowed to proceed for 2 h at room temperature or overnight at 4 °C. After a 5-min centrifugation at 100000*g* (Airfuge, Beckman) at room temperature, supernatants were collected. Unreacted antibodies present in supernatants were assayed by ELISA against the corresponding peptides coated onto microtiter plates as previously described (Brandolin et al., 1989).

Purification of Proteolytic Fragments. Cleavage products of the ADP/ATP carrier arising from incubation of proteases with SMP–BA particles were solubilized in Triton X-100, and purified on a hydroxyapatite (HTP) column (Riccio et al., 1975). The excluded protein fraction was delipidated (Boulay et al., 1983), dissolved in formic acid, and freeze-dried. About 1 mg of the HTP-purified proteolytic fragments was loaded on a 0.75-mm-thick SDS–polyacrylamide gel. Following electrophoresis, the gel was stained for 5 min with 0.3 M $CuCl_2$. The stained bands were excised, destained with 0.25 M EDTA and 0.25 M Tris, pH 9, as described by Lee et al. (1987), and then incubated for 5 min in the electroelution medium consisting of 100 mM ammonium bicarbonate and 0.01% SDS. This was followed by electroelution of the protein material for 5 h at 4 °C in an ISCO apparatus (membrane Spectrapor no. 2, MW cutoff 12K–14K) at 50 mA/cupel. To remove SDS from the eluate, 4 volumes of cold acetone were added, and the protein was allowed to precipitate for 2 h at −20 °C. The precipitate was collected by high-speed centrifugation, dissolved in 70% HCOOH, and freeze–dried. After lyophilization, it was either dissolved in 2% SDS for SPAGE or dissolved in 70% HCOOH followed by incubation for 50–60 h at 37 °C for acidolytic cleavage.

Transfer on the PVDF Membrane (Immobilon) for the Amino Acid Sequence. When low amounts of proteins were handled, transfer on Immobilon (PVDF membrane) by the procedure described by Matsudaira (1987) was used. The gel was first submitted to a 30-min preelectrophoresis, the electrophoresis medium being supplemented with 0.1 mM thioglycolate. Samples corresponding to about 70 μg per lane were loaded on the gel. After electrophoresis, the gel was soaked for 5 min in the transfer buffer consisting of 20 mM sodium carbonate and 20% methanol, pH 10.9. The PVDF membrane was first wetted with methanol, rinsed with water, and then equilibrated for 5 min in the transfer buffer. The gel was electroblotted for 75 min at 250 mA in a Hoeffer Transphor electrophoresis apparatus. The membrane was then rinsed for 2–3 min with distilled water, stained with 0.1% Coomassie blue in 50% methanol, and destained for 5 min in 50% methanol and 10% CH_3COOH. After a final washing for 5 min in water, it was allowed to dry at room temperature. The bands to be analyzed were excised and directly processed for amino acid sequence in a gas-phase sequenator (Applied Biosystem 477A).

Results

General Pattern of Enzymatic Proteolysis of the Membrane-Bound ADP/ATP Carrier. Demonstration of Proteolytic Sites on Inverted Submitochondrial Particles. Four types of mitochondrial particles, namely, mito–CATR, mito–BA, SMP–CATR, and SMP–BA (cf. Experimental Procedures), were used to study the accessibility of the peptide chain of the ADP/ATP carrier to the following proteases: *Staphylococcus aureus* V8 protease, proline C endoprotease, lysine C endoprotease, trypsin, thrombin, α-chymotrypsin, and thermolysin. In a screening assay, the proteolytic efficiency of the above enzymes was checked at 30 °C with a protease to particle protein ratio of 1/20 for periods of time of 10 min to 6 h at the optimal pH for each enzyme. The cleavage products of the carrier protein were separated by SPAGE, transferred onto a nitrocellulose sheet, and immunodetected with antibodies prepared against the SDS-denatured carrier protein (cf. Experimental Procedures). No fragmentation could be detected with protease V8, proline endoprotease, thrombin, and thermolysin. In contrast, trypsin and lysine C endoprotease were able to generate in SMP–BA a discrete number of peptides at 10 °C, using an enzyme to membrane protein ratio as low as 1/200. No cleavage by trypsin and lysine C endoprotease could be detected with mito–CATR, mito–BA, and SMP–CATR. Interestingly, a modest yield of cleavage was found with untreated SMP, which was considerably increased by the addition of BA. This was taken as evidence that some peptide bonds of the carrier are accessible to trypsin and lysine C endoprotease from the matrix side of the inner mitochondrial membrane and that access to these proteases is facilited when the carrier is in the BA conformation. SMP–BA were therefore used for the mapping studies to be described now.

