Orci, L., Rufener, C., Malaisse-Lagae, F., Blondel, B., Amherdt, M., Bataille, D., Freychet, P., & Perrelet, A. (1975) *Isr. J. Med. Sci. 11*, 639–655.

Petruzzelli, L., Herrera, R., & Rosen, O. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327–3331.

Russell, D. S., Gherzi, R., Johnson, E. L., Chou, C.-K., & Rosen, O. M. (1987) *J. Biol. Chem. 262*, 11833–11840.

Schlessinger, J., Schechter, Y., Willingham, M. C., & Pastan, I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2659–2663.

Schlessinger, J., Van Obberghen, E., & Kahn, C. R. (1980) *Nature (London) 286*, 729–731.

Shia, M. A., Rubin, J. B., & Pilch, P. F. (1983) *J. Biol. Chem. 258*, 14450–14455.

Sweet, L. J., Wilden, P. A., Spector, A. A., & Pessin, J. E. (1985) *Biochemistry 24*, 6571–6580.

Sweet, L. J., Morrison, B. D., & Pessin, J. E. (1987a) *J. Biol. Chem. 262*, 6939–6942.

Sweet, L. J., Morrison, B. D., Wilden, P. A., & Pessin, J. E. (1987b) *J. Biol. Chem. 262*, 16730–16738.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liai, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.

Van Obberghen, E., & Kowalski, A. (1982) *FEBS Lett. 143*, 179–182.

Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 945–949.

Yarden, Y., & Schlessinger, J. (1987) *Biochemistry 26*, 1443–1451.

Yu, K. T., Pessin, J. E., & Czech, M. P. (1985) *Biochimie 67*, 1081–1094.

Orientation of the N-Terminal Region of the Membrane-Bound ADP/ATP Carrier Protein Explored by Antipeptide Antibodies and an Arginine-Specific Endoprotease. Evidence That the Accessibility of the N-Terminal Residues Depends on the Conformational State of the Carrier†

Gérard Brandolin,* François Boulay, Pascal Dalbon, and Pierre V. Vignais

Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, France

Received June 7, 1988; Revised Manuscript Received August 23, 1988

ABSTRACT: Two peptides corresponding to the amino acid sequences 1–11 (N-terminal peptide) and 288–297 (C-terminal peptide) of beef heart ADP/ATP carrier have been synthesized. After coupling to ovalbumin, they were injected into rabbits to raise polyclonal antibodies. The specificities of the generated antibodies were tested by enzyme-linked immunosorbent assay (ELISA) and (or) Western blot. Anti-N-terminal antibodies and anti-C-terminal antibodies reacted specifically with the corresponding peptide. However, only anti-N-terminal antibodies reacted with the isolated ADP/ATP carrier; they also reacted with the membrane-bound carrier in freeze–thawed mitochondria and mitoplasts, indicating that the first 10 amino acid residues of the membrane-bound carrier in mitochondria face the cytosol. On the basis that the ADP/ATP carrier can adopt two conformations, one trapped by carboxyatractyloside (CATR conformation) and the other by bongkrekic acid (BA conformation), the reactivity of the anti-N-terminal antibodies to the ADP/ATP carrier in mitoplasts or freeze–thawed mitochondria was tested for each conformation of the carrier. Only in the CATR conformation was the N-terminal region of the membrane-bound carrier reactive to the N-terminal antibodies; the contrasting weak reactivity of the carrier in the BA conformation suggested that the transition from the CATR conformation to the BA conformation results in a restricted conformation of the peptide chain corresponding to the first 10 amino acid residues or its partial burying in the lipid bilayer. These immunological data were complemented by enzymatic data pertaining to proteolysis of the membrane-bound ADP/ATP carrier by an arginine-specific endoprotease. Enzymatic cleavage of the carrier occurred in inside-out submitochondrial particles, but not in right-side-out particles, yielding a large fragment of $M_r \simeq 25\,000$ that was immunodetected on Western blot by anticarrier antibodies but not by anti-N-terminal antibodies. These results demonstrated that the arginine-specific endoprotease had access to the matrix face of the inner mitochondrial membrane, at Arg 30 or at Arg 59. Thus, it appears that in intact mitochondria the sequence of the ADP/ATP carrier including Arg 30 or Arg 59 protrudes into the matrix space, whereas the N-terminal segment corresponding to the first 10 amino acid residues protrudes into cytosol, and the intermediate, rather hydrophobic, sequence spanning residues 9–28 transverses the lipid bilayer of the inner mitochondrial membrane.

Much interest is currently devoted to the analysis of the spatial organization of membrane-embedded proteins by chemical modification, proteolysis, and the use of site-specific immunological probes [for review, see Ovchinnikov (1987)]. These approaches are presently applied in our laboratory to the topographical study of the ADP/ATP carrier, an intrinsic protein of the inner mitochondrial membrane that catalyzes the import of cytosolic ADP into the matrix space of mito-

† This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS/UA No. 1130 alliée à l'INSERM) and the Faculté de Médecine, Université Joseph Fourier de Grenoble.

0006-2960/89/0428-1093$01.50/0

chondria and the concomitant export of mitochondrial ATP toward cytosol [for review, see Vignais et al. (1985)]. The beef heart ADP/ATP carrier is ideally suited since a wealth of structural data is presently available. The protein, which is 297 amino acids long, has been sequenced (Aquila et al., 1982). The isolated protein, solubilized in Triton X-100 and complexed to the specific inhibitory ligands (CATR and BA),[1] behaves as a dimer, as shown by ultracentrifugation (Hackenberg & Klingenberg, 1980) and neutron diffraction studies (Block et al., 1982). The existence of two different conformations of the carrier probed by CATR (or ATR) and BA has been demonstrated, and there is strong evidence that during the course of transport a transition between the two conformations occurs (Block et al., 1983; Brandolin et al., 1985). A model for the transmembrane carrier topography, based on a hydropathy plot, has been proposed (Saraste & Walker, 1982; Runswick et al., 1987). This model does not, however, predict which regions of the carrier are exposed to the cytosol or the matrix space.

