Membrane Topography and Near-neighbor Relationships of the Mitochondrial ATP Synthase Subunits e, f, and g*

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The well characterized subunits of the bovine ATP synthase complex are the α, β, γ, δ, and ε subunits of the catalytic sector, F1; the ATPase inhibitor protein; and subunits a, b, c, and d, OSCP (oligomycin sensitivity-conferring protein), F6, and A6L, which are present in the membrane sector, F0 and the 45-Å-long stalk that connects F1 to F0. It has been shown recently that bovine ATP synthase preparations also contain three small polypeptides, designated e, f, and g, with respective molecular masses of 8.2, 10.2, and 11.3 kDa. To ascertain their involvement as bona fide subunits of the ATP synthase and to investigate their membrane topography and proximity to the above ATP synthase subunits, polyclonal antipeptide antibodies were raised in the rabbit to the COOH-terminal amino acid residues 57–70 of e, 75–86 of f, and 91–102 of g. It was shown that (i) e, f, and g could be immunoprecipitated with anti-OSCP IgG from a fraction of bovine submitochondrial particles enriched in oligomycin-sensitive ATPase; (ii) the NH2 termini of f and g are exposed on the matrix side of the mitochondrial inner membrane and can be curtailed by proteolysis; (iii) the COOH termini of all three polypeptides are exposed on the cytosolic side of the inner membrane; and (iv) f cross-links to A6L and to g, and e cross-links to g and appears to form an e-e dimer. Thus, the bovine ATP synthase complex appears to have 16 unlike subunits, twice as many as its counterpart in Escherichia coli.

Early examination of the polypeptide composition of highly purified bovine ATP synthase complex indicated the presence of at least 15 unlike polypeptides that could be separated by two-dimensional gel electrophoresis (1). Recent studies have suggested, however, that bovine ATP synthase may contain 16 unlike polypeptides (2). Those previously characterized are the α, β, γ, δ, and ε subunits of the catalytic sector, F1; the ATPase inhibitor protein that binds to a β subunit; and subunits a, b, c, and d, OSCP, F6, and A6L, which are contained in the membrane sector, F0 and the 45-Å-long stalk that connects F1 to F0. Subunits a and c are largely membrane-intercalated as in the case of their counterparts in the Escherichia coli ATP synthase complex (3). Also as in the E. coli enzyme, there are two b subunits that form the principal stem of the stalk (Refs. 4 and 5; see, however, Ref. 6). Each b subunit is considered to tra-

verse the membrane twice through hydrophobic stretches of amino acids near its NH2 terminus, with its NH2-terminal end (−30 residues) emerging from the membrane on the F1 side (6).

The remainder of each of the two b subunits (−130 residues) extends from F0 to F1. To these extramembranous segments of the b subunits as well as to F1 are attached OSCP (oligomycin sensitivity-conferring protein), d, and the two copies of F6. A6L is anchored to the membrane via its NH2-terminal 25–30 hydrophobic residues. The remainder of the molecule (total number of residues = 66) is extramembranous on the F1 side and resides near subunit d (5).

The newly characterized polypeptides that are found in preparations of bovine ATP synthase are three small molecules, designated e, f, and g, with respective molecular masses of 8.2, 10.2, and 11.3 kDa (7). Collinson et al. (2) have shown that an ATP synthase fraction prepared after treatment of bovine SMP1 with 3.3 M guanidine HCl, followed by solubilization with n-dodecyl β-D-maltoside and multiple column chromatographies, is devoid of F1 subunits and OSCP, but contains all the other F0 and stalk subunits mentioned above, including e, f, and g. Indeed, F1 subunits and OSCP are the only components of the bovine ATP synthase that are easily removed, as was shown previously by Racker and Horstman (8) with SMP (ASU particles) and by Galante et al. (1) with purified bovine ATP synthase. In addition, both of these preparations, i.e. the ASU particles and the (F1 + OSCP)-depleted ATP synthase, could be reconstituted with added F1 and OSCP, indicating that the removal of these components could be achieved without damage to the remainder of the ATP synthase complex (1, 9).

Whether the preparation of Collinson et al. (2) is capable of a similarly functional reconstitution with F1 and OSCP is not known. However, the fact that this purified preparation contains e, f, and g suggested that these polypeptides should be considered as possible subunits of the bovine ATP synthase complex.

