Oligomycin Sensitivity Conferring Protein of Mitochondrial ATP Synthase: Deletions in the N-Terminal End Cause Defects in Interactions with F_1, while Deletions in the C-Terminal End Cause Defects in Interactions with F_o[†]

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ABSTRACT: The structure/function relationships of oligomycin sensitivity conferring protein (OSCP) of bovine mitochondrial ATP synthase were studied by nested deletion mutagenesis, followed by analyses of the resultant OSCPs for their ability to restore partial reactions of ATP synthesis in OSCP-depleted F_1-F_o complexes. Our results indicate that, from the N-terminus of OSCP, up to 13 amino acid residues could be deleted without any effect on OSCP coupling activity. However, deletion of 16 or more residues led to a slow decline in the ability of resultant mutant forms to restore ATP synthesis. Compared to the wild-type form of OSCP, deletion mutant ND-28 (deletion of residues 1−28) is 50% as active in its ability to reconstitute ATP−P_i exchange activity. Detailed analyses of mutant ND-28 revealed that it was able to bind to the membrane segment (F_o) of ATP synthase and restore oligomycin-sensitive ATPase activity in OSCP-depleted F_1-F_o complexes. However, it did not bind to soluble segment F_1, nor did it confer cold stability to either soluble F_1 or reconstituted F_1-F_o complex. On the other hand, studies on nested deletions on the C-terminal end indicate that three residues could be deleted without compromising the energy-coupling activity of OSCP. However, truncations of five or more residues caused an impairment in the ability of resultant mutant forms to restore ATP−P_i exchange activity in OSCP-depleted complexes. Mutant CD-10 (deletion of amino acids 181−190) was completely ineffective as a coupling factor. Detailed analyses of this mutant revealed that the subunit was able to bind to soluble F_1 segment and confer cold stability to the enzyme but was neither able to associate with the membrane segment (F_o) nor able to reconstitute high oligomycin sensitivity in depleted F_1-F_o complexes. We take these data to suggest that the N-terminal end of OSCP corresponding to residues G16−N28 is essential for binding of the coupling factor to soluble F_1 but not for coupling the energy of proton translocation to the synthesis of ATP; on the other hand, the carboxyl-terminal end of OSCP containing amino acids K181−M186 is important for F_o−OSCP interactions as well as for the coupling of the energy of $\Delta\mu H^+$ during the synthesis of ATP. These results suggest a model for OSCP in which the N-terminus is associated with the F_1 segment and the C-terminus is associated with the F_o segment, while the central part of the polypeptide forms three or more helices constituting the stalk in the intact F_1F_o enzyme.

The ATP synthases (F_1F_o-type ATPase, H^+ ATPase) are membrane-bound enzymes that catalyze synthesis as well as hydrolysis of ATP by coupling the transmembrane movement of protons down the electrochemical gradient. These enzymes are found in the membranes of mitochondria, chloroplasts, and bacteria and show good conservation with respect to their overall structure and function. As the name implies, the F_1F_o ATPases consist of two segments: an extrinsic segment, F_1,[1] that contains the catalytic site(s) for the synthesis and hydrolysis of ATP and a membrane-spanning hydrophobic segment, F_o, that contains a transmembrane channel for proton transport [reviewed in Cross (1981), Senior (1988, 1990), Fillingame (1990), Penefsky and Cross (1991), and Boyer (1993)]. F_1 can be readily removed from the membrane, and in the soluble form it functions essentially as an ATP hydrolase. The ATPase activity of soluble F_1, unlike that of intact F_1F_o enzyme, is cold-labile and insensitive to energy-transfer inhibitors (oligomycin, DCCD) that are known to bind at the F_o segment [Vadineau et al., 1976]. Upon removal of F_1 the membrane fraction functions principally as a passive proton channel. F_1 is composed of five unlike subunits in a stoichiometric ratio of $\alpha_3\beta_3\gamma\delta\epsilon$ in all species examined. The