Photolabeling of the membrane-bound carrier with nonpermeant derivatives of both ATR (Boulay et al., 1983) and ADP (Dalbon et al., 1988) has allowed the identification of a common binding center for ATR (or CATR) and ADP. The sequence Phe 153–Met 200 belongs to this center and is accessible from cytosol. An additional site for ADP binding has been revealed by photolabeling, extending from Tyr 250 to Met 281. However, the photolabeling method is restricted to a limited number of photoprobes derived from substrates and inhibitory ligands. Chemical modification with the alkylating permeant reagent NEM has shown that NEM binds to Cys 56 of the ADP/ATP carrier in the BA conformation (Boulay & Vignais, 1984). Modification of lysine residues by pyridoxal phosphate in mitochondria and inside-out submitochondrial particles has also been used to probe the orientation of the carrier chain (Bogner et al., 1986). There are, however, drawbacks inherent to chemical modifications, including the absence of strict specificity and often uncontrolled permeability.

Two other approaches, namely, interaction with antibodies directed against specific peptide sequences and cleavage of exposed peptide bonds by specific proteases, do not suffer from these drawbacks. In the present work, we have used antibodies directed against synthetic peptides corresponding to the N- and C-terminal sequences of the beef heart mitochondrial carrier, and digestion of the membrane-embedded carrier by an endoprotease specific for arginine residues. The results provide new insight into the transmembrane arrangement of the N-terminal portion of the membrane-bound ADP/ATP carrier and the dependence of this arrangement on the conformational state of the carrier.

Experimental Procedures

Materials. Boc amino acid derivatives were obtained from Peninsula Laboratories and Fluka, and 1% cross-linked chloromethylated styrene–divinylbenzene (Merrifield polymer) was from Fluka. The sources of other chemicals were as follows: Sepharose 4B and Sephadex G-25, Pharmacia; acrylamide and *N,N'*-methylene bisacrylamide, BDH; ovalbumin, 3,3',5,5'-tetramethylbenzidine, and peroxidase-conjugated anti-rabbit IgG, Miles Scientific; peroxidase-conjugated protein A, NEN; ^{125}I-labeled protein A, Amersham; Arg-C endoprotease and carboxyatractyloside, Boehringer. Bongkrekic acid was isolated as described (Lauquin & Vignais, 1976).

Biological Preparations. Beef heart mitochondria were prepared according to the method of Smith (1967). For antibodies assays, we used either freshly prepared beef heart mitoplasts or mitochondria stored in liquid nitrogen and thawed just before use. Intact mitoplasts, corresponding to mitochondria from which the outer membrane and the intermembrane space components were removed, were prepared as described by Burnette and Batra (1985). In brief, freshly prepared beef heart mitochondria were suspended in an hypotonic medium consisting of 60 mM sucrose and 0.1 M EDTA, pH 7.2. Digitonin (5 mg/mL) was then added to a final ratio of 0.8–1 mg digitonin/mg of protein and incubation was allowed to proceed for 15 min at room temperature. The integrity of the resulting mitoplasts was assessed by measurement by activity of the mitochondrial malate dehydrogenase, an enzyme specific for the matrix space. In addition to mitoplasts, freeze–thawed mitochondria were used. Freeze–thawed mitochondria have a damaged outer membrane but an intact inner membrane. They proved to be as useful as mitoplasts for testing the accessibility of antibodies to the inner membrane. Inside-out submitochondrial particles were obtained by differential centrifugation after ultrasonic irradiation of beef heart mitochondria (Beyer, 1967; Lauquin et al., 1977a).

The ADP/ATP carrier protein from beef heart mitochondria was purified by chromatography on hydroxylapatite gel in Triton X-100 (Riccio et al., 1975). Subsequent chemical modifications and acidolytic cleavage have been described (Dalbon et al., 1988). Overnight electrophoretic separation of peptides was performed in 20% acrylamide slab gels (Cabral & Schatz, 1979). The protein concentration was determined by the method of Zak and Cohen (1961) with addition of sodium dodecyl sulfate (Dulley & Grieve, 1975; Chin-Sun & Smith, 1975).

Proteolytic Digestion. Freshly prepared mitoplasts from beef heart mitochondria and inside-out submitochondrial particles in 200 μL of 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA, and 5 mM Tris, pH 7.4, were treated with an arginine-specific endoprotease for 1 h at 37 °C. The reaction was stopped by addition of 20 μL of 20% (w/v) NaDodSO$_4$, 50% (v/v) glycerol, 25% β-mercaptoethanol, and traces of bromophenol blue and heating for 2 min at 100 °C. Aliquots of 15 μL corresponding to about 30 μg of protein were loaded on slab SPAGE for further characterization of the peptide fragments.

Synthesis of Peptides. The N-terminal sequence of the beef heart ADP/ATP carrier corresponding to residues 1–11 (Ac-Ser-Asp-Gln-Ala-Leu-Ser-Phe-Leu-Lys-Asp-Phe) and the C-terminal sequence corresponding to residues 288–297 (Val-Leu-Tyr-Asp-Glu-Ile-Lys-Lys-Phe-Val) 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 phenylalanine at the C-terminal end of the N-terminal peptide to facilitate coupling to the ovalbumin carrier. The C-terminal peptide, spanning amino acid residues 288–297, was synthesized on a benzydrylamine resin; it therefore contained an amide group on the C-terminal residue.

[1] Abbreviations: PBS, phosphate buffer saline consisting of 0.14 M NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, 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; TEA, triethanolamine; TFA, trifluoroacetic acid; Arg-endoprotease, arginine-specific endoprotease; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; CATR, carboxyatractyloside; BA, bongkrekic acid.

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 N-terminal peptide was finally acetylated by treatment of the resin with a mixture of 5 mmol of acetic anhydride and 5 mmol of TEA in 15 mL of dimethylformamide for 30 min at room temperature. 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 10% anisole (v/v) and 5% (v/v) ethanedithiol as scavengers (Stewart & Young, 1984). The released peptide was desalted over Sephadex G-25 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 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 1328 and 1252 for the N- and C-terminal peptides, respectively.