Immunochemical Detection of the Cleavage Products Obtained by Treatment of SMP–BA with Trypsin and Lysine C Endoprotease. SMP–BA were subjected to trypsin treatment, using a trypsin to particles ratio of 1/200, at 10 °C, and then lysed with SDS as described above. The cleavage products in the SDS lysate were separated by SPAGE and immunodetected by antibodies directed against the SDS-denatured ADP/ATP carrier protein. The time course of hydrolysis of the membrane-bound carrier is illustrated in Figure 2, lanes 4–8. Lane 9 corresponds to a control not treated by trypsin. A 25-kDa band appeared after only 10 min of contact of SMP–BA with trypsin. An additional band of 20 kDa was revealed after 30 min (lane 5), which increased in intensity at the expense of the 25-kDa band (lanes 6–8), indicating that the 25-kDa fragment was an intermediate cleavage product. After 2 h, two distinct peptides could be detected in the 20-kDa region (lane 8). When a similar immunodetection technique was applied to mito–CATR, mito–BA, and SMP–CATR treated by trypsin for 4 h at 10 °C (lanes 1–3), there was no evidence of cleavage of the ADP/ATP carrier protein in these particles.

In addition to the SDS-carrier antiserum, the antisera raised against the P40 peptide and the N-ter peptide were used to analyze the cleavage sites of the ADP/ATP carrier in SMP–BA (Figure 3). The conditions of incubation were the same as those used in the experiment of Figure 2. In Figure 3, lanes 1–3 correspond to a control assay showing that the 25- and 20-kDa fragments arising by trypsin cleavage are both immunodetected by the SDS-carrier antiserum. In contrast, the anti-N-ter peptide antiserum reacted with the 25-kDa fragment (Figure 3, lanes 5 and 6), but not with the 20-kDa fragment, indicating that the N-terminal region of the ADP/ATP carrier is present in the 25-kDa fragment but not in the 20-kDa fragment. Both the 25-kDa and 20-kDa fragments were immunodetected by the P40 antiserum (Figure 3, lanes 10–12). Therefore, the epitope corresponding to residues 40–50 is common to the 20- and 25-kDa fragments.

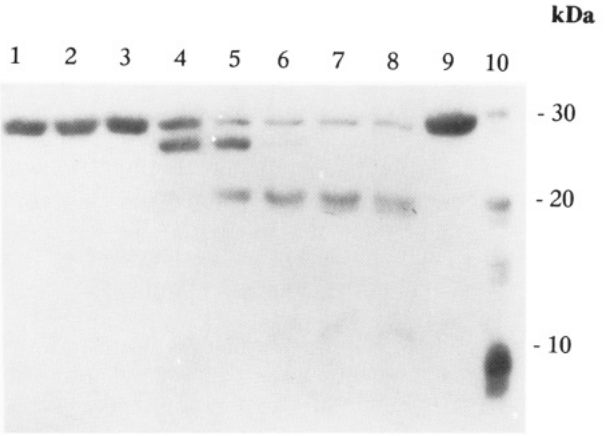

FIGURE 2: Immunodetection of the tryptic fragments of the membrane-bound ADP/ATP carrier in mitoplasts or in SMP. Mitoplasts or SMP in which the ADP/ATP carrier was trapped either in the CATR or in the BA conformation (see Experimental Procedures) were treated with trypsin at 10 °C as described under Experimental Procedures, using a trypsin to mitochondrial protein ratio of 1/200 (w/w). The samples were then lysed with 2% $NaDodSO_4$, and proteins were separated by SPAGE. This was followed by electroblotting and incubation of the nitrocellulose sheets with anti SDS-denatured carrier antiserum (1/100). The immunoreactive peptides were detected by autoradiography after reaction with ^{125}I-labeled protein A. Lane 1, mito–CATR, incubated for 4 h with trypsin; lane 2, mito–BA, incubated for 4 h with trypsin; lane 3, SMP–CATR, incubated for 4 h with trypsin; lanes 4–8, SMP–BA, incubated with trypsin for 10 min, 30 min, 1 h, 2 h, and 4 h, respectively; lane 9, SMP–BA, incubated for 4 h without trypsin; lane 10, ATP/ATP carrier after acidolytic cleavage. Samples contained 10 μg of total protein/lane for lanes 1–9 and 2 μg of protein for lane 10.