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[1] Abbreviations: F_1, membrane-extrinsic portion of the proton-translocating ATP synthase; F_6, coupling factor 6 of mitochondrial ATP synthase; EDTA, ethylenediaminetetraacetic acid; DCCD, *N,N′*-dicyclohexylcarbodiimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; P_i, inorganic phosphate; OSCP, oligomycin sensitivity conferring protein; TEP, 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 0.001% phenylmethanesulfonyl fluoride (PMSF); IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TBT·Cl, tributyltin chloride; buffer A, 50 mM Tris·SO_4, pH 8.0, 150 mM KCl, 50 mM NaCl, 1 mM ATP, 0.5 mM EDTA, and 0.05% Tween 20; WT, the wild-type form of recombinant OSCP; ECF_1F_o, F_1F_o from *Escherichia coli*.

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composition of F_o is species-dependent—the *Escherichia coli* F_o has the simplest structure and contains three subunits in a ratio of $a_1b_2c_{10-12}$ [Fillingame, 1990; Senior, 1990], whereas the one from bovine mitochondria is more complex and shows ten subunits (a, b, c, d, e, f, g, A6L, OSCP, and F_6) in a somewhat uncertain stoichiometry [Collinson et al., 1994a]. Low-resolution electron microscopy studies show that the F_1 and F_o parts are linked by a slender stalk approximately 45 Å long and 25−30 Å in diameter [Fernandez-Moran et al., 1964; Gogol et al., 1987; Lucken et al., 1990]. Although the subunit composition of stalk is not fully established, the one from bovine mitochondrial F_1F_o is believed to contain parts of subunits γ, δ, and ϵ of F_1 and OSCP, F_6, and subunits b and d of F_o [Collins et al., 1994 a−c; Abrahams et al., 1994]. A recent high-resolution X-ray structural study of bovine F_1 also reveals the presence of a 40-Å long stem at the base of F_1 particle which is considered to be part of the stalk and presumably represents the contribution of F_1 subunits to the makeup of the stalk [Abrahams et al., 1993].

As a result of investigations in the last two decades it is clear that (i) the synthesis of ATP at the catalytic sites takes place with virtually no change in free energy and (ii) the need for the energy of electrochemical proton gradient is primarily for the release of newly formed product (ATP) from one catalytic site and simultaneous binding of substrates (ADP and P_i) at the other catalytic site. There is further consensus that the coupling of proton movement down the electrochemical gradient in F_o to the synthesis/hydrolysis of ATP at the catalytic sites in F_1 is indirect and involves long-range conformational changes that are propagated via stalk-forming subunits of the enzyme [Boyer, 1989, 1993]. Thus, in view of the key role played by the stalk subunits in energy coupling, an elucidation of their structure/function relationships would be crucial in reconstructing the sequence of events that precede the synthesis of ATP.