This paper shows that e, f, and g are present in highly purified preparations of the ATP synthase complex with fully oligomycin-sensitive ATPase and uncoupler-sensitive ATP −32P exchange activities and can be immunoprecipitated from a SMP fraction enriched in ATP synthase with antibodies specific for OSCP. It also shows data regarding the membrane topography of these polypeptides in SMP as well as their proximity to one another and to other ATP synthase subunits.

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§ The abbreviations used are: SMP, submitochondrial particle(s); DST, disuccinimidyl tartarate; sulfo-DST, bis(sulfosuccinimidyl) tartarate; DSS, disuccinimidyl suberate; sulfo-DSS, bis(sulfosuccinimidyl) suberate; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EEDQ, N-(ethoxycarbonyl)-2-ethoxy-2-ethylhydroquinone; SANPAH, N-succinimidyl 6-(4-azido-2-nitrophenylamino)hexanoate; PAGE, polyacrylamide gel electrophoresis; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; TLCK, N-tosyl-L-lysine chloromethyl ketone; MOPS, 4-morpholinopropane-sulfonic acid.
EXPERIMENTAL PROCEDURES

Materials—Disuccinimidyl tartarate (DST), bis(sulfosuccinimidyl) tartarate (sulfo-DST), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (sulfo-DSS), 1-ethyl-3-[3-(dimethylamino)propyl]carbodimide (EDAC), N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EDQ), and N-succinimidyl-4-(5-azido-2-nitrophenyl)anisole (SANPAH) were obtained from Pierce. Nitrocellulose membrane (0.2-μm pore size) was from Schleicher & Schuell. SDS, acrylamide, precasted SDS-PAGE standards (broad range), and Bio-Gel A-5m were from Bio-Rad. TPCK-treated trypsin, TLCK-treated chymotrypsin, proteinase K, digitonin, Tween 20, and molecular mass standards for gel filtration were from Sigma. Goat horseshadish-conjugated anti-rabbit IgG was from Calbiochem, and [125I]-labeled protein A and enhanced chemiluminescence reagents were from Amersham Corp. Bovine ATP synthase was prepared according to Galante et al. (10), and bovine SMP according to Matsuno-Yagi and Hatefi (11). The latter and Saccharomyces cerevisiae SMP were supplied by Dr. A. Matsuno-Yagi, and rat liver SMP were kindly donated by Dr. P. L. Pedersen (Johns Hopkins University, Baltimore, MD).

Preparation of Mitochondria and Mitoplasts—Heavy bovine heart mitochondria were prepared according to Hatefi and Lester (12), and mitoplasts according to Krebs et al. (13). The milligram ratio of digitonin to mitochondrial protein for the preparation of mitoplasts was 0.24. The activities of monoamine oxidase (outside membrane enzyme) and malate dehydrogenase (matrix enzyme) in the mitoplast preparations were 90 and 95%, respectively, of the total activities of the original sample of mitochondrial protein.

Antipeptide Antibodies—Peptides corresponding to the COOH-terminal residues 57–70 of e (14), 75–86 of f, and 91–102 of g (2) plus an NH₂-terminal Cys for coupling to keyhole limpet hemocyanin were synthesized, linked to keyhole limpet hemocyanin, and used to immunize rabbits as previously described (5). Positively reacting IgG appeared in the sera after the third injections. The antibodies were affinity-purified using the respective peptides coupled to CNBr-Sepharose (Pharmacia Biotech Inc.) or antigens immobilized on nitrocellulose filters as described before (5). The affinity-purified IgG were stored at −20°C in small aliquots. Their potencies were tested by enzyme-linked immunosorbent assays, and their specificities by immunoblotting. In each case, the immunospecificity of the affinity-purified IgG was confirmed by blotting mitoplasts with the IgG preparation preincubated with the respective peptide antigen. Rabbit polyclonal antibodies against other ATP synthase subunits have been described elsewhere (5).