Coupling of Peptide to Ovalbumin and Generation of Rabbit Anti-Peptide Antibodies. The N-terminal peptide (residues 1–11) and C-terminal peptide (residues 288–297) were coupled with bisdiazotized benzidine (BDB) to ovalbumin through the hydroxyl group of Tyr 11 (replacing Phe 11) and Tyr 290, respectively. The coupling reaction was performed as described by Tamura and Bauer (1982). To allow specific coupling at the tyrosine residues, the two synthetic peptides were citraconylated at lysine residues (Boulay et al., 1983). Routinely, 0.1 μmol of ovalbumin was reacted with 3 μmol of N- or C-terminal peptides. The ovalbumin–peptide conjugate was first dialyzed for 2 h against 5% acetic acid for deprotection of the NH_2 group of lysine, and a further dialysis step against PBS was performed to remove excess BDB and free peptide. The binding stoichiometry of peptide with respect to ovalbumin (mol/mol) was between 15 and 20. 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.

Antibodies directed to the N-terminal peptide were purified by affinity chromatography according to the following procedure. First, the ADP/ATP carrier protein (20 mg) was coupled to CNBr-activated Sepharose 4B by using the method described by Axen et al. (1967). The carrier-coupled Sepharose column (1.5 × 10 cm) was equilibrated with PBS. Antiserum against the N-terminal peptide was layered onto the top of the column. After an overnight recycling perfusion at 4 °C, the N-terminal peptide antibodies were eluted with a medium consisting of 3 M KSCN and 0.5 M NH_4OH; the pH of the eluted fractions was immediately adjusted to 7.4. The recovered antibodies were extensively dialyzed against PBS and stored at −20 °C.

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 carrier protein in a carbonate buffer was added to each well of the plate. The carrier protein recovered from hydroxylapatite chromatography in 0.1% Triton X-100 was used, without further purification, after dilution in 0.05 M $NaHCO_3/Na_2CO_3$, pH 9.6, to the desired concentration. 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 unspecific sites. After washing 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 peroxidase-conjugated anti-rabbit IgG diluted 2000-fold with PBS-T was added to each well. After another 2-h incubation at room temperature followed by washing, 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).

To titrate antibodies against the membrane-bound carrier, the wells of the microtiter plates were coated overnight, at 4 °C, with freeze–thawed mitochondria or with mitoplasts, preincubated with CATR or BA when indicated, and then diluted at the appropriate concentration with a medium consisting of 0.12 M KCl, 10 mM MOPS, and 1 mM EDTA, final pH 6.7. The next steps were as described above, except that a protein A–peroxidase conjugate was substituted for the peroxidase-conjugated anti-rabbit IgG.

Back-titration of antibodies by ELISA was used in particular to analyze the effect of the CATR or BA conformation of the membrane-bound carrier on accessibility of the N-terminal segment. Freeze–thawed mitochondria were diluted at appropriate concentrations in a medium consisting of 0.12 M KCl, 10 mM MOPS, and 1 mM EDTA, final pH 6.7. After incubation with saturating concentrations of BA or CATR for 1 h at 4 °C in the presence of ADP, antiserum was added and incubation was pursued for another 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 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 ADP/ATP carrier protein and the derived fragments. The electrotransfers on nitrocellulose were performed as previously described (Boulay et al., 1986) except that PBS was replaced by PBS supplemented with 0.05% (w/v) Tween 20.

Results

Characterization of Peptide Antibodies by ELISA. The presence of peptide-specific antibodies in the serum of rabbits immunized against the ovalbumin-conjugated N- or C-terminal peptide was assayed by ELISA, using microtiter plates with the peptides coated on the wells. The data presented in Figure 1 illustrate the potent reactivity of the antisera against the respective peptides. No reaction was found with the preimmune sera. To ascertain the specificity of the antibodies, controls were performed, in which the anti-C-terminal and anti-N-terminal antisera were assayed against the coated N-terminal peptide and C-terminal peptide, respectively. No cross-reaction was detected. Furthermore, no reaction of the

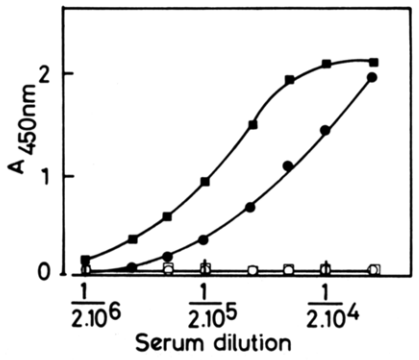

FIGURE 1: Reactivity of anti-N-terminal peptide antiserum and anti-C-terminal peptide antiserum to N-terminal and C-terminal peptides assessed by ELISA. Microtiter plates were coated with the N-terminal peptide or the C-terminal peptide at 200 ng/mL in PBS. The immobilized peptides were incubated with the corresponding antisera added at different dilutions. This was followed by peroxidase-conjugated rabbit antibody, hydrogen peroxide as substrate, and TMB as chromogenic indicator (cf. Experimental Procedures). The data in ordinate represent values of absorbance at 450 nm. (□) Preimmune serum; (■) anti-C-terminal peptide antiserum; (○) preimmune serum; (●) anti-N-terminal peptide antiserum.

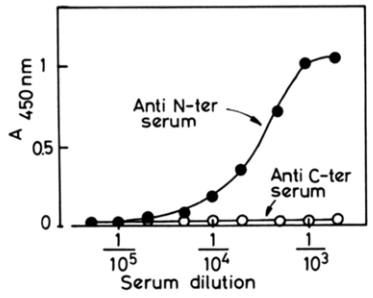

FIGURE 2: Reactivity of anti-N-terminal and anti-C-terminal peptide antisera to the CATR–carrier complex assessed by ELISA. Microtiter plates were coated with the purified CATR–carrier complex at 174 ng/mL. The immobilized CATR–carrier complex was then incubated with either the anti-N-terminal antiserum or the anti-C-terminal antiserum. Immunoreactivity of the antisera was revealed as in Figure 1.