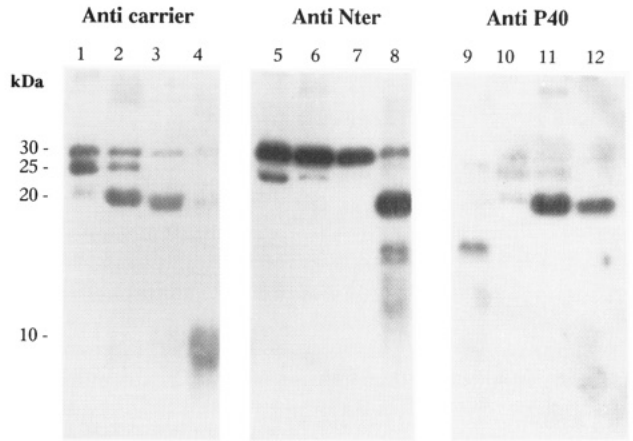

FIGURE 3: Reactivity of the tryptic fragments of the ADP/ATP carrier towards the SDS-treated ADP/ATP carrier antiserum, anti-N-terminal antiserum, or anti-P40 antibodies. SMP-BA were treated with trypsin at 10 °C, and the resulting fragments of the ADP/ATP carrier were purified by HTP chromatography as described under Experimental Procedures. After SPAGE and Western blot, the nitrocellulose sheets were incubated with anti-SDS-denatured carrier antiserum (1/100), with anti-N-ter antiserum (1/80), or with anti-P40 antibodies (1/10) as indicated. The immunoreactive fragments were detected by autoradiography after incubation of the nitrocellulose sheet with ^{125}I-labeled protein A. Lanes 1–10, 10-min trypsin cleavage (10 μg/lane); lanes 2–11, 30-min trypsin cleavage (10 μg/lane); lanes 3–12, 2-h trypsin cleavage (10 μg/lane); lanes 4–9, acidolytic fragments of the ADP/ATP carrier (2 μg/lane).

It is noteworthy that the P40 antiserum was more immunoreactive toward the 20-kDa fragment than toward the whole carrier protein and the 25-kDa fragment. This may be explained by the release of constraints in the peptide chain of the membrane-embedded carrier resulting from peptide bond cleavage in the N-terminal region of the ADP/ATP carrier.

Similarly to trypsin, lysine C endoprotease at a low concentration (enzyme to particle ratio of 1/200) efficiently attacked the ADP/ATP carrier protein, essentially in SMP–BA (Figure 4). A 25-kDa fragment was immunodetected by the SDS-carrier antiserum after 10 min of enzymatic digestion at 30 °C (Figure 4, lane 4). After 4 h, an additional band corresponding to a 15-kDa peptide was immunodetected (Figure 4, lane 8). The 25-kDa fragment reacted with the P40 antiserum (Figure 5, lane 8), but not with the N-ter peptide antiserum (Figure 5, lane 4). The 15-kDa fragment was not revealed either by the P40 antiserum or by the N-ter peptide antiserum. On the other hand, both the 15-kDa fragment and the 25-kDa fragment were immunodetected by the SDS-carrier antiserum (Figure 4, lane 8, and Figure 5, lane 2) and the C-ter peptide antiserum (Figure 5, lane 6); yet the immuno-

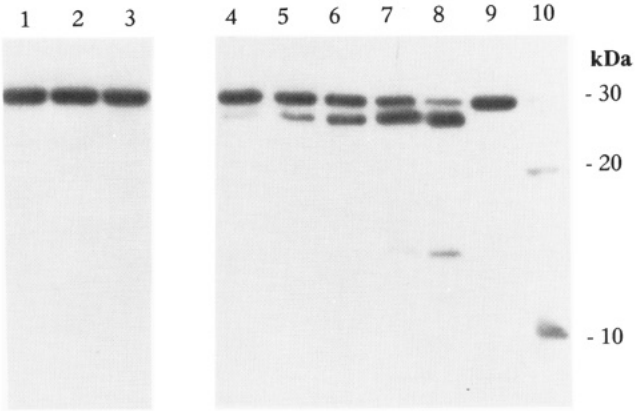

FIGURE 4: Immunodetection of fragments of the membrane-bound ADP/ATP carrier after cleavage with lysine C endoprotease. After treatment with CATR or BA, samples of mitoplasts or SMP were treated by lysine C endoprotease as described in the legend of Figure 2 for the trypsin cleavage, except that the temperature was 30 °C. Proteolytic fragments of the ADP/ATP carrier were detected as described in Figure 2. Lane 1, mito–CATR, incubated for 4 h with lysine C endoprotease; lane 2, mito–BA, incubated for 4 h with lysine C endoprotease; lane 3, SMP–CATR, incubated for 4 h with lysine C endoprotease; lanes 4–8, SMP–BA, incubated with lysine C endoprotease for 10 min, 30 min, 1 h, 2 h, and 4 h, respectively; lane 9, SMP–BA, incubated for 4 h at 30 °C without lysine C endoprotease; lane 10, ADP/ATP carrier after acidolytic cleavage. Samples contained 10 μg of protein/lane for lanes 1–9 and 2 μg of protein for lane 10.

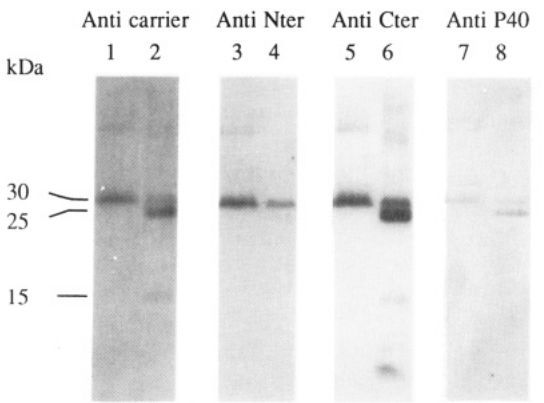

FIGURE 5: Immunoreactivity of the fragments resulting from the cleavage of the membrane-bound ADP/ATP carrier by lysine C endoprotease. SMP were treated with BA and then incubated with endoprotease Lys-C at 30 °C for a period of 6 h. The fragments resulting from the cleavage of the ADP/ATP carrier were collected as described under Experimental Procedures and submitted to SPAGE analysis. After blotting, the peptides were immunodetected by antisera directed against either the SDS-treated ADP/ATP carrier, the N-terminal peptide, the C-terminal peptide, or P40 as indicated on the figure, followed by treatment with ^{125}I-labeled protein A and autoradiography. Lanes 1, 3, 5, and 7, purified ADP/ATP carrier (2 μg/lane); lanes 2, 4, 6, and 8, fragments of the ADP/ATP carrier after cleavage with lysine C endoprotease (10 μg/lane).