Isolation of the ATPase-rich Fraction of SMP and Immunoprecipitation—Both SMP were solubilized with deoxycholate in the presence of 1 mM KCl, conditions that solubilize all the respiratory chain and ATP synthase complexes (15, 16). The solubilized SMP were centrifuged for 20 min at 100,000 × g, and the supernatant was applied to a Bio-Gel A-5m column (2.5 × 65 cm; fractionation range of 1 × 10⁹ to 5 × 10¹⁰ Da) as described previously for the purification of the ATP synthase complex (10). Fractions of 3.8 ml were collected at a flow rate of 12 ml/h, as described previously for the purification of the ATP synthase complex (10), and 20 µl of each fraction was electrophoretically transferred to a nitrocellulose membrane, and immunoblotting with antibodies against e, f, and g or with immunoglobulin fractions prepared from the corresponding sera. The column was calibrated using molecular mass standards from Sigma.

Immunoprecipitation of the ATP synthase complex with anti-OSCP antibodies was performed essentially according to Robbins et al. (18), except that the concentration of Triton X-100 did not exceed 0.5% and 0.5 mM phenylmethylsulfonyl fluoride was included in all buffers. Extraction of SMP with 0.1 M Na₂CO₃ or 6 M urea was performed essentially according to Fujiki et al. (19). The buffers contained 0.5 mM phenylmethylsulfonyl fluoride. After 30 min on ice, the solubilized proteins were separated by centrifugation and precipitated with 10% trichloroacetic acid. Then, the insoluble and solubilized proteins were prepared for SDS-PAGE and immunoblotting.

Cross-linking Conditions—Washed SMP were suspended in 0.25 M sucrose containing 50 mM triethanolamine, pH 8.0, and adjusted to 1 mg/ml. Freshly prepared DSS in dimethylsulfoxide was added to a final concentration of 0.5 mM. After 40 min of incubation at room temperature, the reaction was terminated by the addition of ammonium acetate to 50 mM, and the samples were prepared for SDS-PAGE. Cross-linking conditions for DST, EDAC, sulfo-DST, and sulfo-DSS were the same as described above. For cross-linking with EEDQ, SMP were suspended at 1 mg/ml in 0.25 M sucrose containing 25 mM MOPS, pH 7.0. EEDQ dissolved in methanol was added to a final concentration of 10 mM, and after 40 min of incubation at room temperature, the reaction was stopped by the addition of a solution of lysine, pH 6.8, to 0.1 M. After 10 min, the samples were prepared for SDS-PAGE. For cross-linking with SANPAH or N-(5-azido-2-nitrobenzoyloxy)succinimide, SMP in the same buffer as indicated for DSS cross-linking were incubated for 20 min at room temperature in the dark with a 0.1 mM final concentration of the respective reagent freshly dissolved in dimethylformamide. Either excess reagent was removed by centrifugation and resuspension of SMP in buffer, or the modified particles were directly placed under a long wavelength UV lamp (8 watts) at a distance of 5 cm for 10 min on ice, with occasional mixing. Cross-linking of F₆-depleted ASU particles and purified ATP synthase with the above reagents was done similarly. Then, the samples were prepared for SDS-PAGE, electrophoresis to nitrocellulose, and immunoblotting.

Treatment of SMP and Mitoplasts with Proteases—SMP and freshly prepared mitoplasts were suspended in 3 ml of 0.25 M sucrose containing 0.5 M Tris-HCl, pH 7.5. TPCK-treated trypsin or TLCK-treated chymotrypsin was added at a protease/particle protein concentration of 1:100 and 1:10 (w/w), and the suspension was incubated for 30 min at 37°C. Where indicated, the proteolysis mixture also contained 0.4% deoxycholate. Proteolysis was stopped by the addition of 2 mM final concentration of phenylmethylsulfonyl fluoride, and aliquots were prepared for SDS-PAGE and immunoblotting.

Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed according to Laemmli (20), routinely using a separating gel containing 15% acrylamide. Gels were stained with Coomassie Brilliant Blue, or the protein bands were electrotransferred to nitrocellulose sheets according to Towbin et al. (21) and probed with the affinity-purified antibodies of interest as described before (5). The immunoblots were developed using enhanced chemiluminescence reagents.

Interaction of Antibodies with SMP and Mitoplasts—Particles (0.2–0.3 mg/ml) in 0.25 M sucrose containing 50 mM Tris-acetate, pH 7.7, and 0.5% bovine serum albumin were incubated for 2 h at 24°C with 1:100 to 1:10 diluted affinity-purified antibodies against e, f, and g or with immunoglobulin fractions prepared from the corresponding sera. The particles were then washed three times by centrifugation and resuspension in the same buffer. To 500 µl of particle suspension was added an equal volume of 125I-protein A (200,000 cpm), and the mixture was incubated for 1 h at 24°C. The particles were sedimented by centrifugation and washed three times with buffer, and their radioactivity was counted in a Packard Auto-Gamma Counter.

