Topography of Oligomycin Sensitivity Conferring Protein in the Mitochondrial Adenosinetriphosphatase–ATP Synthase†

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ABSTRACT: The topographical organization of oligomycin sensitivity conferring protein (OSCP) in the mitochondrial adenosinetriphosphatase (ATPase)–ATP synthase complex has been studied. The accessibility of OSCP to monoclonal antibodies has been qualitatively visualized by using the protein A–gold electron microscopy immunocytochemistry or quantitatively estimated by immunotitration of OSCP in depolymerized or intact membranes. Besides, OSCP cannot be labeled by 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) which selectively labels the hydrophobic core of membrane proteins. These observations demonstrate an external location of OSCP on the inner face of the inner mitochondrial membrane. The position of OSCP relative to other peptides of the complex has been analyzed by cross-linking experiments using either zero length *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline or 11-Å span dimethyl suberimidate cross-linkers in the ATPase–ATP synthase complex. The OSCP cross-linked products were identified either by immunocharacterization with anti-α, anti-β, or anti-OSCP monoclonal antibodies or by their molecular weight. OSCP was cross-linked with either the α- or β-subunits of F1 or to a subunit of M_r 24 000. Other types of cross-linking were obtained by the labeling of OSCP with [*cysteamine*-^{35}S]-*N*-succinimidyl 3-[[2-((2-nitro-4-azidophenyl)amino)ethyl]dithio]propionate ([^{35}S]SNAP) and reconstitution of SNAP–OSCP with F1 in urea-treated submitochondrial particles. Under these conditions, OSCP is found to be adjacent to two other peptides of molecular weight close to 30 000. A comparison is made between the topology and the organization of the b-subunit of *Escherichia coli* and OSCP, suggesting an analogy between OSCP and the hydrophilic part of the b-subunit.

The mitochondrial adenosinetriphosphatase (ATPase)[1]–ATP synthase (EC 3.6.1.3) (Pedersen, 1983; Senior, 1979) contains as its homologous system in *Escherichia coli* (Fillingame, 1981) and in other organisms (Pedersen, 1983) two main parts, F1 and F0. F1 is water-soluble, contains the nucleotide and phosphate binding sites, and is made of five polypeptides, α, β, γ, δ, and ϵ, found in most species. F0 is membrane-embedded and catalyzes the H^+ conduction across the membrane. The analysis of the genes that code for F0 and F1 subunits in E. *coli* (Downie et al., 1979; Von Meyenburg & Hansen, 1980; Walker et al., 1984; Futai & Kanazawa, 1983) has recently permitted the identification of the peptides involved in the membrane sector F0: subunit a, which is very hydrophobic and therefore embedded in the lipid phase of the membrane; subunit b, which contains a small hydrophobic tail inserted in the membrane and a large hydrophilic section interacting with F1 subunits (Cox et al., 1981); subunit c, also called DCCD binding protein, mainly embedded in the membrane (Sebald & Hoppe, 1981). The subunits a and c or their equivalents have been identified in many organisms. In yeasts and mammalian species these peptides are coded by the mitochondrial DNA. The equivalent of subunit b of *E. coli* is not well-defined in other organisms. The ATPase–ATP synthase of eucaryotes is more complex than that of bacteria. It contains additional peptides such as the oligomycin sensitivity conferring protein (OSCP) (Mac Lennan & Tzagoloff, 1968), an inhibitory peptide IF1 (Pullman & Monroy, 1963), probably other peptides such as factor B (Joshi et al., 1979), factor F6 (Knowles et al., 1971), and the uncoupler binding protein (Hanstein & Hatefi, 1975), and eventually some other peptides (Capaldi, 1982).

Very little is known about the role of most of these peptides in the structure and the function of the ATPase–ATP synthase. The availability of monoclonal antibodies specific to OSCP in our laboratory (Archinard et al., 1984) has permitted us to analyze the topography of this peptide in the complex. These antibodies have been used to demonstrate the accessibility of OSCP to the aqueous phase in inverted submitochondrial particles and to identify the nearest neighbors of OSCP in the mitochondrial membrane after reticulation of the ATPase–ATP synthase with cross-linking reagents. The chosen reagents were different either in length [zero length such as *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline (EEDQ) or 11 Å long such as dimethyl suberimidate] or in reactivity toward amino acids: amino groups, carboxyl groups, or nonspecific groups (azido derivative). Unsuccessful attempts

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[1] Abbreviations: OSCP, oligomycin sensitivity conferring protein; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; [^{35}S]-SNAP, [*cysteamine*-^{35}S]-*N*-succinimidyl 3-[[2-((2-nitro-4-azidophenyl)amino)ethyl]dithio]propionate; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline; DMS, dimethyl suberimidate; ETP, phosphorylating submitochondrial particles; ATPase, adenosinetriphosphatase; AS particles, ETP depleted from the natural F1-ATPase inhibitor peptide IF1; ASU particles, ETP depleted from F1-ATPase and from IF1; ASUA particles, ETP depleted from F1-ATPase, IF1, and OSCP; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; kDa, kilodalton; Tris, tris(hydroxymethyl)aminomethane.

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to label OSCP from the hydrophobic core of the membrane also contribute to define OSCP as a rather extrinsic peptide located on the matrix side of the inner mitochondrial membrane.

Experimental Procedures

Materials

$Na^{125}I$, [^{35}S]SNAP, and [^{125}I]TID were obtained from the Commissariat à l'Energie Atomique and from Amersham. Specific chemicals were purchased from the following sources: EEDQ, Aldrich; dimethyl suberimidate, Pierce; protein A, IBF; Sepharose–protein A, Pharmacia; horseradish peroxidase conjugated antimouse immunoglobulin sheep antibody and antimouse immunoglobulin rabbit antibody, Biosys; CM-Trisacryl, IBF; IODO-GEN, Pierce.