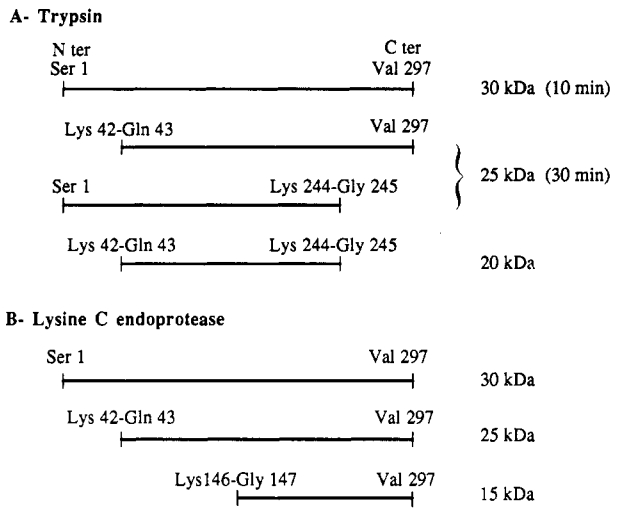

FIGURE 6: Alignment of the fragments resulting from the proteolysis of membrane-bound ADP/ATP carrier in SMP. The ADP/ATP carrier embedded in the membrane of SMP pretreated with BA was cleaved with trypsin (A) or with lysine C endoprotease (B). Tryptic cleavage generated first two peptides of 25 kDa whose sequences extended from Gln-43 to Val-297 and from Ser-1 to Lys-244. Subsequent cleavage of these peptides resulted in the accumulation of a 20-kDa fragment spanning Gln^{43}–Lys^{244}. Proteolysis of the ADP/ATP carrier with endoprotease Lys-C gave rise to a 25-kDa fragment comprising the sequence Gln^{43}-Val^{297} and to a 15-kDa peptide starting at Gly-147 and ending at Val-297.

reactivity of the 15-kDa fragment was much less intense than that of the 25-kDa fragment, possibly due to a restricted accessibility of the epitope. The significance of the cleavage resulting in the accumulation of the 15-kDa fragment was ascertained by the fact that this cleavage occurs only with SMP–BA. The 25-kDa fragment generated by tryptic cleavage of the carrier in SMP–BA (cf. Figure 3) was also immunodetected by the C-ter peptide antiserum (data not shown). Since the N-ter antibodies and the C-ter antibodies were able to react with a 25-kDa fragment arising by tryptic cleavage, it is inferred that two peptides of similar size, one of which contains the N-ter sequence and the other the C-ter sequence, are generated by trypsin cleavage of the membrane-embedded carrier in SMP–BA.

To localize the sites of enzymatic cleavages, the cleavage products, together with the uncleaved carrier protein, were separated from other mitochondrial proteins by HTP chromatography and analyzed for N-terminal amino acid sequences. In the case of trypsin cleavage, the following sequences, NH_2-Gln-Ile-Ser-Ala and NH_2-Gly-Ala-Asp-Ile, were determined, which, by comparison with the overall sequence of the ADP/ATP carrier, allowed the localization of the two cleavage sites at positions Lys^{42}-Gln^{43} and Lys^{244}-Gly^{245} in the membrane-bound carrier (Figure 6A). The N-terminal serine residue of the ADP/ATP carrier protein is acetylated (Aquila et al., 1982), and the N-terminal region of the carrier could, therefore, not be sequenced. Consequently, the two 25-kDa fragments generated by trypsin (cf. above) were identified as follows. The fragment containing the N-ter residue of the carrier (acetylated serine) was terminated by Lys-244 at its carboxylic end, and that containing the C-ter residue of the carrier was terminated by Gln-43 at its amino end. An additional tryptic cleavage yielded the 20-kDa peptide Gln^{43}-Lys^{244}. The identity of the minor peptide that appeared, secondary to the main peptide in the doublet corresponding to a mass of 20 kDa (cf. Figure 2), could not be ascertained because of the limited amount of this product. Amino acid sequence analysis was also performed with the 25- and 15-kDa fragments generated by lysine C endoprotease (Figure 6B). The N-terminal sequence of the 15-kDa fragment, Gly-Ala-Ala-Gln-Arg, indicated that the site of cleavage was located between Lys-146 and Gly-147. The N-terminal sequence of the 25-kDa protein was identical to that obtained by trypsin cleavage, indicating that cleavage by lysine C endoprotease occurred between Lys-42 and Gln-43.