The oligomycin sensitivity conferring protein (OSCP) is a small molecular weight (20 097 Da; 190 amino acid residues) subunit of mitochondrial ATP synthase, present in the stalk region between the F_1 and F_o segments [Kagawa & Racker, 1966; McLennan & Tzagoloff, 1968; Van de Stadt et al., 1972; Dupuis & Vignais, 1985; Pedersen & Carafoli, 1987]. Recent crystal structure studies of bovine heart F_1 indicate the presence of a 40-Å long stem at the base of the F_1 particle [Abrahams et al., 1993]. Next to the stem is a pit that extends 35 Å into F_1 and is believed to be occupied by OSCP and/or subunit b. It has been proposed that the stalk, stem, and central helical domain representing γ subunit of F_1 are all part of a mechanism for transfer of energy from F_o to the catalytic sites in the $\alpha_3\beta_3$ core. OSCP is considered to be homologous to subunit δ of *E. coli* F_1 on the basis of overall similarities in the predicted secondary structure and certain functional properties for the two polypeptides [Walker et al., 1982]. It has no intrinsic catalytic activity but is absolutely essential for restoration of ATP synthesis as well as for conferral to F_1 of the ability to be inhibited by oligomycin and DCCD in OSCP-depleted F_1F_o complexes [Senior, 1971; Joshi & Huang, 1991; McLennan & Tzagoloff, 1968; Kagawa & Racker, 1966]. It is well-known that OSCP can complex with either soluble F_1 [Dupuis et al., 1985] or isolated F_o [Dupuis & Vignais, 1987] but is not obligatory for F_1-catalyzed ATP hydrolysis or for F_o-mediated passive H^+ conduction [Pringle et al., 1990]. Furthermore, it is evident that OSCP is not necessary for binding of F_1 to the membrane [Pringle et al., 1990]. Nevertheless, OSCP is crucial for restoration of ATP synthesis in OSCP-depleted complexes. Thus, the primary role of OSCP is presumably in the transmission of energy of proton translocation, thereby facilitating the coupling of the otherwise uncoupled enzyme. *In vitro* reconstitution experiments to reassemble the stalk and stalk + F_1 complexes have shown that, of the subunits examined, OSCP is the only one that shows direct interactions with F_1; all others require OSCP before they can enter into interactions with the subunits of the catalytic segment [Collinson et al., 1994c]. These observations demonstrate that OSCP or subunits analogous to OSCP are central for correct F_1 and F_o interactions and energy coupling during ATP synthesis.

In our efforts to get a better understanding of the role of OSCP in mitochondrial energy coupling, we initiated a systematic study to localize domains of OSCP of functional and/or structural importance using a nested deletion strategy. The deletion forms were expressed *in vitro* in a heterologous expression system and the mutant forms of OSCP were analyzed for their ability (or inability) to reconstitute partial steps in ATP synthesis in OSCP-depleted complexes. Our previous studies indicated that amino acid residues K181−L190, the last 10 residues at the C-terminus (or some of the residues in this region), are critical for restoration of ATP synthesis in OSCP-depleted F_1F_o preparations [Joshi et al., 1992]. Our present data suggest that the N-terminal end is important for OSCP−F_1 interactions while the C-terminal end is crucial for OSCP−F_o interactions. On the basis of results of present experiments and previously published secondary predictions for OSCP, a model has been proposed to indicate its orientation in intact F_1F_o ATP synthase.

EXPERIMENTAL PROCEDURES

DNA polymerase I (Klenow fragment) and Sequenase version II were obtained from U.S. Biochemical Corp. (Cleveland, OH), and *Thermus aquaticus* DNA polymerase from Perkin-Elmer (Norwalk, CT). All other DNA-modifying enzymes and restriction endonucleases were obtained from New England Biolabs (Beverly, MA) and were used according to manufacturer's protocols. α-^{35}S-DeoxyATP and L-^{35}S-methionine were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively. dNTPs were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and acrylamide gel reagents were from Bio-Rad (Richmond, CA). All other chemicals were from Sigma (St. Louis, MO). Expression plasmid pRKOSCPWT and host cell strain *E. coli* BL21 (DE3) were generous gifts of Dr. John E. Walker (The Medical Research Council, Cambridge, U.K.).

Nomenclature of OSCP Mutants and Construction of OSCP Expressed Plasmids. In order to maximize recombinant OSCP expression, the target coding sequence was isolated from the original OSCP expression plasmid pKOSCP WT and recloned into a pET-based expression vector pMW7 [Joshi et al., 1992; Collinson et al., 1994c]. The new construct, pRKOSCP WT, yielded 50−80 mg of purified OSCP/L of cell culture. Mutant forms of OSCP having truncations were named on the basis of number of residues deleted from the specified terminus. Thus mutants ND-7, ND-8, ND-9, ND-11, ND-13, ND-16, ND-24, and ND-28 represent respectively deletion of 7, 8, 9, 11, 13, 16, 24, or 28 amino acid residues from the N-terminus; likewise

mutants CD-1, CD-2, CD-3, CD-5, CD-7, and CD-10 represent, respectively, deletion of 1, 2, 3, 5, 7, or 10 amino acid residues from the C-terminus of intact OSCP.