Methods

Biological Preparations. Previously described procedures were used to obtain pig heart mitochondria (Godinot et al., 1969), F1-ATPase (Penin et al., 1979), ETP, and ATPase–ATP synthase (Penin et al., 1982) from mitoplasts (Maïsterrena et al., 1974), ETP-U from ETP and A particles [Fessenden & Racker (1966) as modified by Higashiyama et al. (1975)], AS and ASU particles (Horstman & Racker, 1967), ASUA particles (Van de Stadt et al., 1972), and monoclonal antibodies directed against the α- and β-subunits of F1-ATPase (Moradi-Améli & Godinot, 1983) and against OSCP (Archinard et al., 1984). The monoclonal antibodies were purified by affinity chromatography on protein A–Sepharose according to the procedure of Ey et al. (1978). Protein concentration was estimated by the method of Lowry et al. (1951). ATPase activity and oligomycin sensitivity were measured as described (Penin et al., 1982).

Purification of OSCP. OSCP was purified from a new method derived from those of Mac Lennan and Tzagoloff (1968) and Senior (1979). When the latter method was applied to the purification of OSCP from pig heart mitochondria, low yields were obtained. The difficulty in improving the purification procedure of OSCP comes from the absence of known intrinsic enzymatic activity of this protein. The availability in our laboratory of monoclonal antibodies directed specifically against OSCP has permitted us to set up a new protocol: the efficiency of each purification step was tested after separation of the proteins by SDS–PAGE (Laemmli, 1970), transfer onto nitrocellulose sheets (Towbin et al., 1979), and estimation of the amount of OSCP present in each fraction by immunodetection (as will be discussed). This study has indicated that the preparation of submitochondrial particles and the sodium bromide extractions used in the original procedures (Mac Lennan & Tzagoloff, 1968; Senior, 1979) led to severe loss of OSCP and could be advantageously replaced by alkaline and salt treatments of mitochondria. The ion-exchange chromatography step was also modified to speed up and improve the efficiency of the purification procedure. The new protocol is reported here. All steps were carried out at 0–4 °C. Frozen pig heart mitochondria were suspended at a protein concentration of 15 mg/mL in 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5. The pH was quickly brought to about 11.5 by the addition of 6 N KOH (final concentration 50 mM), under vigorous shaking with a magnetic stirrer. One minute later, the pH was lowered to 9.2 with 10 N acetic acid. After centrifugation for 10 min at 25000g, the supernatant that contained the natural F1-ATPase inhibitor peptide, IF1, was saved for eventual further purification by the method of Horstman and Racker (1970). The pellet was homogenized in the above medium. The same alkaline treatment was repeated once. The pellet was homogenized at a protein concentration of 15 mg/mL in 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 0.2 M KCl, and 10 mM Tris base, pH 9.2, and centrifuged for 30 min at 100000g. This KCl treatment was repeated once. The pellet was homogenized in the same buffer at a protein concentration of 10 mg/mL, and ammonia (0.9 specific gravity NH_3 solution) was added to give a final concentration of 0.4 N. The suspension was stirred for 10 min and centrifuged for 1 h at 300000g. The clear supernatant was adjusted to pH 8.0 with 10 N acetic acid and centrifuged for 30 min at 100000g. The supernatant diluted with 5 volumes of cold distilled water was eventually readjusted to pH 8.0 and passed on a sintered glass funnel containing 60 mL of CM-Trisacryl Ultrogel, at a high flow rate (1 L/h). The CM-Trisacryl gel had been first washed with 2 volumes of 1 M ammonium acetate and 2 M KCl, pH 8.0, and then with at least 3 volumes of equilibrating buffer prepared exactly under the same conditions as those used for the preparation of the protein solution before chromatography (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 0.2 M KCl, 10 mM Tris base, pH 9.2; addition of ammonia and acetic acid and dilution with 5 volumes of cold distilled water). Under these conditions, OSCP was completely retained by the CM-Trisacryl gel. Then, one gel volume was suspended in 2 volumes of equilibrating buffer and the slurry poured into a 3.2 cm diameter column. The column was washed with 3 volumes of buffer containing 0.15 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 15 mM Tris–H_2SO_4, pH 7.5, at a flow rate of 2 mL/min. OSCP was then eluted with 0.4 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 15 mM Tris–H_2SO_4, pH 7.5, at a flow rate of 1 mL/min. OSCP was then concentrated by addition of solid ammonium sulfate to obtain a 70% saturation at 0 °C. After at least 1 h at 0 °C the precipitate collected by centrifugation at 100000g for 20 min was dissolved in a minimal volume of 20 mM Tris–H_2SO_4, pH 8.0. After centrifugation for 5 min at 10000g to eliminate any aggregated material, the OSCP solution (free of contaminants as shown by electrophoresis, Figure 1) could be stored as such in liquid nitrogen or as an ammonium sulfate suspension, saturated at 70%. The yield was of 8–10 mg of pure OSCP starting from 10 g of mitochondria, that is, about 2 times the yield described by Senior for beef heart mitochondria and at least 5 times the yield obtained in our hands with the Senior method applied to pig heart mitochondria. The OSCP prepared under these conditions had exactly the same capacity as the OSCP prepared with the Senior method for the reconstitution of net ATP synthesis in the presence of ASU or ASUA particles and purified F1-ATPase (not shown).

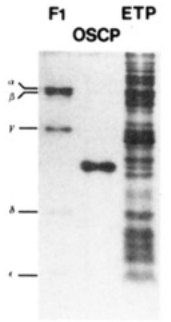

FIGURE 1: Purification of OSCP. (Lane 1) F1, 10 μg of protein; (lane 2) OSCP, 5 μg of protein; (lane 3) ETP, 20 μg of protein.