C-terminal and N-terminal peptides was detected with the rabbit antiserum directed to ovalbumin.

Reactivity of Anti-N-Terminal and Anti-C-Terminal Peptide Antisera against the CATR–Carrier Complex. Anti-N-terminal and anti-C-terminal peptide antisera were assayed by ELISA against the purified CATR–carrier complex coated on the wells of the microtiter plates. As shown in Figure 2, the anti-N-terminal peptide antiserum, but not the anti-C-terminal peptide antiserum, reacted with the CATR–carrier complex. These results were corroborated by immunoblotting, using the membrane-bound ADP/ATP carrier in a $NaDodSO_4$ lysate of beef heart mitochondria (Figure 3); the anti-N-terminal peptide antiserum (lane 1), but not the anti-C-terminal peptide antiserum (lane 3), labeled a zone of $M_r \simeq 30\,000$ corresponding to the ADP/ATP carrier protein. Absence of immunological reactivity of the anti-C-terminal peptide antiserum might be due to the lack of accessibility of the C-terminal region, as discussed later (see Discussion). Mitochondrial proteins with a M_r higher than 30 000 reacted with anti-N-terminal and anti-C-terminal antisera. These proteins were not related to the ADP/ATP carrier, as they disappeared when antibodies were purified by affinity chromatography (cf. Experimental Procedures).

The identity of the N-terminal region of the carrier reacting with the anti-N-terminal peptide antiserum was ascertained by immunoblot analysis of peptide fragments generated by acidolytic cleavage of the purified ADP/ATP carrier protein.

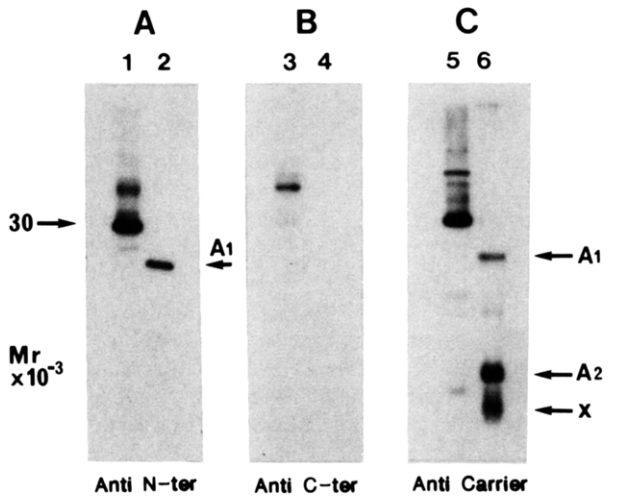

FIGURE 3: Binding of antibodies to the ADP/ATP carrier protein and its acidolytic fragments explored by Western blot analysis. Samples of lysed mitochondria (lanes 1, 3, 5) and purified ADP/ATP carrier protein after acidolytic cleavage (see Experimental Procedures) (lanes 2, 4, 6) were electrophoresed on a 20% polyacrylamide gel in the presence of 0.5% $NaDodSO_4$. After electroblotting, the nitrocellulose sheets were treated with the anti-N-terminal peptide antiserum (A), the anti-C-terminal peptide antiserum (B), and the anti-$NaDodSO_4$-treated carrier antiserum (C). After incubation with ^{125}I-labeled protein A, the immunoreactive proteins were detected by autoradiography as described under Experimental Procedures. The two acidolytic cleavage products are designated A_1 and A_2. The radioactive band (X) with an M_r of $\simeq 8000$, occasionally found, is probably a secondary cleavage product. Note that the antisera reacted not only with the carrier protein in the mitochondrial lysate but also with several other proteins of higher molecular weight. Immunological reaction with these contaminant proteins disappeared when anticarrier antibodies were prepared by affinity chromatography (cf. Results).

Acidolytic cleavage of the carrier protein takes place exclusively at the Asp 203–Pro 204 bond, thus generating an N-terminal fragment of M_r 20 000 (A1) and a C-terminal fragment of M_r 10 000 (A2) (Boulay et al., 1986). Only the A1 fragment reacted with the anti-N-terminal antiserum (Figure 3, lane 2). Yet, as shown in Figure 3, lane 6, both fragments A1 and A2 were immunodetected by anti-$NaDodSO_4$ carrier antiserum. The lack of reactive bands at the level of A1 or A2 to the anti-C-terminal antiserum (Figure 3, lane 4) is consistent with the lack of reactivity of the whole carrier to this antiserum.

Reactivity of the N-Terminal Region of the Membrane-Bound ADP/ATP Carrier Protein toward the Anti-N-Terminal Antiserum. Having shown that the anti-N-terminal antibodies reacted with the isolated carrier protein, we further examined whether they were able to react with the membrane-bound carrier.

Additional series of immunotitration, based on ELISA, were performed with coated mitoplasts or coated freeze–thawed mitochondria. Both types of particles yielded similar results, which is not surprising because in both cases accessibility of antibodies to the inner mitochondrial membrane was made possible by removal or damage of the outer membrane. As illustrated in Figure 4, the binding of anti-N-terminal antibodies to freeze–thawed mitochondria increased with the amount of particles coated on the microtiter plates to a plateau value. Furthermore, the N-terminal peptide added together with the antiserum inhibited efficiently the binding of anti-N-terminal antibodies. In contrast, no reaction was observed with the anti-C-terminal antiserum. These results indicate that the N-terminal region of the membrane-bound carrier is accessible to specific antibodies from the cytosolic face of the inner mitochondrial membrane. It could be argued that

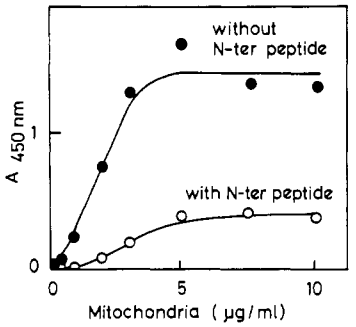

FIGURE 4: Reactivity of the anti-N-terminal peptide antiserum toward the membrane-bound ADP/ATP carrier in mitochondria assessed by ELISA. Microtiter plates were coated with increasing concentrations of freeze–thawed beef heart mitochondria. The immobilized mitochondria were incubated with the anti-N-terminal peptide antiserum diluted 1000-fold in the absence or in the presence of competing N-terminal peptide at 2.5 µg/mL. Binding of antibodies was detected by a chromogenic reaction (cf. Figure 1 and Experimental Procedures). Plotted data in ordinate represent values of absorbance at 450 nm.