Deletions at the N-terminal end were performed by a progressive unidirectional exonucleolytic degradation approach according to Henikoff [1984]. Briefly, first a unique *Sph*I site was introduced in the OSCP coding sequence and a *Hin*dIII site was eliminated from the multiple coding region of the vector such that the resultant plasmid had unique *Nde*I, *Sph*I, and *Hin*dIII sites at positions −3, 3, and 10, respectively, in recombinant OSCP coding sequence. The mutations were performed using the Overlap Extension Technique involving PCR (Joshi et al., 1992). The mutagenized DNA samples were next treated with *Hin*dIII and *Sph*I, giving rise to a linear fragment with a unique 3′ recessive end. Samples obtained were next incubated with exonuclease III in order to achieve progressive nucleolytic degradation in the 3′ to 5′ direction in the strand with the unique 3′ recessive end. Aliquots were withdrawn at several time points and were incubated first with mung bean S1 nuclease, to degrade single-stranded DNA, followed by T_4 DNA ligase to recircularize the resultant double-stranded DNA. This strategy resulted in retention of codon for the initiating methionine but in elimination of codons for the next three residues from OSCP coding sequence prior to any DNA degradation by exonuclease III. Thus, the recombinant OSCPs so produced had a minimum deletion of three residues from the N-terminus. The C-terminal deletion mutants were produced by replacing the native codon by stop codon TAA at the deletion site. For deletion mutants CD-5, CD-7, and CD-10, an additional *Eco*RI site was introduced downstream of the new stop codon.

Transformation of E. coli KP3998 and BL21 (DE3) and Expression of OSCP Gene. In order to establish the DNA sequence of replaced codons or confirm the absence of deleted codons in the mutagenized forms of the OSCP gene, *E. coli* strain KP3998 was transformed with various expression plasmids. The transformants were grown in LB agar containing 100 μg of ampicillin/mL. The substitution of native codons at the deletion/substitution site(s) was determined by dideoxy sequencing [Sanger et al., 1977]. In order to induce protein expression, *E. coli* strain BL21 (DE3) was transformed with OSCP expression plasmids and the transformants were grown overnight at 37 °C in LB agar containing 100 μg of ampicillin/mL [Studier et al., 1990]. The next day a single colony of transformed cells was inoculated into 1 L of 2× TY medium containing 100 μg of ampicillin/mL and the culture was grown at 37 °C to an A_{600} of 0.6 (7−9 h). At that point IPTG was added to a final concentration of 0.6 mM together with additional ampicillin to 100 μg/mL, and growth was resumed for 4−5 h [Collinson et al., 1994c].

In Vivo Radiolabeling of OSCP. Mutant forms ND-28 and CD-10 and WT form of OSCP were radiolabeled *in vivo* using ^{35}S-methionine (1.01 Ci/μmol; 10 mCi/mL) essentially according to Mukhopadhyay et al. (1992). Briefly, cells were grown at 37 °C in M9 medium containing 100 μg/mL ampicillin to A_{600} of 0.6. To 10 mL of the above culture IPTG was added to 0.7 mM concentration and ampicillin to 100 μg/mL of culture, and the incubation was continued for another hour. Rifampicin (200 μg/mL) was added and growth was continued for 1.5 h. Next, 150 μCi of ^{35}S-methionine was added. After overnight incubation at 37 °C, the cells were collected by centrifugation and stored at −20 °C until use.