Radiolabeling of Proteins. Protein A, purified monoclonal antibodies, OSCP, and F1 were iodinated with ^{125}I in the presence of IODO-GEN (Fracker & Speck, 1978) as described previously (Moradi-Améli & Godinot, 1983).

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) using a stacking gel containing 4% acrylamide and a separating gel routinely containing 12.5% acrylamide. After separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the subunits were electrophoretically transferred from the gels onto nitrocellulose sheets (10 × 15 cm) by the method of Towbin et al. (1979).

Immunodecoration of OSCP or α- or β-Subunits of F1. The cross-linked products formed between the various peptides, OSCP, α, and β, were characterized by immunodecoration using a second antibody labeled with horseradish peroxidase: after electrotransfer of the proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the nitrocellulose sheets were incubated overnight in phosphate–saline buffer (10 mM P_i, 0.9% NaCl, pH 7.2) containing 0.05% Tween 20. The nitrocellulose sheets were then successively incubated as follows: for 3–4 h with the culture supernatants containing the monoclonal antibodies or with the purified monoclonal antibodies diluted in phosphate–saline buffer and 0.05% Tween 20; for 6 × 10 min in phosphate–saline buffer and 0.05% Tween 20; for 1 h with horseradish peroxidase conjugated anti-mouse immunoglobulin raised in sheep; for 5 × 10 min in phosphate–saline buffer and 0.05% Tween 20; for 10 min in distilled water to remove the Tween 20 which inhibits the peroxidase. Peroxidase activity linked to the immune complexes on the nitrocellulose sheets was revealed by staining with α-naphtol. The α-naphtol (0.3%) dissolved just before use in 10 mL of methanol was diluted with 40 mL of distilled water containing 100 μL of H_2O_2 (30%).

Total Immunotitration of OSCP in Various Membranes. The amount of OSCP present in the various membrane preparations was estimated by counting the amount of ^{125}I-labeled purified monoclonal antibodies specific to OSCP that could bind to nitrocellulose sheets after electrotransfer of the membrane proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The counts measured were then compared to a standard curve of OSCP made exactly under the same conditions, on the same slab (F. Penin et al., unpublished observations).

Solid-Phase Competitive Radioimmunoassays. Flexible polyvinyl plates (96 wells, Falcon) were incubated with 0.1% poly(L-lysine) hydrobromide for 4 h at room temperature and rinsed with distilled water. In each well, 0.5 μg of OSCP in 0.1 M sodium phosphate, pH 8.0, was incubated overnight. The wells were emptied and dried with cool air and washed 4 times for 10 min with phosphate–saline buffer containing 1% BSA.

The antibodies adequately diluted (in order to observe 80% of their maximal binding capacity) were preincubated for 1 h either with pure OSCP or with the various membranes at various concentrations. This incubation mixture was transferred to the 96-well plates on which OSCP had been previously bound. After a 1-h incubation, the plates were washed 3 times for 10 min in phosphate–saline buffer containing 1% BSA and incubated again with ^{125}I-protein A (2×10^5 cpm per well) in phosphate–saline buffer for 1 h at 37 °C. After three more washes, the wells were cut out and counted in a γ-counter (Packard).

Immunoelectron Microscopy with Gold-Labeled Protein A and Negative Staining. Nickel grids (200 mesh) covered with a carbon-coated collodium film (200 Å) (Bradley, 1965) were floated on a droplet of ETP homogenized at 0.5 mg of protein/mL in 10 mM sodium phosphate buffer, pH 7.5. After 2 min, the excess solution was drained out on a filter paper, and the grids were floated at room temperature on droplets of monoclonal antibodies in culture supernatants, of culture medium, or of normal mouse serum diluted at 1/200 in phosphate–saline buffer (controls). After a 3-h incubation, the grids were drained, washed 4 times by floating for 10 min on droplets of phosphate–saline buffer containing 1% BSA, incubated for 1 h with gold-labeled protein A prepared according to Bendayan et al. (1980), and washed again 4 times with phosphate–saline buffer and finally for 5 min with distilled water. The grids were slightly stained with 4% aqueous uranyl acetate for 15–30 s (Gottschalk, 1976), dried on a filter paper, and rapidly examined with an electron microscope Jeol 1200 Ex. operating at 80 kV. During the entire procedure, care was taken to prevent drying of the grids.

Cross-Linking of ATPase–ATP Synthase. The enzyme was diluted at a protein concentration of 2.5 mg/mL 0.1 M triethanolamine, pH 8.0, and incubated for 30 min at 20 °C with 7 mM dimethyl suberimidate. The reaction was stopped by addition of 20 mM lysine, 2.5% sodium dodecyl sulfate, and 6.25% β-mercaptoethanol.

In a parallel experiment, ATPase–ATP synthase was diluted at 2.5 mg of protein/mL of 50 mM MOPS, pH 6.5. A concentrated methanolic solution of EEDQ was added to give a final concentration of 1 mM and a methanol concentration of less than 1%. After 30 min at 30 °C, cross-linking was stopped by addition of 4% sodium dodecyl sulfate and 10% β-mercaptoethanol.

Samples cross-linked by dimethyl suberimidate or by EEDQ were then analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) with a gradient of acrylamide (10–18%).

To identify the components of the cross-linked products between OSCP and other subunits, the proteins separated on polyacrylamide gels were electrophoretically transferred onto nitrocellulose sheets and analyzed by immunodetection, as described previously, after incubation with culture supernatants containing monoclonal antibodies specific to the α- or β-subunits of F1 (20-D6 or 14-D5, respectively) (Moradi-Améli & Godinot, 1983) or specific to OSCP (2B1-B1 or 5 D6-D1) (Archinard et al., 1984).