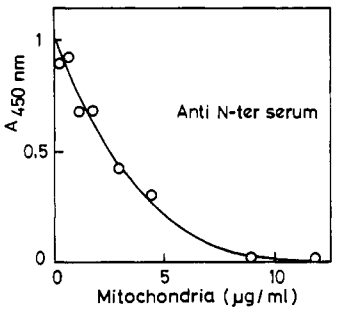

FIGURE 5: Back-titration by ELISA of anti-N-terminal antibodies after reaction with the membrane-bound ADP/ATP carrier in mitochondria. The anti-N-terminal antiserum diluted 1000-fold was incubated at 25 °C with increasing concentrations (0.2–10 µg/mL) of freeze–thawed beef heart mitochondria. After 2 h, the mitochondria were sedimented by centrifugation. Unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the N-terminal peptide at 100 ng/mL. After an incubation of 2 h at 25 °C, the bound antibodies were assayed by a chromogenic reaction (cf. Figure 1 and Experimental Procedures).

coating the freeze–thawed mitochondria or mitoplasts on the microtiter plates could disorganize the membrane structure, resulting in access of N-terminal peptide antibodies to initially nonexposed epitopes of the membrane-bound carrier. The following experiment was devised to test this possibility.

Various amounts of freshly prepared mitoplasts or freeze–thawed mitochondria were left in contact with a fixed concentration of anti-N-terminal antiserum. After centrifugation, the antibodies remaining in the supernatant were back-titrated by ELISA, using microtiter plates coated with N-terminal peptide. As shown in Figure 5, the binding of the anti-N-terminal antibodies to the coated N-terminal peptide markedly decreased with the amount of freeze–thawed mitochondria, full inhibition being obtained with less than 10 µg of mitochondrial protein. Conversely, with the same procedure, freeze–thawed mitochondria were unable to prevent the reaction of the anti-C-terminal peptide antiserum with the C-terminal peptide. Similar results were obtained with mitoplasts.

Complementary experiments with inside-out submitochondrial particles were inconclusive. This was probably due to contamination of the inverted particles by right-side-out particles in which the carrier had the same orientation as in mitochondria, as will be discussed later.

Effect of the CATR and BA Conformations on the Binding of Anti-N-Terminal Antibodies to the Membrane-Bound ADP/ATP Carrier. The ADP/ATP carrier can adopt two

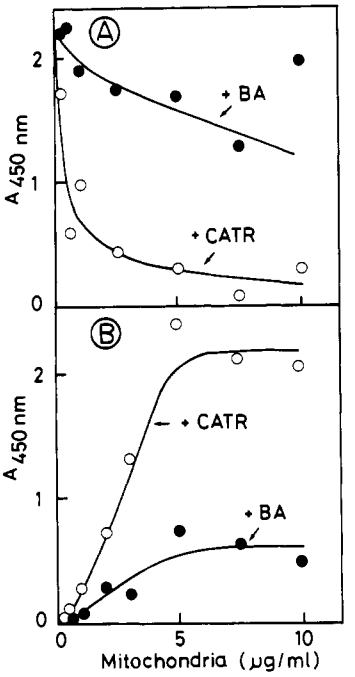

FIGURE 6: Reactivities of the CATR and BA conformations of the membrane-bound carrier to anti-N-terminal peptide antibodies. (A) Freeze–thawed mitochondria at increasing concentrations (0.2–10 µg/mL) were incubated with 15 µM CATR plus 2 µM ADP or with 20 µM BA plus 2 µM ADP in 0.12 M KCl, 1 mM EDTA, and 10 mM Mops, pH 6.6, for 1 h at 25 °C. This was followed by addition of the anti-N-terminal peptide antiserum diluted 1000-fold in PBS. After a further incubation of 2 h at 25 °C, the mitochondria were sedimented. Unreacted antibodies present in the supernatants were assayed by ELISA, using microtiter plates coated with the N-terminal peptide at 100 ng/mL. (B) In another experiment, the mitochondria treated by CATR or BA as described above were coated overnight onto microtiter plates. The immobilized mitochondria were incubated for 2 h at 25 °C with the anti-N-terminal peptide antiserum diluted 1000-fold. In both (A) and (B) bound antibodies were assayed by a chromogenic reaction (cf. Figure 1 and Experimental Procedures).

conformational states that are trapped by the inhibitors CATR and BA, respectively. CATR is a nonpermeant inhibitor that binds to a region of the carrier exposed to cytoplasm, whereas BA has to enter the inner mitochondrial membrane to bind the carrier [for review, cf. Vignais et al. (1985)]. The transition between the CATR conformation and the BA conformation is triggered by the specific substrates ADP and ATP and is thought to reflect topographical changes of the carrier protein occurring during transport. To examine which of the two conformations had the highest affinity for anti-N-terminal antibodies, freeze–thawed mitochondria were first incubated with either CATR or BA added at saturating concentrations to stabilize the carrier in the CATR or BA conformation and then allowed to react with anti-N-terminal antiserum. In a first experiment, the nonreacted antibodies were back-titrated by ELISA. As shown in Figure 6A, the reactivity of the N-terminal region of the membrane-bound carrier in mitochondria toward anti-N-terminal peptide antibodies was much higher when the carrier was in the CATR conformation than when it was in the BA conformation. This result was fully corroborated by direct ELISA titration in a separate experiment in which the reactivity of coated mitochondria having the ADP/ATP carrier in either the CATR conformation or the BA conformation was assayed with anti-N-terminal antibodies (Figure 6B).