Purification of Recombinant OSCP. Cells were harvested by centrifugation for 15 min at 7000 rpm in a Sorvall centrifuge (GSA rotor). The sediment from each liter of culture was suspended in 25 mL of TEP buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 0.001% PMSF) and the cells were lysed by passing twice through a French press at 14 000 lb/in^2 at 0−4 °C. The lysate was centrifuged for 20 min at 18 000 rpm in an SS34 rotor in order to sediment the inclusion bodies, which contain recombinant OSCP. The sediments were washed three times by resuspension in TEP buffer followed by centrifugation. The pellets were stored at −20 °C. Solubilization of recombinant OSCP and its further purification were conducted essentially according to Collinson et al. (1994c). Briefly, inclusion bodies isolated from 0.5 L of culture were resuspended by homogenization in 100 mL of OSCP buffer (50 mM Tris·HCl, pH 7.0, containing 5 mM 2-mercaptoethanol, 0.001% PMSF, and 1 mM EDTA) containing 0.2 M NaCl and 6.0 M guanidine hydrochloride. Insoluble material, if any, was removed by centrifugation of the suspension for 10 min at 8000 rpm in a Sorvall centrifuge. The supernatant fraction was subjected to overnight (minimum 18 h) dialysis against 4 L of the same buffer as above but without guanidine hydrochloride, using Spectrapor 6 dialysis tubing (MWCO = 1000). Dialyzed material was subjected to brief centrifugation (10 min at 8000 rpm) to remove any precipitate developed during dialysis. The clear supernatant was applied to an S-Sepharose Fast Flow column (10 × 2.6 cm i.d.) equilibrated with buffer containing 0.2 M NaCl. The column was developed with a linear gradient between 100 mL of OSCP buffer containing 0.2 M NaCl and 100 mL of OSCP buffer containing 1.0 M NaCl. The elution rate was approximately 2−2.5 mL/min, and fractions (approximately 4.0−5.0 mL) were collected 2 min apart. Fractions were analyzed by UV at 276 nm and the ones showing absorbance >0.05 were subjected to SDS−PAGE in order to assess protein purity. The fractions of interest were pooled and stored either at 4 or at −20 °C.

N-Terminal deletion mutants were purified by a modified procedure. The supernatant fraction obtained after solubilization of inclusion bodies was dialyzed against OSCP buffer that contained 50 mM NaCl instead of 200 mM NaCl. The dialyzed material was first applied to a DEAE-Sephadex column equilibrated with the same buffer as the recombinant protein. Fractions were analyzed by UV at 260 as well as at 276 nm. The ones showing absorbance of >0.05 at 276 nm but <0.005 at 260 nm were pooled and applied next to an S-Sepharose column equilibrated with the same buffer as the resuspension buffer for initial OSCP inclusion bodies. The column was developed with a linear gradient between 100 mL of a buffer containing 50 mM NaCl and 100 mL of buffer containing 300 mM NaCl. Samples enriched in OSCP as judged by A_{276} were pooled.

Purification of ^{35}S-OSCP. The cells of an *in vivo* labeled culture were collected and lysed by French press, and ^{35}S-OSCP was purified essentially as described for purification of unlabeled OSCP except for the following modifications. The inclusion body pellet obtained from radiolabeled cells was diluted with the inclusion body fraction, derived from a 200-mL culture of corresponding unlabeled OSCP. The pellet from mixed cells was solubilized in 10 mL of OSCP buffer containing 0.2M NaCl and 6.0 M guanidine hydrochloride and subjected to overnight dialysis. The supernatant

fraction from the dialysate was applied to a 2.5 × 0.5 cm S-Sepharose column equilibrated with OSCP buffer containing 0.2 M NaCl. After the unadsorbed protein was washed down, the column was developed directly with buffer containing 0.7 M NaCl. Fractions were monitored by A_{276} as well as by counting radioactivity.

Other Methods. All standard DNA manipulations [Sambrook et al., 1989; Wood et al., 1985; Ausubel et al., 1987] and electrophoretic procedures [Joshi & Burrows, 1990] were carried out according to published protocols unless otherwise stated. Oligodeoxynucleotides were synthesized with a Milligen/BioSearch cyclone DNA synthesizer in accordance with standard automated phosphoramidite chemistry.