Cross-Linking of OSCP upon Reconstitution with F1 and ETP-U Particles. [^{35}S]SNAP (6.7 μCi) was incubated for 20 min in the dark with OSCP diluted at a concentration of 0.2 mg of protein/mL of 10 mM triethanolamine, pH 8.0, at a molar ratio of 2 SNAP/1 OSCP. The reaction was stopped by addition of 10 mM lysine. A control was made omitting OSCP. The OSCP–SNAP (4 μg) was reconstituted with 400 μg of ETP-U particles and 100 μg of purified F1 and incubated for 30 min at 30 °C in 0.25 M sucrose, 10 mM triethanolamine, and 10 mM Mg ATP, pH 7.5. The samples were sedimented at 105000*g* for 4 min in an Airfuge (Beckman) and washed by filtration–centrifugation through a Sephadex G-50 column equilibrated in the same buffer. Under these conditions, free OSCP was retained by the column. The reconstituted complex was then photoirradiated at 30 °C for 20 min with a 250-W lamp situated at 15 cm. The samples were protected from UV radiations by a glass filter. After photoirradiation, the samples were depolymerized in the presence of 6.25% β-mercaptoethanol, 1.66% SDS, and 8 M urea. These conditions were adequate both to depolymerize the proteins and to cleave the disulfide bridge of the SNAP. The [^{35}S]cysteamine remained linked to the peptides to which OSCP was originally cross-linked while the migration of these peptides was not significantly modified [Schwartz et al., 1982]. After polyacrylamide gel electrophoresis in the presence of SDS, the gels were dried and submitted to autoradiography

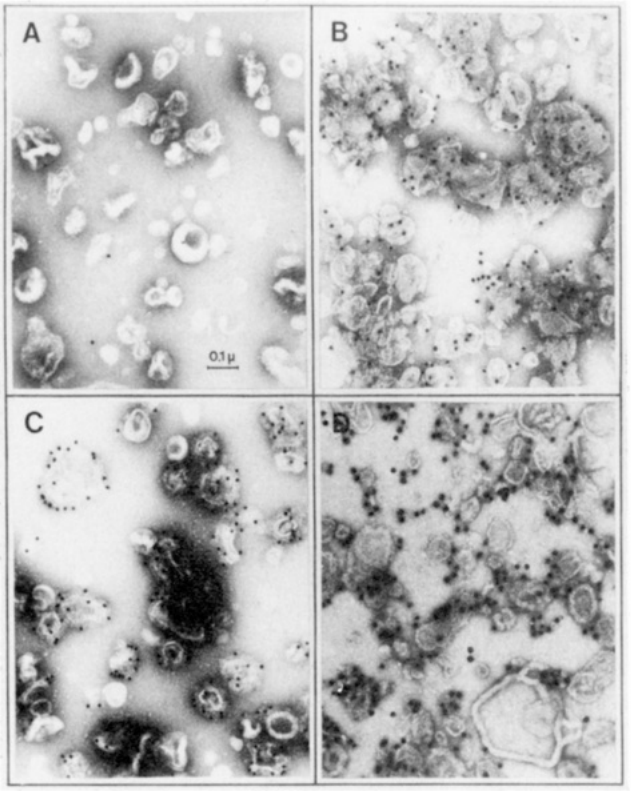

FIGURE 2: Localization of OSCP in pig heart submitochondrial particles (ETP) by protein A–gold electron microscopic immunocytochemistry. Incubation of ETP with control culture medium (A), culture supernatants containing the monoclonal antibodies $2B_1$-B_1 [(B) anti-OSCP], $7B_3$ [(C) anti-α], $14D_5$ [(D) anti-β]. The size of the gold particles varied from one preparation to the other between 10 (A–C) and 12–15 nm (D).

at −70 °C, using Kodak direct exposure films.

Photolabeling with [^{125}I]TID. ETP were diluted at 1 mg of protein/mL of 0.25 M sucrose and 10 mM Tris-HCl, pH 7.5. [^{125}I]TID (10 Ci/mmol) (Brenner & Semenza, 1981) was incubated at 1 μM for 5 min with the ETP at room temperature and photoirradiated in a quartz cuvette situated at 5 cm from a 60-W mercury lamp. During photolysis, samples were protected by a plexiglass filter [$\lambda < 320$ nm] and continuously stirred. After irradiation for 5 min, the samples were washed 5 times by centrifugation in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.5, depolymerized, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and autoradiographed as above.

RESULTS

Accessibility of OSCP to Monoclonal Anti-OSCP Antibodies in Mitochondrial Membranes. ETP (inverted submitochondrial particles) were adsorbed on electron microscopic grids, incubated first with monoclonal anti-OSCP antibodies and then with gold-labeled protein A (Bendayan, 1984). Under these conditions, the binding of the antibodies to OSCP present in the membranes could be directly visualized, as shown by the large number of electron-dense gold particles seen on Figure 2B as compared to the controls (Figure 2A). Several other types of controls were performed: the culture supernatants containing the antibodies could be replaced by culture supernatants of cells which did not produce antibodies, by purified normal mouse immunoglobulins diluted in the culture medium, or by nonimmune mouse serum. The pig heart mitochondrial membranes could be replaced by rat liver mitochondrial membranes which were not recognized by our anti-OSCP monoclonal antibodies (Archinard et al., 1984).