Localization of an Arginine Endoprotease Cleavage Site in the Membrane-Embedded Carrier. For a further insight into the transmembrane arrangement of the carrier chain in

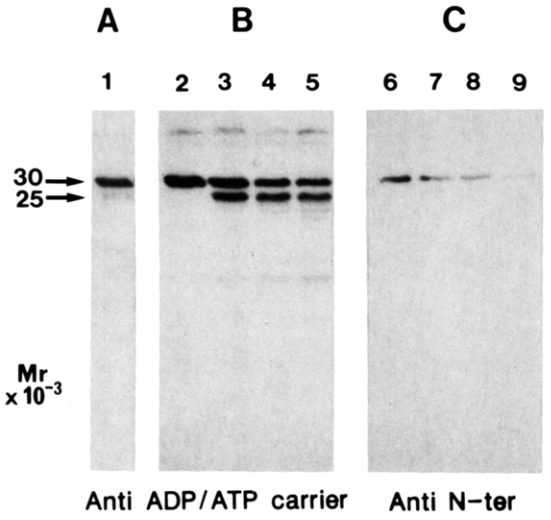

FIGURE 7: Reactivity of anti-carrier antibodies and anti-N-terminal peptide antibodies to the carrier protein and the Arg-endoprotease-derived fragments in mitoplasts and inside-out submitochondrial particles, explored by Western blot analysis: (A) Mitoplasts; (B and C) inside-out particles. Mitoplasts (30 μg) were incubated with 8 μg of Arg-endoprotease. Inside-out particles (33 μg) were treated with increasing concentrations of Arg-endoprotease at the following ratios of protease to particles (w/w): (Lanes 2 and 6) 0; (lanes 3 and 7) 1/10; (lanes 4 and 8) 1/5; (lanes 5 and 9) 1/4. After 1 h of incubation at 30 °C, the particles were lysed by 2% $NaDodSO_4$ and proteins were separated by polyacrylamide gel electrophoresis in the presence of 0.5% $NaDodSO_4$. This was followed by electroblotting and detection of the immunoreactive peptides on the nitrocellulose sheets by the anti-$NaDodSO_4$-treated carrier antiserum diluted 50-fold (A and B) and affinity-purified anti-N-terminal peptide antibodies diluted 50-fold (C), followed in both cases by reaction with ^{125}I-labeled protein A and exposure to X-ray film. An autoradiogram of each gel is presented. The 25 000 M_r protein corresponds to the immunoreactive large fragment released from the carrier protein after cleavage by the Arg-endoprotease.

the inner mitochondrial membrane, enzymatic digestion of the ADP/ATP carrier in freshly prepared mitoplasts and inside-out submitochondrial particles was combined with an immunological approach. In a previous study, polyclonal antibodies raised against the solubilized ADP/ATP carrier protein were used to detect immunologically reactive cleavage fragments in the carrier by Western blot analysis (Boulay et al., 1986). In the present study, we took advantage of the reactivity of the N-terminal antiserum to map cleavage sites with an arginine-specific endoprotease.

The Arg-endoprotease added to mitoplasts was unable to cleave the carrier protein, as evidenced by the absence of cleavage products in immunoblot analysis with anti-$NaDodSO_4$ carrier antiserum, even after 1 h of incubation at 37 °C, using a ratio of enzyme to mitochondrial protein of 1/4 (w/w) (Figure 7A). In contrast, the ADP/ATP carrier in inside-out particles was efficiently cleaved by the Arg-endoprotease, resulting in accumulation of a large fragment of M_r 25 000 ± 1000, reactive against the anti-$NaDodSO_4$ carrier antiserum in immunoblots (Figure 7B). Taken together, these results lead to the conclusion that the cleavage site is located close to an extremity of the peptide chain of the membrane-bound carrier on the matrix face of the inner mitochondrial membrane.

When Arg-endoprotease cleavage products of the ADP/ATP carrier were blotted onto nitrocellulose and treated with anti-N-terminal antiserum, no reaction occurred (Figure 7C), indicating that the cleavage site for the Arg-endoprotease is located close to the N terminus of the peptide chain of the carrier. There are two arginine residues in this region of the carrier, Arg 30 and Arg 59. Cleavage at either of these arginine residues is compatible with the M_r of 25 000 ± 1000 of the resulting product.

Discussion

Immunological and enzymatic approaches have been used to localize emerging segments in a number of membrane-embedded proteins including 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). In the present work, antibodies were raised against two peptides corresponding to the N-terminal and C-terminal regions of the beef heart ADP/ATP carrier. These antibodies were used to probe the orientation of the corresponding epitopes in the ADP/ATP carrier of beef heart right-side-out and inside-out particles. The access of an arginine-specific endoprotease to the peptide chain of the membrane-embedded carrier was also studied. We shall first consider some of the methodological aspects related to the immunological and enzymatic approaches used. Then, we shall discuss the dependence of the immunological reactivity of the N-terminal portion of the carrier chain on the carrier conformation.

Methodological Aspects. Both N-terminal peptide and C-terminal peptide conjugated to ovalbumin were able to generate anti-N-terminal and anti-C-terminal antibodies in rabbits. Whereas the anti-N-terminal antiserum reacted toward the isolated or membrane-bound carrier protein, the anti-C-terminal antiserum was inefffective. This result was unexpected since the anti-C-terminal antiserum reacted efficiently with the C-terminal peptide, and the C-terminal region of the carrier contains strong antigenic determinants (Boulay et al., 1986). Furthermore, the N- and C-terminal regions are usually located on the surface of proteins; they are accessible to solvent and often flexible (Chavez & Sheraga, 1979; Thornton & Sibanda, 1983). It cannot be excluded, however, that the conformation or the flexibility of the C-terminal region of the carrier protein differs from that of the isolated C-terminal peptide and that, thereby, its accessibility to antibodies is hampered. It is also possible that coupling of the C-terminal peptide by means of Tyr 290 to ovalbumin results in decrease in immunogenicity by shortening the C-terminal reactive region to only eight amino acid residues.