OSCP-depleted submitochondrial particles (AE-P) were isolated according to Fessenden and Racker (1967) except for conducting the depletion at pH 9.8 instead of 9.2. Purified membrane preparations depleted of F_1 and OSCP (UF_o) were isolated according to Pringle et al. (1990). Bovine heart mitochondria [Joshi & Sanadi, 1979], OSCP [Senior, 1971], and F_1-ATPase [Horstman & Racker, 1970] were isolated as described previously. The F_1-ATPase had a specific activity of 55−70 μmol min^{-1} mg^{-1}. It was stored at 4 °C in 50% saturated ammonium sulfate. Before use, F_1-ATPase was centrifuged for 5 min in a microcentrifuge at 14 000 rpm, dissolved in the desired buffer, and desalted on a Sephadex G-25 column equilibrated with the buffer of interest. SDS−polyacrylamide gel electrophoresis, electrotransfer, staining for protein, and Western blotting were carried out as described previously [Joshi & Burrows, 1990].

Circular Dichroism Analyses. CD spectra were carried out on an Aviv 62DS spectrometer (Aviv Associates, Lakewood, NJ) with a temperature control accessory (Hewlett-Packard, Palo Alto, CA). OSCP was suspended in 20 mM Tris·HCl, pH 7.5, to 0.20 mg/mL and spectra were recorded from 250 to 180 nm at room temperature in a 0.05-cm quartz cell. Stability of OSCP folding conformation to temperature changes was examined by monitoring the CD spectra at different temperatures. Spectra recorded were averages of three scans and were corrected for baseline contribution due to suspension buffer.

Reconstitution of Membrane-Bound ATPase Activity. Membrane-bound ATPase complexes were reconstituted by adding F_1 and OSCP to membrane fractions depleted of F_1 and OSCP (UF_o) as described in Pringle et al. (1990). Briefly, aliquots of UF_o (50 μg) were incubated at 30 °C in a total volume of 50 μL containing F_1 (12.5 μg), varying amounts of OSCP, and 0.25 M sucrose, 50 mM Tris·acetate, 10 mM DTT, and 0.02% Tween 20 (pH 7.5 buffer). After 30 min, 50 μL of 20 mM Tris·HCl buffer, pH 8.5, was added. The ATPase activity was measured on a 10-μL aliquot as described before [Pringle et al., 1990].

Reconstitution of $^{32}P_i$-ATP Exchange. For reconstitution of $^{32}P_i$−ATP exchange activity, 200-μg aliquots of OSCP- and F_1-depleted submitochondrial particles (AE-P) were incubated with 40 μg of F_1-ATPase and 0.2 μg of recombinant OSCP for 10 min at 23 °C in the presence of 50 μmol of Tricine·KOH buffer (pH 8.0) containing 0.25 μmol of $MgCl_2$, 0.5 μmol of DTT, and 1.25 mg of bovine serum albumin in a total volume of 0.25 mL. The exchange activity was initiated by adding 200 μL of a solution containing 7.5 μmol of ATP, 2.5 μmol of ADP, 10 μmol of $MgCl_2$ (pH 7.5), and 50 μL of a solution containing 10 μmol of potassium phosphate buffer (pH 8.0) and 500 000 cpm of $^{32}P_i$. Samples were incubated at 37 °C. After 15 min the enzymatic activity was terminated by adding 0.25 mL of 20% trichloroacetic acid. [γ-^{32}P]ATP was separated from $^{32}P_i$ as described previously [Joshi et al., 1985].