Protein Assay—Protein concentration was determined according to Lowry et al. (22), using bovine serum albumin as a standard. For mitochondria, mitoplasts, and SMP, protein concentration was estimated by the biuret method (23) in the presence of 0.1% potassium deoxycholate.

RESULTS AND DISCUSSION

Immunodetection of e, f, and g in SMP and the ATP Synthase Complex—Antipeptide antibodies were raised in the rabbit to keyhole limpet hemocyanin-linked synthetic peptides corresponding to the COOH-terminal 14 residues of e (Glu-57–Lys-70), 12 residues of f (Tyr-75–Tyr-86), and 12 residues of g (Ile-91–Val-102). Immunoblots of whole bovine heart mitochondria showed that each affinity-purified IgG was specific for the polypeptide it was intended and that each could be completely blocked by the synthetic peptide against which it was raised. Other experiments showed that e, f, and g were firmly bound to the mitochondrial inner membrane, and like subunits a and c of the ATP synthase complex, they could not be extracted with 6 M urea or at high pH (0.1 M Na₂CO₃). By comparison, F₁ subunits, OSCP, and d were extracted with 6 M urea, and F₁ subunits, OSCP, d, and F₆ with 0.1 M Na₂CO₃. In these extractions, F₁ and OSCP were removed completely, and d and F₆ partially (data not shown). As is shown in Fig. 1, e, f, and g are present in highly purified preparations of the bovine ATP synthase complex. They are difficult to distinguish on the SDS gel (lane 1), but are clearly exhibited when the ATP synthase complex is blotted with each affinity-purified antiserum (lanes 2–4). It can be seen that e (lane 2), f (lane 3), and F₆ (lane 5), with respective molecular masses of 8.2, 10.2, and 9.0 kDa, move very close to one another on the Laemmli-type SDS gel (15% acrylamide) and that g (lane 4) and A₆L (lane 6), with respective molecular masses of 11.3 and 8.0 kDa, band together. That the relative mobilities of e, f, and g on the SDS gel do not correspond to their molecular masses has been noted.
previously (2). Fig. 2 shows the cross-reactivity of antipeptide antibodies to bovine e, f, and g with polypeptides in rat liver SMP. It can be seen in lanes 4 and 6 of Fig. 2 that antigens recognized by our antipeptide antibodies to bovine g and f (lanes 3 and 5), respectively, are also present in rat liver sub-mitochondrial particles, with the mobility of rat liver f being clearly different from that of bovine heart. Assuming that the reactive epitope of rat liver f is also at the COOH terminus and that this polypeptide has not suffered proteolysis during preparation of rat liver SMP, the faster mobility of rat liver f may mean that its matrix-side extramembranous segment is shorter than its bovine counterpart (see below). The COOH-terminal peptide of bovine e against which our anti-e antibodies were raised has the amino acid sequence RELAEAEVDIFK (24). The corresponding segment of e from rat liver has the sequence ERELAEEDVSIFK. As seen in Fig. 2, our anti-e IgG did not recognize the rat protein, which appears to be referable to the differences in their COOH-terminal heptapeptides. In addition, our antipeptide antibodies to e, f, and g failed to recognize any polypeptides in mitochondria from S. cerevisiae.