Immunological assays by ELISA or Western blots yielded clear-cut results with the freeze–thawed mitochondria and the mitoplasts; however, no definite conclusions could be drawn from the assays carried out with inside-out submitochondrial particles obtained by sonication. This is most likely due to the fact that the population of sonicated particles was heterogeneous, with a small fraction of the particles being in the right-side-out configuration (Lauquin et al., 1977b). Because of the extreme sensitivity of ELISA, a small contamination of the population of inverted particles by right-side-out particles would introduce considerable uncertainty into the assessment of immune titration data. The uncertainty concerning the orientation of the carrier chain in inside-out particles was removed by the use of the Arg-endoprotease. This enzyme, which attacks selectively peptide bonds at arginine residues, cleaved the membrane-bound carrier protein in inside-out particles, but not in mitoplasts.

The use of Arg-endoprotease made it possible to answer the question of whether the subunits of the membrane-bound

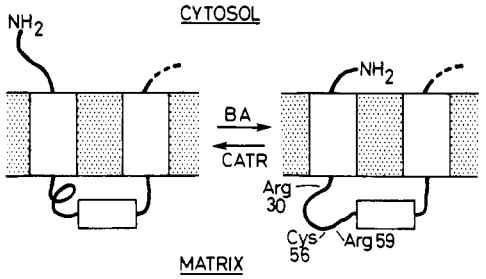

FIGURE 8: Scheme depicting a possible motion of the N-terminal segment of the ADP/ATP carrier chain during the reversible transition from the CATR conformation to the BA conformation. The first two helices of the five that have been recently postulated (Dalbon et al., 1988) are represented in the scheme. The N-terminal segment protrudes further into cytosol in the CATR conformation, and conversely the segment containing Arg 30, Cys 56, and Arg 59 protrudes further into the matrix in the BA conformation (for details, see text).

oligomeric ADP/ATP carrier (Block & Vignais, 1984) are oriented in a parallel or antiparallel manner. The asymmetric cleavage at the level of Arg 30 or Arg 59 excludes an antiparallel orientation of the subunits.

Orientation of the N-Terminal Portion of the Membrane-Bound ADP/ATP Carrier and Dependence on the Conformational State. Combining the immunological and enzymatic approaches gives rise to three main results from this study. (1) The N-terminal portion of the membrane-bound ADP/ATP carrier in mitochondria devoid of the outer membrane is reactive to anti-N-terminal antibodies. (2) In inside-out particles, but not in mitochondria, the ADP/ATP carrier is cleaved at the level of Arg 30 or Arg 59 by an Arg-endoprotease. (3) The reactivity of the N-terminal portion of the ADP/ATP carrier to anti-N-terminal antibodies is favored when the carrier is in the CATR conformation. The first two results demonstrate a cytosolic exposure of the N-terminal region of the ADP/ATP carrier corresponding to the first 10 amino acid residues and a matrix exposure of the segment including Arg 30 or Arg 59. This is consistent with structure predictions based on hydropathy plots suggesting that the hydrophobic sequence extending from residues 9 to 28 transverses the lipid bilayer of the mitochondrial membrane (Runswick et al., 1987). The matrix exposure including Arg 30 or Arg 59 is in agreement with the selective reactivity of Cys 56 to *N*-ethylmaleimide (NEM), a permeant alkylating reagent (Boulay & Vignais, 1984). The striking influence of the binding of CATR on the reactivity of the N-terminal segment of the membrane-bound carrier implies that large conformational changes occur upon binding of CATR, since CATR binds to a segment of the carrier extending from Cys 159 to Met 200 (Boulay et al., 1983), quite remote from the N terminus. Conversely, the decreased immunological reactivity of the N-terminal segment of the carrier in the BA conformation might be explained by the fact that this segment adopts a restricted conformation and (or) has a decreased flexibility due possibly to interaction with other segments of the carrier chain exposed to cytosol. Alternatively, this segment might become partially buried in the phospholipid bilayer. Along this line, it should be stressed that the greater reactivity of Cys 56 in the BA conformation (Boulay & Vignais 1984) is the mirror image of the greater reactivity of the N-terminal region of the carrier chain in the CATR conformation. One might imagine that the transition from the CATR conformation to the BA conformation includes a pulling motion of the N-terminal region of the carrier toward the matrix, through the first membrane-embedded α-helix spanning residues 9–28, as illustrated in Figure 8. The reverse motion would occur when the carrier adopts the CATR conformation. On the basis of the present work, no conclusion can be drawn concerning possible motion of the other membrane-bound helices. However, freeze-fracture electron microscopy studies of the purified ADP/ATP carrier protein in phospholipid vesicles have shown that in the presence of CATR the carrier molecules are anchored preferentially at the external surface of the vesicles, and addition of BA results in an increased percentage of carrier molecules at the internal surface of the vesicles (Brandolin et al., 1980). It was suggested that the events corresponding to the reversible transition from the CATR conformation to the BA conformation, and thereby to ADP/ATP transport, might include a slight inside-directed movement of the carrier due to unmasking of polar groups induced by BA on the inside-facing region of the carrier. Further work is obviously required to delineate in detail the different changes that occur on both faces of the carrier when this protein adopts the CATR and BA conformations.

ACKNOWLEDGMENTS

We thank Jeannine Bournet for excellent secretarial assistance.

Registry No. Ac-Ser-Asp-Gln-Ala-Leu-Ser-Phe-Leu-Lys-Asp-Phe, 117918-54-4; Val-Leu-Tyr-Asp-Glu-Ile-Lys-Lys-Phe-Val, 117918-55-5.

REFERENCES

Aquila, H., Misra, D., Eulitz, M., & Klingenberg, M. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 345–349.

Axen, R., Porath, J., & Ernback, S. (1967) *Nature (London) 214*, 1302–1304.

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

Beyer, R. E. (1967) *Methods Enzymol. 10*, 186–194.