Effect of Trypsin Cleavage on Binding of [^{3}H]BA to SMP. As BA binds to the inner face of the ADP/ATP carrier, SMP were utilized to study BA binding and release under conditions of limited proteolysis by trypsin. A first experiment was devised to study the effect of trypsin on the release of membrane-bound [^{3}H]BA. SMP were incubated with [^{3}H]BA at increasing concentrations up to 20 μM. The pellet recovered by centrifugation was rinsed. The particles were resuspended and then subjected to trypsin treatment, using a trypsin to particle protein ratio of 1/200, at 10 °C for periods of time ranging from 5 min to 2 h, and the remaining bound radioactivity was determined. The remaining bound radioactivity was similar to that found in the nontrypsinized particles (data not shown).

In the second experiment, SMP were subjected to trypsin proteolysis as described above and then supplemented with [^{3}H]BA used at increasing concentrations up to 20 μM. Similar [^{3}H]BA binding curves were obtained for trypsin-treated SMP and control SMP, suggesting that binding of [^{3}H]BA to the internal face of the membrane-bound ADP/ATP carrier does not require an intact polypeptide chain.

Discussion

The approach used in the present study to explore the topography of the beef heart ADP/ATP carrier peptide chain combined the proteolytic attack of the membrane-bound carrier chain and the microsequencing of the resulting fragments. A similar approach has recently been reported for different membrane-bound proteins, including the erythrocyte glucose carrier (Cairns et al., 1987), the Na^+,K^+–ATPase (Ovchinnikov et al., 1987), and bacteriorhodopsin (Fimmel et al., 1989). As an additional approach, we used immunodetection with a set of antibodies directed against 3 different synthetic peptides: (1) a 10 amino acid peptide corresponding to the N-terminal region of the carrier (Brandolin et al., 1989): (2) a 10 amino acid peptide spanning residues 40–50 in the carrier chain; and (3) a 13 amino acid peptide corresponding to the C-terminus of the carrier.

Methodological Aspects Concerning the Use of Mitoplasts (Right-Side-Out Particles) and Sonic Particles (Inside-Out Particles) To Assess the Cytosolic or Matrix Exposure of the Extramembrane Segments of the ADP/ATP Carrier Chain. The ratio of protease to protein particles should be low enough (<1/100) to avoid unwanted effects of trace amounts of contaminating enzymes, such as phospholipases which may attack the phospholipid bilayer, and therefore make the carrier protein freely accessible to protease. The conclusions concerning the internal or external exposure of given segments of the carrier chain to one side of the other or the mitochondrial membrane were ascertained by comparing the cleavage efficiency of different specific proteases. Two types of mitochondrial particles were used, namely, mitoplasts, i.e., mitochondria in which the outer membrane was intentionally damaged by freeze–thawing, and inside-out submitochondrial particles obtained by sonication of freeze–thawed mitochondria. Whereas virtually all of the freeze–thawed mitochondria have their inner membrane in the right-side-out conformation, the percentage of SMP with the inside-out conformation could be approximated to 75–80%, the remaining particles having their membrane in the same orientation as

Table I: Summary of Topographical Data Concerning the Reactivity of the Membrane-Bound ADP/ATP Carrier Protein to Photolabels, Chemical Reagents, Antibodies, and Proteolytic Enzymes

treatment	cytosolic exposure	matrix exposure	reference
[^{3}H]NAP$_4$-ATR labeling	Cys^{159}–Met^{200}		Boulay et al. (1983)
[^{3}H]NEM binding		Cys-56	Boulay & Vignais (1984)
2-azido-ADP binding	Phe^{153}–Met^{200} and Tyr^{250}–Met^{281}		Dalbon et al. (1988)
binding of anti-N-terpeptide antibodies to mitochondria (+CATR)	N-terminal region		Brandolin et al. (1989)
SMP treated by arginine endoprotease		Arg-30 and/or Arg-59	Brandolin et al. (1989)
SMP (+BA) treated by trypsin and lysine C endoprotease		Lys^{42}–Gln,^{43} Lys^{146}–Gly,^{147} Lys^{244}–Gly^{245}	this paper

in mitoplasts (right-side-out) (Lauquin et al., 1977). Consequently, when mitoplasts are treated with proteases, immunodetection of cleavage products of the peptide chain may be interpreted unambiguously as the result of a direct access of exposed segment(s) of the peptide chain to proteases. On the other hand, the enzymatic cleavage of the carrier protein in SMP should be considered as meaningful only when mitoplasts are not responsive; this was indeed the case in the present work.

The effect of proteases was routinely tested with mitoplasts treated by CATR or with SMP treated by BA. This experimental procedure relies on the previous finding that CATR and BA bind to regions of the ADP/ATP carrier exposed to the outer face and the inner face of the mitochondrial membrane, respectively (Vignais et al., 1985). It also relies on the fact that binding of antibodies raised against the N-terminal portion of the carrier protein in mitoplasts is considerably amplified when the mitoplasts are pretreated with CATR (Brandolin et al., 1989). On the basis of these considerations, the work reported here will be discussed in light of results derived from previous work (Dalbon et al., 1988; Brandolin et al., 1989).