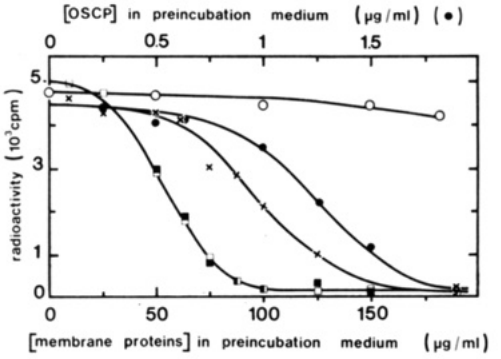

FIGURE 3: Competition for anti-OSCP antibody between bound OSCP and either soluble OSCP or various submitochondrial particles. The wells of a microtiter plate were incubated with 500 ng of purified OSCP as described under Experimental Procedures. After removal of excess OSCP by washing, each well was incubated with 50 μL of anti-OSCP antibody culture supernatant diluted (1/2) in the presence of mitochondria (○), ETP (□), ASU (■), ASUA (×), or OSCP (●) at the indicated concentrations. The ELISA assay and the membrane treatments were performed as described under Experimental Procedures. To calculate the amount of OSCP present in the membranes, the OSCP concentration corresponding to 50% binding is determined on the OSCP curve and compared to the membrane protein concentrations producing a 50% binding.

In all these cases, the density of electron-dense gold particles observed was as low as that observed in the control of Figure 2A. Figure 2B was obtained with the anti-OSCP 2B1-B1, but the results of 5D6-D1 were similar (not shown). These results suggested that the epitopes of OSCP recognized by both antibodies were directly accessible from the matrix face of mitochondrial membranes. Parts C and D of Figure 2 show that the binding of antibodies specific to the α- and β-subunits can be visualized with gold-labeled protein A in a similar manner to that observed in the case of OSCP.

Experiments were conducted to check whether OSCP might at least partly become artifactually exposed during incubations and washings of membranes. The principle of the experiment was to compare the amount of OSCP accessible to antibodies at the surface of membranes to the total amount of OSCP present in membranes. The comparison was made either in membranes exhibiting a high rate of ATP synthesis and full oligomycin sensitivity or in membranes more or less depleted from F1 by urea and ammonia treatments.

The total amount of OSCP present in the membranes was titrated as detailed by Penin et al. (1985), according to the following principle: the membranes depolymerized in 8 M urea and 1% SDS, were submitted to electrophoresis, and then were transferred to nitrocellulose. The determination of the amount of OSCP present in the blots was measured by the binding of iodinated purified antibody and comparison to a standard curve of purified OSCP treated exactly under the same conditions at the same time. A value of 16.6 μg of OSCP/mg of protein of ETP was found. In a second series of experiments, the amount of OSCP accessible at the surface of the membranes was measured by competitive radioimmunoassay by comparison with purified OSCP. The results are shown in Figure 3: one can calculate that 19 μg of OSCP/mg of mitochondrial protein was accessible to the antibodies in inverted submitochondrial particles treated or not with urea (ETP or ASU) while 11.4 μg of OSCP could be titrated in ASUA particles. It was checked that the oligomycin sensitivity of the ATPase activity did not decrease during the incubation of ETP with the antibodies, whether the membrane protein concentration was from 10 to 100 μg/mL (not shown). This indicates that, under the conditions used, F1 was not dissociated from F0.

Table I: Comparison between Total Amounts of OSCP in Dissociated Membranes and the Amount of OSCP Accessible to Monoclonal Antibodies at the Surface of Mitochondrial Membranes after Various Treatments

membrane	total OSCP[a] (μg/mg of protein)	accessible OSCP[b] (μg/mg of protein)
mitochondria	12.1 ± 2.3 (26)	no competition
ETP	16.6 ± 3 (10)	19 ± 2.5 (4)
ASU particles	17.8 ± 1.4 (4)	19 ± 2.4 (4)
ASUA particles	13.5 ± 2.3 (8)	11.4 ± 2.3 (4)

[a] Total OSCP was measured after electrophoresis of dissociated membranes, electrotransfer to nitrocellulose, and immunotitration with ^{125}I-labeled anti-OSCP monoclonal antibody (average of n experiments ± SD). The number of determinations is given in parentheses. [b] OSCP accessible at the surface of the membranes was estimated by competitive radioimmunoassay (cf. Figure 3).

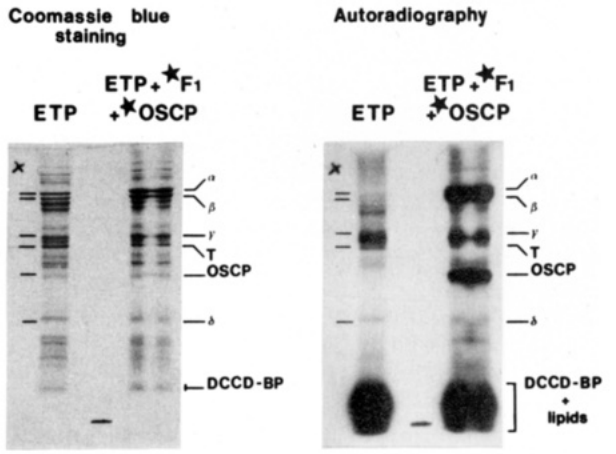

FIGURE 4: Identification of peptides of ETP labeled with [^{125}I]TID: (Left panel) Coomassie Blue staining of the gel. (Right panel) Autoradiography. Track 1, ETP labeled with [^{125}I]TID. Track 2, ETP labeled with [^{125}I]TID comigrating with purified ^{125}I-labeled F1 and ^{125}I-labeled OSCP. This comigration was made to facilitate the identification of the positions of F1 subunits and of OSCP on the autoradiogram. Experimental conditions as described under Experimental Procedures. α, β, γ, δ, OSCP, T, and DCCD-BP represents the subunits of F1, OSCP, the adenine nucleotide translocator, and the DCCD-binding protein, respectively.