Although e, f, and g are present in our highly purified bovine ATP synthase and also in the (F1 + OSCP)-depleted ATP synthase preparation of Collinson et al. (2), it was still desirable to see whether they can be immunoprecipitated together with other ATP synthase subunits, using antibodies to a well established ATP synthase subunit. For this purpose, bovine heart SMP were solubilized with deoxycholate (0.3 mg/ml of protein) in the presence of 1 M KCl, a procedure that solubilizes all five enzyme complexes of the mitochondrial electron transport/oxidative phosphorylation system (15, 16). Then, after a brief centrifugation at 100,000 × g, the supernatant was placed on a column of Bio-Gel A-5m as described under “Experimental Procedures,” and the fraction with the highest oligomycin-sensitive ATPase activity was collected. Using antibodies specific for OSCP, the ATP synthase complex was immunoprecipitated from this fraction and was shown by immunoblotting to contain e, f, and g (Fig. 3, lanes 1–3, respectively). Lane 4 of Fig. 3 is a control showing that these antigens were absent when preimmune serum was used for immunoprecipitation. The results of Fig. 3 demonstrate that e, f, and g occur in a fraction of sub-mitochondrial particles enriched in oligomycin-sensitive ATPase activity and can be immunoprecipitated therefrom with antibodies specific for OSCP. Hence, it could be concluded that e, f, and g are closely associated with the ATP synthase complex and may be considered as subunits of this enzyme complex. If so, this would mean that the mammalian ATP synthase complex is composed of 16 unlike subunits, twice as many as the unlike subunits of the bacterial ATP synthase complex.

Topography of e, f, and g in SMP—Although our affinity-purified antipeptide antibodies reacted strongly with e, f, and g in immunobLOTS (Figs. 1–3) as well as with the corresponding free peptides in enzyme-linked immunosorbent assays (data not shown), they exhibited no reactivity with their respective antigens in SMP and mitoplasts. We had encountered this situation with antibodies specific for other ATP synthase subunits and had shown that the membrane-bound antigens unreactive with antibodies were nevertheless accessible to proteases (4). With e, f, and g also, proteases provided the means
for studying their membrane topography, as summarized in Fig. 4. In Fig. 4, each vertical lane is a separate experiment, the conditions for which are given above the immunoblots. Thus, bovine SMP (lanes 1-7) or mitoplasts (lanes 8-11) were treated with a low (L) or high (H) concentration of trypsin or chymotrypsin in the absence or presence of deoxycholate, as specified under "Experimental Procedures," and then subjected to SDS gel electrophoresis and immunoblotting with affinity-purified anti-e, anti-f, or anti-g antipeptide antibodies. Lane 1 is a control showing immunoblots of e, f, and g. Conditions for each lane are shown at the top, where + and - indicate the absence and presence, respectively, of the material indicated on the left. The three sets of blots marked e, f, and g show only the appropriate immunoreactive molecular mass regions, which were cut from the remainder of each blot that was devoid of any immunoreactive bands. The broadening of the bands in lane 9 for g and in lane 10 for f may mean partial proteolysis of the COOH termini of these subunits without removal of their epitopes for antibody recognition.

The effect of chymotrypsin on f was small, but was much greater on g (lanes 10 and 11). Trypsin did not appear to degrade g in mitoplasts (see, however, the legend to Fig. 4). It is clear from the results of Fig. 4 that the carboxyl termini of e, f, and g are all exposed on the cytosolic side of the mitochondrial inner membrane and can be cleaved off by proteolysis (lanes 8-11). Furthermore, f and g appear to be transmembrane, with segments on the matrix side of the inner membrane that can be curtailed by chymotrypsin (lanes 5 and 6; see also the effect of trypsin on g in lanes 2 and 3). However, there is no indication from these results whether e is also transmembrane and exposed on the matrix side of the inner membrane. We also checked the effect of proteinase K on e, f, and g, and the results were essentially the same as those shown in Fig. 4 for chymotrypsin. Proteinase K hydrolyzed f and g in SMP, resulting in the formation of antigenic fragments, but had no effect on e from the matrix side of the inner membrane. Removal of F1 from SMP changed the kinetics of proteolysis of the remaining ATP synthase subunits, but not the final proteolysis results.

Near Neighbors of e, f, and g—Fig. 5 shows the results of attempts by cross-linking experiments to identify the near neighbors of e, f, and g in SMP. The cross-linking reagents used were EEDQ in Fig. 5A, DSS in Fig. 5B, and SANPAH in Fig. 5C, with respective cross-linking distances of 0, 11.4, and 18.2 Å. SMP were treated with the cross-linking reagents and then subjected to SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose sheets, and immunoblotting as described under "Experimental Procedures" and in the legend to Fig. 5. The affinity-purified antipeptide antibodies with which the nitrocellulose strips were blotted are indicated in Fig. 5 at the top of each pair of lanes, of which the left lane (odd-numbered lane) is the control SMP not subjected to cross-linking. Treatment of SMP (as well as (F1 + OSPC)-depleted particles and purified ATP synthase with EEDQ resulted in the appearance of a product with a relative molecular mass of 22 kDa, which was recognized by our anti-e antibodies only (Fig. 5A, lane 2). Because this product was not recognized by antibodies to other ATP synthase subunits (we do not have antibodies to the e subunit of F1), we assume that it is the result of cross-linking between two molecules, possibly an e-e dimer. EEDQ treatment of SMP did not produce any cross-linked products involving f and