Molecular Basis for a Model of the Arrangement of the Peptide Chain of the ADP/ATP Carrier in the Mitochondrial Membrane. The results presented in this paper and previous results as summarized in Table I can be interpreted as follows:

(1) Only in SMP pretreated by BA, but not in mitoplasts, is the ADP/ATP carrier chain susceptible to proteolytic attack. Cleavage of the Lys^{42}-Glu^{43} bond and the Lys^{244}-Gly^{245} bond by trypsin and lysine C endoprotease occurred at relatively low enzyme concentrations and after short periods of incubation. Additional cleavage between Lys^{146}-Gly^{147} required a longer period of incubation and may be a subsequent event to the first two cleavages. In fact, one can imagine that cleavage at Lys^{42}-Gly^{43} and at Lys^{244}-Gly^{245} might loosen some constraints in the central region of the carrier protein. It should be stressed that the cleavage at Lys^{146}-Gly^{147} is obtained only with SMP–BA, which emphasizes the significance of this cleavage. One can therefore conclude that the three sites of cleavage of the membrane-embedded carrier are exposed to the matrix compartment of mitochondria when the ADP/ATP carrier is in the BA conformation. This property inherent to the BA conformation adds to the list of properties recognized by the immunological and chemical approches that characterize the two conformations assumed by the ADP/ATP carrier, namely, the CATR conformation and the BA conformation (Buchanan et al., 1976; Block et al., 1983; Brandolin et al., 1985, 1989).

The matrix exposure of residues Lys^{42}-Glu^{43}, close to Cys-56, is consistent with the matrix localization of Cys-56 determined by reactivity with the permeant SH reagent *N*-ethylmaleimide (Boulay & Vignais, 1984) and with the cleavage of the carrier at Arg-30 or Arg-59 in SMP treated by arginine C-endoprotease (Brandolin et al., 1989). The equidistance between the three peptide bonds, namely, Lys^{42}-Gln^{43}, Lys^{146}-Gln^{147}, and Lys^{244}-Gly^{245}, susceptible to proteolysis, would be in accordance with the presence of three repeats in the peptide chain of the ADP/ATP carrier (Runswick et al., 1987).

(2) On the basis of photolabeling of discrete sequences of the membrane-embedded ADP/ATP carrier in mitochondria with 2-azido-ADP and azidoatractyloside, two nonpermeant compounds which in principle react with amino acid residues accessible from the cytosol, it has been concluded that the segments spanning Phe^{153}-Met^{200} and Tyr^{250}-Met^{281} are accessible from the outside. It is interesting to note that the segments of the peptide chain of the carrier that have been labeled by 2-azido-ADP and by azidoatractyloside are adjacent to two of the conformation-sensitive proteolytic sites of the carrier. This strongly suggests the appartenance of these regions to the translocation path.

(3) Since the membrane-bound ADP/ATP carrier in mitoplasts, but not in SMP, is able to react with antibodies directed against an 11 amino acid segment of the ADP/ATP carrier at the N-terminus, it was concluded that the N-terminal portion of the carrier chain protrudes into cytosol, the exposure to the cytosol being accentuated when the carrier is in the CATR conformation (Brandolin et al., 1989).

(4) In ELISA, we found that an antiserum directed against a 13 amino acid peptide, corresponding to the carboxylic end of the ADP/ATP carrier protein (cf. Experimental Procedures), was reactive against SMP and mitochondria coated onto microtiter plates. However, the back-ELISA titration (see Experimental Procedures) indicated higher immunoreactivity of SMP compared to freeze–thawed mitochondria. These results could not lead to an unequivocal conclusion, contrary to those obtained with the anti-N-ter peptide antiserum (Brandolin et al., 1989). A tentative explanation was that the C-ter region is located within the bilayer and is accessible from both faces, depending on experimental conditions.

On the basis of the above data and the theoretical structural predictions, tentative models of arrangement of the peptide chain of the carrier can be proposed (Figure 7A,B). A first model, with 6 transmembrane α helices, would fit with the presence of 3 repeats of about 100 amino acid residues each (Runswick et al., 1987), and with the presence of 3 equidistant sites of enzymatic cleavage, located at Lys^{42}-Glu^{43}, Lys^{146}-Gly^{147}, and Lys^{244}-Gly^{245}, oriented toward the matrix space of mitochondria. Six transmembrane α helices would be consistent with the exposure of the N-ter region of the carrier to the cytosol (Brandolin et al., 1989), with the exposure of Cys-56 to the matrix space (Boulay & Vignais, 1984), and with the cytosolic exposure of the C-ter region of the ADP/ATP carrier. A similar arrangement with six transmembrane α helices was reported for the phosphate carrier (Capobianco et al., 1991) and the uncoupling protein (Eckerskorn & Klingenberg, 1987), which would be consistent with the fact that the three proteins possess structural homology (Runswick et al., 1987). A second model of the arrangement of the peptide chain of the carrier with only five transmembrane segments has been proposed (Dalbon et al., 1988), taking into