Table I shows that the amount of OSCP accessible to antibodies in various membranes more or less depleted from F1 is very similar to that obtained by titration of the total amount of OSCP present in the same membranes except in the case of mitochondria for which no OSCP is accessible to the antibodies. Therefore, it can be concluded that the epitopes of OSCP, which are recognized by our antibodies, are fully accessible in the ETP and that urea and ammonia treatments reputed to remove F1 did not unmask OSCP. It should be noticed that a direct comparison of the amount of OSCP relative to the protein content of the various membranes is somewhat misleading since the different treatments applied to these membranes can remove a variety of proteins in addition to F1 or OSCP. Therefore, the ASU and ASUA particles are more depleted in OSCP than the figures could suggest. Nevertheless, the ASUA particles used here are not completely depleted from their initial OSCP content.

Attempts To Label OSCP from the Hydrophobic Core of the Membrane with [^{125}I]TID. In order to determine if OSCP contains domains that penetrate the lipid core of the membrane, [^{125}I]TID, a lipid-soluble photoactivable carbene-generating reagent (Brenner & Semenza, 1981), has been used. This compound has been shown to label in a highly specific manner those parts of membrane proteins that are embedded

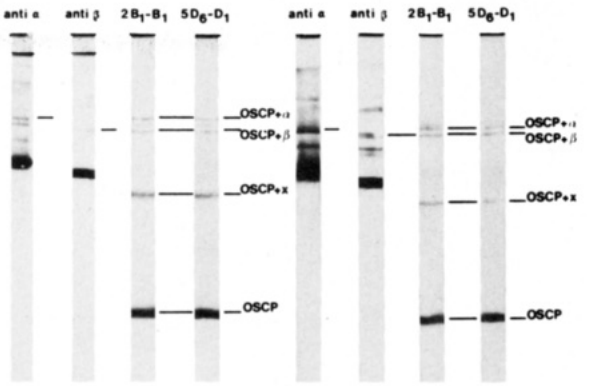

FIGURE 5: Transfer of cross-linked products of ATPase–ATP synthase complex from polyacrylamide gel slab onto nitrocellulose sheets followed by immunodecoration with anti-OSCP $2B_1$-B_1 or 5 D_6-D_1, anti-α, and anti-β subunit monoclonal antibodies. Cross-linking, electrophoresis, transfer, and immunodecoration are described under Experimental Procedures. Cross-linking reagents: DMS (left panel) or EEDQ (right panel).

in the lipid bilayer. Figure 4 shows that the treatment of ETP with [^{125}I]TID did not produce any labeling of peptides of molecular weight close to that of OSCP. On the contrary a strong labeling is observed for peptides of M_r ~30 000. The main labeling is found near the migration front of the gel where the DCCD-binding protein and the lipids are located. In addition, the γ-subunit of F1 also seems to be labeled. A weak labeling can also be detected at the level of the α- and β-subunits of F1. But the autoradiogram is remarkably clear at the level of OSCP even after 2 months of exposure of the film to the gel containing the labeled membranes.

Topography of OSCP in the ATPase–ATP Synthase Complex. Cross-linking experiments have been conducted to determine which subunits of the ATPase–ATP Synthase complex interact with OSCP. Three reagents differing widely in the length of their arm and in their reactivity have been studied: DMS, EEDQ, and SNAP. DMS reacts primarily with amino groups and has two reactive groups separated by an 11-Å arm (Wang & Moore, 1977). EEDQ is a zero-length cross-linker able to react with carboxyl and amino groups (Belleau & Malek, 1968). SNAP can form a covalent derivative mainly with amino groups of OSCP and then reacts with a variety of neighboring amino acids upon photoactivation (Schwartz et al., 1982). Figure 5 shows that the major cross-linked products obtained by treating oligomycin-sensitive ATPase with DMS or EEDQ are identical. The same results were obtained with submitochondrial particles (ETP; not shown). The anti-OSCP antibodies react mainly with three cross-linked products of apparent molecular weight of about 75 000, 72 000, and 45 000.

The peptide of M_r 75 000 also reacts with the anti-α antibody. That of M_r 72 000 also reacts with the anti-β antibody. Since the molecular weight of OSCP and α- and β-subunits of F1 have been estimated at 21 000 (Ovchinnikov et al., 1983), 53 300 (Knowles & Penefsky, 1972), and 51 300 (Runswick & Walker, 1984), respectively, all data prove that the 75 000 and 72 000 cross-linked products can be identified as OSCP-α and OSCP-β, respectively. The cross-linked product of M_r 45 000 (OSCP–X) could not be identified with certitude. Its migration could be clearly distinguished from an OSCP dimer obtained with ^{125}I-radiolabeled purified OSCP (not shown). It must be a cross-linked product between OSCP and a peptide of about 24 kDa. The intensity of the latter cross-linked product was always higher than that corresponding to the α- and β-subunits, as shown in Figure 5. The intensities of OSCP–α and OSCP–β bands were not significantly different.

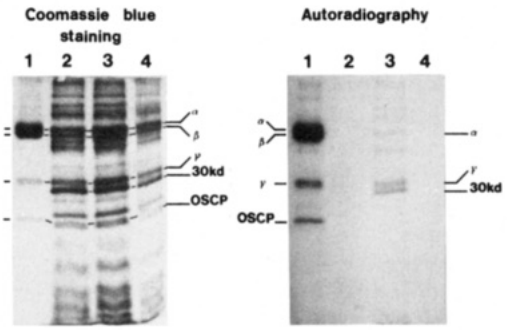

FIGURE 6: Identification of peptides adjacent to OSCP during its reconstitution with F_1 and ETP-U particles after labeling of OSCP with [^{35}S]SNAP and photoirradiation of the reconstituted complex. (Left panel) Coomassie Blue staining. (Right panel) Autoradiography. Track 1, iodinated F_1 and iodinated OSCP; track 2, ETP + [^{35}S]-SNAP–OSCP; track 3, ETP-U particles + [^{35}S]SNAP–OSCP + F_1; track 4, same as track 3 except that [^{35}S]SNAP was reacted with 10 mM lysine before its addition to the OSCP solution. Experimental conditions as described under Experimental Procedures.