Block, M. R., & Vignais, P. V. (1984) *Biochim. Biophys. Acta 767*, 369–376.

Block, M. R., Zaccai, G., Lauquin, G. J. M., & Vignais, P. V. (1982) *Biochem. Biophys. Res. Commun. 109*, 471–477.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1983) *Biochemistry 22*, 2202–2208.

Bogner, W., Aquila, H., & Klingenberg, M. (1986) *Eur. J. Biochem. 161*, 611–620.

Boulay, F., & Vignais, P. V. (1984) *Biochemistry 23*, 4807–4812.

Boulay, F., Lauquin, G. J. M., Tsugita, A., & Vignais, P. V. (1983) *Biochemistry 22*, 477–484.

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

Brandolin, G., Doussière, J., Gulik-Krzywicki, T., Lauquin, G. J. M., & Vignais, P. V. (1980) *Biochim. Biophys. Acta 592*, 592–614.

Brandolin, G., Dupont, Y., & Vignais, P. V. (1985) *Biochemistry 24*, 1991–1997.

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

Cabral, F., & Schatz, G. (1979) *Methods Enzymol. 56*, 602–613.

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.

Chin-Sun, W., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.

Dalbon, P., Brandolin, G., Boulay, F., Hoppe, J., & Vignais, P. V. (1988) *Biochemistry 27*, 5141-5149.
Davies, S., Meeran, K., Cairns, M. T., & Baldwin, S. A. (1987) *J. Biol. Chem. 262*, 9347-9352.
Dulley, J. R., & Grieve, P. A. (1975) *Anal. Biochem. 64*, 136-141.
Hackenberg, H., & Klingenberg, M. (1980) *Biochemistry 19*, 548-555.
Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595-598.
Lauquin, G. J. M., Duplaa, A. M., Klein, G., Rousseau, A., & Vignais, P. V. (1976) *Biochemistry 15*, 2323-2327.
Lauquin, G. J. M., Villiers, C., Michejda, J. W., Hryniewiecka, L. V., & Vignais, P. V. (1977a) *Biochim. Biophys. Acta 460*, 331-345.
Lauquin, G. J. M., Devaux, P. F., Bienvenue, A., Villiers, C., & Vignais, P. V. (1977b) *Biochemistry 16*, 1202-1208.
Ovchinnikov, Y. A. (1987) *Trends Biol. Sci. (Pers. Ed.) 12*, 434-438.
Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986) *Biochemistry 25*, 2633-2643.
Riccio, P., Aquila, H., & Klingenberg, M. (1975) *FEBS Lett. 56*, 133-138.
Runswick, M. J., Powell, S. J., Nyren, P., & Walker, J. E. (1987) *EMBO J. 6*, 1367-1373.
Saraste, M., & Walker, J. E. (1982) *FEBS Lett. 144*, 250-254.
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.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Vignais, P. V., Block, M. R., Boulay, F., Brandolin, G., & Lauquin, G. J. M. (1985) in *Structure and Properties of Cell Membranes* (Bengha, G., Ed.), Vol. II, pp 139-179, CRC, Boca Raton, FL.
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.
Zak, B., & Cohen, J. (1961) *Clin. Chim. Acta 6*, 665-670.

Nature of Proton Cycling during Gramicidin Uncoupling of Oxidative Phosphorylation†

Siro Luvisetto and Giovanni Felice Azzone*
CNR Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, University of Padova, 35100 Padova, Italy

Received June 6, 1988; Revised Manuscript Received August 22, 1988

ABSTRACT: Addition of gramicidin D to liver mitochondria, incubated in low- or high-salt media, results in stimulation of respiration in the absence or presence of depression of $\Delta\tilde{\mu}_H$, respectively. Gramicidin D concentrations 2 orders of magnitude higher are required in the low-salt media with full uncoupling at 1 nmol of gramicidin·mg^{-1}. The stimulation of respiration is not accompanied by increased passive proton influx in low-salt media. In high-salt media, the extent of respiratory stimulation and the extent of $\Delta\tilde{\mu}_H$ depression differ according to the nature and concentration of cation. The flow–force relationship is very steep when gramicidin D induced uncoupling occurs in low-salt media and much less steep in high-salt media. A multiplicity of flow–force relationship, respiratory rate vs $\Delta\tilde{\mu}_H$, is obtained, the slope of which depends on the nature and concentration of cation, and which can be reproduced by computer simulation by introducing a variable extent of proton cycling either in the membrane or in the pump. The *apparent* proton conductance, as analyzed in the relationship of $J_e/\Delta\tilde{\mu}_H$ vs $\Delta\tilde{\mu}_H$, increases in the so-called ohmic and nonohmic regions according to whether gramicidin D is added in high-salt or low-salt media, respectively. Titration with antimycin of the respiratory control ratio (RCR) in gramicidin D treated mitochondria leads to a depression of the RCR in high-salt but not in low-salt media. The view is discussed that in low-salt media the gramicidin D induced uncoupling is due to a cycling of protons within a proton domain operationally located at or near the proton pump. This leads to a $\Delta\tilde{\mu}_H$-independent uncoupling different from the $\Delta\tilde{\mu}$-dependent uncoupling due to proton cycling through the lipid bilayer.

The mechanism of uncoupling is one of the most fundamental problems of oxidative phosphorylation and as such has always received utmost attention in studies of energy coupling. In fact, if any acceptable mechanism of oxidative phosphorylation must explain how ATP is made, it must provide an equally satisfactory explanation as to why ATP is not made. Both questions have been answered by the chemiosmotic hypothesis, and this has been the main reason for its success.

That uncoupling agents increase the conductance for protons of the inner mitochondrial membrane and of black lipid membranes may be considered an established fact (Mitchell & Moyle, 1967; Hopfer et al., 1968). Whether the mem-

† This work was aided in part by a grant from the Regione Veneto and by a fellowship of ASSNE to S.L.
* Address correspondence to this author at the Institute of General Pathology, University of Padova, via Loredan 16, 35100 Padova, Italy.

0006-2960/89/0428-1100$01.50/0