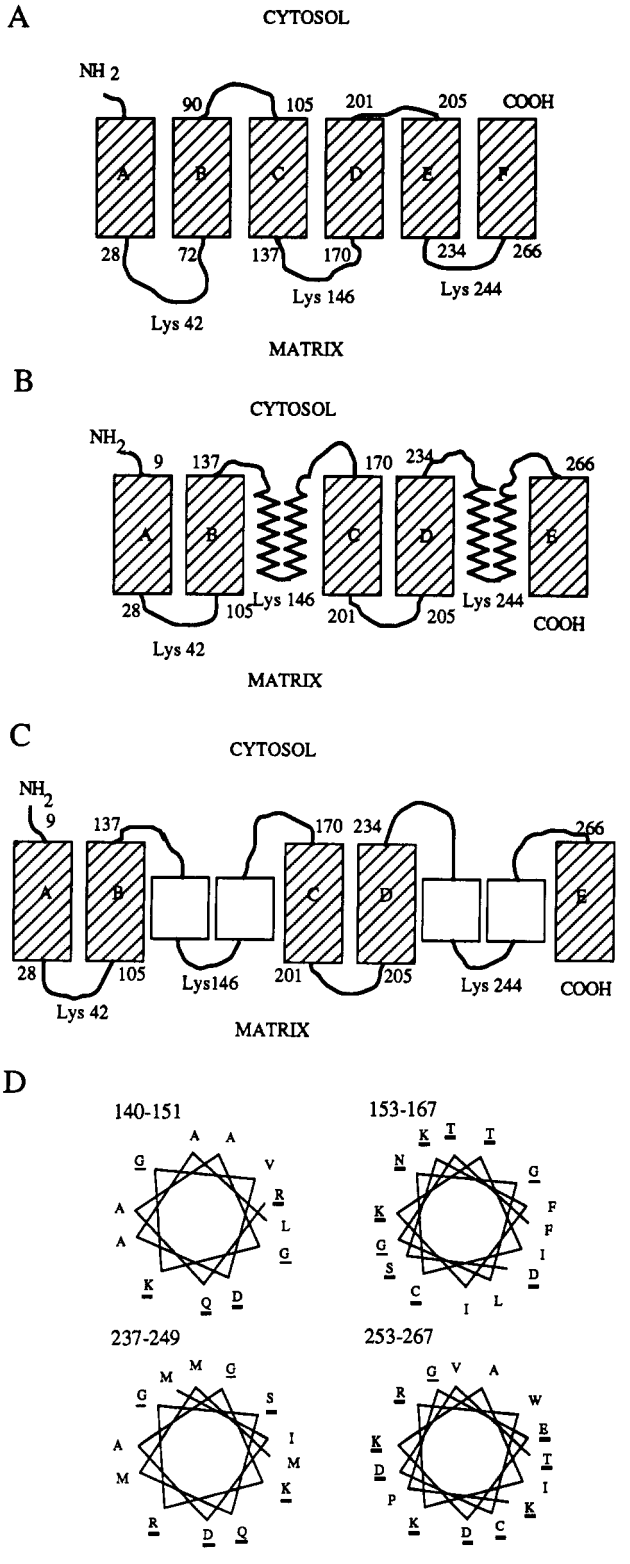

FIGURE 7: Scheme illustrating the postulated arrangement of amphipathic helices corresponding to the following sequences: residues 140–151, 153–167, 237–249, and 253–267. The scheme shows the insertion of the above sequences in the phospholipid bilayer and their possible arrangement shown in front view (A, B, and C). Scheme A illustrates a classical model of the ADP/ATP carrier with six transmembrane α helices. In schemes B and C is shown a model of carrier with five transmembrane α helices, together with the relationship between the four amphipathic segments 140–151, 153–167, 237–249, and 253–267 and the proteolytic attack of the carrier in the BA conformation. The arrangement of the four amphipathic segments either in β sheets or in short α helices is illustrated in schemes B and C, respectively. Scheme D is an Edmunson-wheel diagram of the short α helices illustrated in scheme C, with heavy dashes underlining the typical hydrophilic residues located in the helices.

account the fact that the rather weakly hydrophobic character of the region extending from residues 70–100 in the sequence (containing almost 40% polar amino acids) is not typical of a transmembrane segment. In the case of a model with five transmembrane α helices, the C-terminal extremity of the chain would be oriented toward the matrix compartment. However, as discussed above, no definite conclusion can be drawn concerning the orientation of the C-ter region of the ADP/ATP carrier, and therefore a choice between the two models cannot be made.