SNAP is a cross-linking reagent that could be covalently bound to OSCP through its succinimide group. The SNAP–OSCP adduct could then be reassociated with F1 and with submitochondrial particles partially depleted from F1 and OSCP (ETP-U particles) under conditions permitting the reconstitution of an oligomycin-sensitive ATP synthase. After elimination of the free SNAP–OSCP from the reconstituted complex, the samples were photoirradiated. Upon photolysis, the azido group of SNAP was converted to a highly reactive nitrene that could induce the formation of a covalent cross-linked product either between OSCP and adjacent peptides or between the original site of OSCP and a second site of OSCP. Since the SNAP contains a disulfide bridge, the cross-linked produce could be cleaved by dithiothreitol. The adjacent peptides could be recognized after separation in SDS gel electrophoresis and autoradiography because the [^{35}S]-SNAP sulfur of the cysteamine moiety is involved in the disulfide bond. Figure 6 shows the autoradiogram obtained under these conditions. A faint radioactivity can be detected at the level of the α-subunit (track 3) as observed with other cross-linking reagents, but the main radioactive bands are located at the levels of two peptides close to 30 kDa. They can correspond to the γ-subunit of F1, to the adenine nucleotide translocator (Vignais, 1976), or to the phosphate carrier (Wohlrab, 1980). When [^{35}S]SNAP–OSCP was added to ETP that were not depleted from their endogenous amount of OSCP (track 2), no detectable radioactivity could be found, indicating that if unspecific binding of [^{35}S]SNAP–OSCP occurs, cross-linked products with unspecific peptides could not be detected in the absence of available OSCP binding sites. This indicates that nonspecific cross-linking must be of limited importance if it exists. In the same way, no radioactivity was detected if the [^{35}S]SNAP reacted with lysine before addition of OSCP, which shows that there is no unspecific reaction of free [^{35}S]SNAP upon photoactivation (track 4).

Discussion

External Position of OSCP in the ATPase–ATP Synthase Complex. In most textbook models, OSCP is presented as a part of the connecting link (stalk) between F1 and F0, suggesting a position of OSCP somewhat buried. The various approaches used in this study rather support a model of the mitochondrial ATPase–ATP synthase complex in which OSCP occupies an external position on the matrix face of the inner mitochondrial membrane as compared to F1 and the membrane. Indeed, the epitopes recognized by our monoclonal antibodies are directly accessible to the medium when inverted submitochondrial particles are incubated with these antibodies. This accessibility could be visualized by protein A–gold electron microscopic immunocytochemistry. It was also indirectly demonstrated by radioimmunoassays. Indeed, the total amount of OSCP titrated after dissociation of these membranes was equal to the amount of OSCP measured by competitive radioimmunoassays using intact inverted membranes in the competition. Treatment of these membranes with urea which has been reported to remove F1 did not increase the accessibility of OSCP to the antibodies. It could be argued that only a small part of OSCP is recognized by the antibodies and that, perhaps, this part protrudes out of the molecule far away from the site of interaction of OSCP with F0 and F1. This is rather unlikely because the size of OSCP is small as compared to that of an immunoglobulin G, and therefore, even if the specific epitope is small, the surface adjacent to the epitope interferes in the binding of the immunoglobulin (Berzofsky & Berkower, 1984). If this surface which normally anchors the antibody on purified OSCP was completely or partly masked in the complex by F1 or F0, the affinity for the antibody would decrease, and the results obtained by comparing the total amount of OSCP to the amount titrated in competition would be different. In addition, in a model such as that proposed by Fernandez-Moran et al. (1964) where OSCP represents the stalk between F0 and F1 with a dimension of 30 Å in diameter and 50 Å in length, it seems difficult to imagine that the two molecules of immunoglobulins bound to the two OSCP's present in the complex (Penin et al., 1985) would be able to insert themselves between F0 and F1 without modifying the rate of ATP synthesis or that of ATP hydrolysis (Archinard et al., 1984). A model where at least a part of OSCP would be externally located is therefore more appropriate. Other observations made in the literature could also be interpreted along the same lines. Hundall and Ernster (1981) have shown that OSCP protects the α-subunit of F1 against trypsin attack while the protection afforded by F1 on OSCP proteolysis is only partial. This would occur if OSCP was used to cover F1 in such a way that trypsin would have to cleave OSCP before reaching F1.

It is known that there are direct interactions between F0 and F1 in the absence of added OSCP (Mac Lennan & Tzagoloff, 1968; Vadineanu et al., 1976). OSCP added after the binding of F1 to F0 restores the capacity of net ATP synthesis and of the oligomycin sensitivity of the ATPase activity in urea- and ammonia-treated particles without important additional binding of F1 (F. Penin et al., unpublished results). In these conditions, OSCP must therefore bind on top of F0 around F1, even if a rearrangement of the peptides can occur afterward.

The fact that [^{125}I]TID was unable to label OSCP suggests that OSCP should not be deeply anchored in the apolar lipid core of the membrane. However, OSCP may interact closely with membrane peptides which would shield OSCP from reaction with TID. Besides, the amino acid side chains of OSCP in contact with the membrane may not readily react with TID. Therefore, the experiments describing the failure to label OSCP with TID must be interpreted with some caution.