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**Fig. 4.** Effects of trypsin and chymotrypsin on e, f, and g in SMP and mitoplasts. SMP or mitoplasts (3 mg/ml) were incubated with a low (L; protease/particle protein concentration of 1:100 (w/w)) or a high (H; protease/particle protein concentration of 1:10 (w/w)) concentration of TPCK-treated trypsin or TLCK-treated chymotrypsin for 30 min at 37 °C. Where indicated at the top, the sample also contained 0.4% deoxycholate (DOC). Proteolysis was terminated by the addition of 2 mM phenylmethylsulfonyl fluoride, and the samples were electrophoresed on three identical 15% SDS gels. The separated proteins were then transferred to nitrocellulose and blotted with affinity-purified antipeptide antibodies to e, f, and g. Conditions for each lane are shown at the top, where + and - indicate the absence and presence, respectively, of the material indicated on the left. The three sets of blots marked e, f, and g show only the appropriate immunoreactive molecular mass regions, which were cut from the remainder of each blot that was devoid of any immunoreactive bands. The broadening of the bands in lane 9 for g and in lane 10 for f may mean partial proteolysis of the COOH termini of these subunits without removal of their epitopes for antibody recognition.

**Fig. 5.** Cross-linking of e, f, and g to other ATP synthase subunits. Conditions for cross-linking, SDS-PAGE, and immunoblotting are described under "Experimental Procedures." A, SMP untreated (lane 1) or treated (lane 2) with 10 mM EEDQ for 40 min and blotted with affinity-purified anti-e antibodies; B, SMP untreated (lanes 1, 3, and 5) or treated (lanes 2, 4, and 6) with 0.5 mM DSS for 40 min and blotted with affinity-purified anti-e (lanes 1 and 2), anti-f (lanes 3 and 4), and anti-g (lanes 5 and 6) antibodies; C, SMP treated with 0.1 mM SANPAH for 20 min and then kept in the dark (lanes 1 and 3) or subjected to UV irradiation (lanes 2 and 4) (see "Experimental Procedures"). After SDS-PAGE and transfer to nitrocellulose, the proteins were blotted with affinity-purified anti-f (lanes 1 and 2) and anti-A6L (lanes 3 and 4) antibodies. SDS gels in A and C received 10 µg of SMP protein/lane, and those in B received 20 µg. The positions of prestained molecular mass markers (in kilodaltons) are shown on the left.
Treatments of SMP with DSS resulted in the appearance of cross-linked products that were recognized by antibodies to e (Fig. 5B, lane 2), e and g (lanes 2 and 6), and f and g (lanes 4 and 6). The cross-linked products e-g and f-g reacted weakly with anti-e and anti-f antibodies, as seen in Fig. 5B (lanes 2 and 4, respectively). For this reason, the amount of SMP applied to each lane of the SDS gel in the experiments shown in panel B was twice the amount used in each lane of the experiments shown in panels A and C. In Fig. 5C, we show the results of cross-linking experiments with the heterobifunctional photoreactive reagent SANPAH, of which the succinimidyl moiety reacts with free amino groups, and the substituted azidonitrophenyl yields a nitrene upon photoreactivation, capable of covalent modification of protein residues in its vicinity. It can be seen in Fig. 5C that the use of this reagent resulted in the formation of a cross-linked product of f and A6L (lanes 2 and 4). A similar band, but in much lower yield, was also produced when N-(5-azido-2-nitrobenzoyloxy)succinimide (cross-linking distance of 7.7 Å) was used instead of SANPAH. Photoliation of rabbit liver SMP after treatment with SANPAH did not cross-link f to A6L. This may be related to the possibility discussed above that the matrix-side extramembranous segment of a tail liver f may be shorter than its bovine counterpart.