At first sight, neither of the two postulated models, with six helices and five helices, appears to accommodate the mapping data obtained through the use of photoreactive specific reagents and limited proteolysis of the carrier. Therefore, we are faced with the following dilemma. The results of photolabeling of the membrane-embedded carrier with the azido derivatives of ADP and ATP suggest a cytosolic orientation for the two peptide segments containing the bonds Lys^{146}-Gly^{147} and Lys^{244}-Gly^{245}, whereas the enzymatic approach by proteolytic attack indicates a matrix orientation of these two segments when the carrier adopts the BA conformation. A solution to this dilemma resides in the recognition that both the segments of the peptide chain characterized by the conformation-sensitive cleavable bonds and the segments photolabeled with 2-azido-ADP and azidoatractyloside typically contain amphipathic segments extending from amino acid residues 140–151 and 153–167, on the one hand, and from 237–249 and 253–267, on the other. These segments are too short to cross the membrane as classical α helices of 50-Å length, yet they can do so in the form of β sheets or in the form of α helices shorter than the thickness of the bilayer (Lodish, 1988). The Edmunson-wheel representation of the four amphipathic segments organized in short α helices clearly shows clustering of polar amino acid residues on one face of the helices (Figure 7D). One may therefore imagine that the amphipathic helices might be packed in order to allow their polar faces to be shielded from the hydrophobic core of the membrane, and to face each other to delineate a hydrophilic channel. A similar arrangement of amphipathic helices was recently suggested by Jähnig (1989) for two membrane proteins: the lactose permease of the *Escherichia coli* inner membrane and the Omp A protein of the *E. coli* outer membrane. However, unlike these two proteins, the ADP/ATP carrier exhibits specific dynamics that reside in the reversible transition between two conformations trapped by BA and CATR, which is probably inherent in the functioning of ADP/ATP transport. It is assumed, in fact, that the amphipathic helices undergo conformational changes that would explain the conformation-sensitive accessibility of bonds Lys^{146}-Gly^{147} and Lys^{244}-Gly^{245}. On the other hand, the presence of a hydrophilic channel would allow the specific labeling of polar regions, accessible from the cytosol, with photolabeling reagents derived from ADP and atractyloside (Dalbon et al., 1988).

As the isolated ADP/ATP carrier complexed to CATR or to BA is dimeric (Hackenberg & Klingenberg, 1980; Block et al., 1982) and probably tetrameric in its functional form in the mitochondrial membrane (Block & Vignais, 1984; Vignais et al., 1989), the possibility should be considered that a hydrophilic space in the bilayer is made by the juxtaposition of two or four monomers, the amphipathic segments being organized in short α helices (or in antiparallel β sheets) (Figure 8). Such a model of the carrier would obviously result in a 2-fold symmetry, whatever the arrangement of the peptide chain in the carrier monomer.

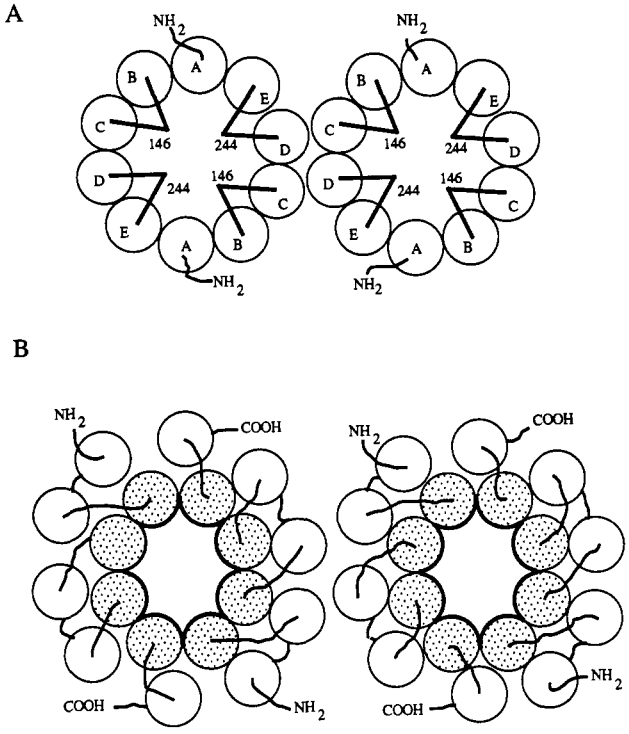

FIGURE 8: Postulated tetrameric organization of the ADP/ATP carrier protein (top view). The figure is a representation of the ADP/ATP carrier protein with five hydrophobic pillars and four amphipathic segments (140–151, 153–167, 237–249, 252–267) per monomer. Because of the uncertainty concerning the sidedness of the C-terminal end of the peptide chain of the carrier (see Discussion), a similar arrangement of the hydrophobic pillars could be imagined with six transmembrane pillars and a 3-fold symmetry. In the top view representation illustrated in the figure, A and B refer to the arrangement of the amphipathic segments as β sheets (heavy lines) and short α helices (shaded circles), respectively. The numbers indicated in the figure correspond to the lysine residues at the trypsin and lysine C endoprotease cleavage sites. Hydrophilic regions corresponding to the short amphipathic α helices in (B) are highlighted.

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