Peptides Adjacent to OSCP. The cross-linking reagents such as DMS and EEDQ mainly react with lysine or carboxyl groups that face the aqueous phase. Therefore, the fact that OSCP gives cross-linking products with subunits of the ATPase–ATP synthase indicates that OSCP and these subunits must possess hydrophilic domains that are close to each other. By cross-linking purified F1 with purified OSCP, Dupuis et

al. (Dupuis & Vignais, 1985; Dupuis et al., 1985) have shown that OSCP can be covalently bound to the α- and β-subunits of F1 with a preferential interaction with the α-subunit. However, these experiments could not ascertain whether the cross-linked products would be the same inside the membrane. In the present work where the experiments have been performed either with purified F0–F1 or with mitochondrial membranes, OSCP was also found to cross-react with both α- and β-subunits of F1, but no significant difference could be detected in the reaction of OSCP with α or β. Besides, another major cross-linking product of OSCP that could not be detected in studies made with soluble F1 and OSCP could be found in the complex. The 45-kDa OSCP–X could be tentatively assigned to an adduct between OSCP and the 24-kDa peptide found in mammalian and yeast complexes. The nature of the 45-kDa OSCP–X remains hypothetical and should be confirmed either by immunological characterization or by specific radiolabeling of the 24-kDa subunit. It is probably similar to the 45 kDa cross-linked product between OSCP and a 24-kDa peptide present in the F0–F1 complex observed by Torok and Joshi (1985). In the latter study, the cross-linking performed by treating purified F0–F1 complex with copper *o*-phenanthroline induced an inhibition of ATP synthesis.

Upon reconstitution of the [^{35}S]SNAP-labeled OSCP, additional cross-linking products were observed with peptides of about 30 kDa. Among these peptides, the γ-subunit of F1 could be a good candidate. Other peptides, such as the adenine nucleotide translocator and the phosphate carrier are very interesting to consider in the view of their possible functional interaction with the ATPase–ATP synthase complex. It should be observed that [^{125}I]TID also heavily labeled the same peptides. It would therefore be tempting to conclude that the cross-linking products observed with SNAP–OSCP are preferentially related to the more hydrophobic peptides. The reactive group of the SNAP involved in the reaction with peptides adjacent to OSCP is a nitrophenylazido group that has a much more hydrophobic character than the reactive groups of dimethyl suberimidate or EEDQ.

Can OSCP Be Considered as Equivalent to the b-Subunit of E. coli? Several data in the literature favor an analogy between OSCP and the b-subunit of *E. coli*. Ovchinnikov et al. (1984a,b) have shown that there is a homology in the amino acid sequence of OSCP and the central part of the *E. coli* b-subunit. The hydrophobicity profile of the two peptides is also similar. The studies of Foster and Fillingame (1982) and of Nielsen et al. (1981) indicate that the b-subunit of *E. coli* is the only peptide of the complex F0–F1 present in two copies. Similarly, Penin et al. (1985) have shown that the mitochondrial membrane contains two copies of OSCP per mole of F1.

Cross-linking experiments have shown that, in *E. coli*, the b-subunit interacts with the a-subunit, with the β-subunit, and presumably with the α-subunit (Aris & Simoni, 1984). In mitochondria, OSCP is adjacent to α, β, and presumably to the 24-kDa peptide, eventually analogous to the *E. coli* a-subunit, since it is also a hydrophobic peptide not easily removed from the membrane. In this respect, OSCP and the b-subunit seem to have a similar position in the complex.

However, the labeling with the hydrophobic probe [^{125}I]TID is different for these two protein. OSCP is not labeled at all with TID. Hoppe et al. (1984) used this reagent to identify the individual amino acid residues of the b-subunit of *E. coli* F0–F1 ATP synthase in contact with the lipid phase of the membranes. They have shown that this reagent labeled almost all amino acids of the N-terminal segment of this subunit, starting at the very beginning of the polypeptide chain and ending at position 27 or 28. They concluded that the b-subunit appears to be anchored to the membrane by this short hydrophobic N-terminal segment. It is interesting to note that the homology of sequence between OSCP and b-subunit aligns at position 1 of OSCP and position 21 of the b-subunit (Ovchinnikov et al., 1984a,b). Therefore, most of the part of the b-subunit anchored in the membrane is missing in OSCP. This can explain the difference in the reactivity of the two peptides toward TID in the membrane.

F1 affords some protection against trypsin proteolysis of either the b-subunit of *E. coli* (Hermolin et al., 1983; Perlin et al., 1983; Hoppe et al., 1983) or OSCP (Hundal et al., 1984). The trypsin cleavable segment of the b-subunit is not required for proton translocation through F0 (Hermolin et al., 1983; Perlin et al., 1983; Hoppe et al., 1983) although the b-subunit is compulsory for the reconstitution of proton translocation (Schneider & Altendorf, 1984). The site of trypsin cleavage of this subunit is assumed to be located at position 35, and the homology of sequence between b-subunit and OSCP starts at position 21 (Ovchinnikov et al., 1984a,b). This would mean that the N-terminal amino acids of *E. coli* b-subunit would be responsible for the role of this subunit in proton translocation while the function of OSCP would only be to keep F1 bound to the membrane in a conformation adequate for proton translocation and ATP synthesis.

In conclusion, the model of the ATPase–ATP synthase that would best explain our results includes an external position of OSCP as compared to F1 and an interaction of OSCP through hydrophilic domains with the α- and β-subunits of F1 and possibly with the 24-kDa peptide of F0. These interactions are analogous to interactions observed for the b-subunit of *E. coli*.

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Registry No. SNAP, 81705-07-9; EEDQ, 16357-59-8; DMS, 29878-26-0; ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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