In previous studies (5), it was found that DST (cross-linking distance of 6.4 Å) was highly effective in producing in SMP dimeric cross-linked products involving α, β, OSCP, b, d, F6, and A6L. In SMP, EDAC cross-linked b to F6, and in F1-depleted SMP, it also cross-linked d to F6. These reagents were ineffective in producing cross-linked products of e, f, and g in SMP. However, in the purified ATP synthase complex, DST produced e-b, f-b, and g-b cross-links. These protein bands exhibited relative molecular masses around 36 and 48 kDa, and the former reacted with anti-F6 antibodies as well (data not shown). Treatment of SMP with EDAC did not produce any cross-linked products involving f and g. In the purified ATP synthase complex, SANPAH produced cross-linked products involving e, g, and h. However, none of the reagents used in this study resulted in any cross-links involving e, f, or g with the F1 subunits α, β, γ, and δ.

Because the results of Fig. 4 indicated that the COOH terminus of e, f, and g as well as most (if not all) of the bulk of e are on the cytosolic side of the mitochondrial inner membrane, cross-linking experiments were also performed with mitoplasts (mitochondria denuded of outer membrane). The cross-linking reagents used were the water-soluble compounds sulfo-DST (cross-linking distance of 6.5 Å), sulfo-DSS (cross-linking distance of 11.4 Å), and EDAC (EEQW was also used; cross-linking distances of 0). The only cross-linked product of e, f, and g detected was a band similar to that in Fig. 5 (A, lane 2; B, lane 2), i.e. a possible e-f dimer. The results of Fig. 4 on the membrane topography of e and f agree with their hydrophathy profiles shown in Fig. 6. The hydrophathy profile of f clearly shows a hydrophobic cluster of 20 residues near its COOH terminus, which could traverse the mitochondrial inner membrane. In that case, the NH2-terminal 45–50 residues of the molecule could be on the matrix side of the inner membrane, and the COOH-terminal 10–15 residues on the cytosolic side. The amino acid sequence of f shows the presence of a tryptophan at position 22, a leucine at position 24, a methionine at position 25, and a phenylalanine at position 28, the carboxyl-terminal side of any one of which could be the site of chymotrypsin cleavage. These cleavages would result in an antigenic fragment –7–7.5 kDa, which agrees with the position of the cleavage product of f seen in Fig. 4 (lane 6). The hydrophathy profile of e also agrees with the data of Fig. 4. Thus, the hydrophilic COOH-terminal 45–50 residues of e could be on the cytosolic side of the inner membrane and could be susceptible in mitoplasts to proteolysis by trypsin or chymotrypsin (Fig. 4, lanes 8–11). As seen in Fig. 6, the NH2-terminal 25–30 residues of e are hydrophobic. This segment could anchor e to the inner membrane without protruding much from the matrix side. In contrast to e and f, the hydrophathy profile of g does not show any hydrophilic segments. However, it is possible that the first 60–65 NH2-terminal residues are extramembranous on the matrix side. Then, the protein enters the membrane and protrudes from the cytosolic side. In that case, multiple trypsin and chymotrypsin cleavage sites are available near the NH2 terminus of g to produce the products seen in lanes 2, 3, 5, and 6 of Fig. 4.

Fig. 7 summarizes in graphic form the results of Figs. 4 and 5. It can be seen that f is near A6L, which was shown earlier to cross-link with d (5), and that g is close to f and e. Because a cross-link between e and f was not detected in our studies, the two subunits have been placed on opposite sides of g in Fig. 7. As mentioned above, DST produced e-b, f-b, and g-b cross-links in ATP synthase preparations. These cross-linked products also reacted with anti-F6 antibodies. These data have not been
included in Fig. 7, however, because the cross-linked products mentioned were not seen with SMP.

Finally, one other point may be considered here. The amino acid sequence of the COOH-terminal half of e has the potential for a coiled-coil structure formation\(^2\) (possibly with another molecule of e), as does the amino acid sequence of the ATPase inhibitor protein (27). The latter has been reported to undergo a \(\beta\)-strand to \(\alpha\)-helix conformation change as the medium pH is changed from 6.5 to 8.0 (28). This conformation change has functional significance because at pH 7.0, the inhibitor protein binds to F\(_1\) in the presence of MgATP and inhibits ATP hydrolysis, and at pH >7.0, it is released (29). Whether e undergoes functionally significant conformation changes with pH also remains to be seen.

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