Binding of ^{35}S-OSCP to F_1-ATPase. Binding of ^{35}S-OSCP to F_1-ATPase was determined by separating F_1-bound OSCP from free OSCP by centrifugation through a spin column filled with a cation exchanger, CM Sephadex C-25 [Dupuis et al., 1985]. Briefly, 38.3-μg aliquots of F_1-ATPase were incubated with ^{35}S-OSCP for 10 min at 30 °C in 115 μL of a buffer containing 50 mM Tris·SO_4, 50 mM NaCl, 1 mM ATP, 0.5 mM EDTA, and 0.05% Tween 20, pH 8.0. In order to separate the F_1-bound OSCP from free OSCP, the incubation mixture was centrifuged through 150 μL of CM Sephadex C-25 medium, equilibrated in the same buffer as described above, and contained in a 1-mL tuberculin syringe. OSCP that is not bound to F_1 is retained by the column due to its net positive charge, while the F_1-bound OSCP passes through the column. The incubation mixture and column eluates were monitored by liquid scintillation counting in order to determine the binding stoichiometry of OSCP to F_1-ATPase. Controls for elution of free OSCP gave values less than 5% of OSCP bound to the column. The biological activity of OSCP and F_1-ATPase remained unchanged in the course of the experiment. For measuring the binding of deletion mutant ND-28, the buffer utilized contained 50 mM Tris·SO_4, 50 mM NaCl, 1 mM ATP, 0.5 mM EDTA, and 0.025% Tween 20.

Binding of ^{35}S-OSCP to UF_o. Binding of WT and deletion mutant forms of OSCP was performed essentially as described by Dupuis and Vignais (1987). Briefly, aliquots of UF_o (400 μg) were incubated for 1 h at 30 °C with ^{35}S-OSCP in the absence or presence of F_1-ATPase (250 μg of F_1/mg of UF_o) in an 800-μL reaction volume of buffer A (50 mM Tris·SO_4, 150 mM KCl, 50 mM NaCl, 1 mM ATP, 0.5 mM EDTA, and 0.05% Tween 20, pH 8.0). Reconstituted particles were sedimented by centrifugation for 30 min in an Eppendorf centrifuge. A 750-μL aliquot was withdrawn from the supernatant fraction for measurement of free ^{35}S-OSCP. The rest of the samples were centrifuged for another 15 min and the tubes were carefully drained in order to remove the last traces of the supernatant. The pellets containing UF_o-bound ^{35}S-OSCP were resuspended in 500 μL of buffer A, and aliquots of suspension were counted for determining the binding stoichiometry of OSCP to UF_o or UF_o·F_1.

Binding Affinity of ^{35}S-OSCP for UF_o and UF_o·F_1 Complexes. In order to determine the affinity of OSCP for UF_o or UF_o·F_1 complex, 200-μg aliquots of UF_o were incubated at 30 °C with 0.48 nmol of ^{35}S-OSCP and, where indicated, with 0.5 nmol of F_1-ATPase in a total volume of 400 μL of buffer A as described in the preceding paragraph. After 1 h, an 80-μL aliquot containing a 10-fold excess of unlabeled OSCP/OSCP buffer was added and incubation was continued for another 30 min. Procedures for separating the UF_o-bound OSCP from unbound OSCP have been described in the preceding paragraph.

Presentation of Data. The data shown in various figures and tables are averages of five independent experiments that have corresponding values in close agreement.

RESULTS

Expression of Mutant Forms of OSCP. The expression of WT as well as mutant forms of OSCP was successful in

E. coli, and the recombinant protein accumulated in inclusion bodies in every single case. Western blots of total cell homogenates of various mutant forms revealed a single band whose SDS−PAGE mobility was found to be consistent with that derived from the coding sequence for the respective transcript (data not presented). This suggested that the truncation products are of the desired size and are indistinguishable from the WT OSCP with respect to stability *in vivo* in the host and subcellular location.

The solubilization of recombinant OSCPs was readily achieved by resuspension of the inclusion body pellet in buffer containing 6.0 M guanidine hydrochloride. The soluble fraction was better than 90% OSCP as judged by Coomassie blue staining of samples subjected to SDS−PAGE. However, this fraction had a much higher absorbance ratio (1.08 ± 0.05) at 260/276 nm compared to the value determined for pure OSCP (0.52 ± 0.05), suggesting that a nonproteinaceous material with a high absorbance at 260 nm was associated with OSCP in the inclusion bodies. The separation of OSCP from this material could be achieved by chromatography on an S-Sepharose column—the nonproteinaceous material appeared in the void volume while OSCP adhered to the cation exchanger. Pure OSCP was eluted at 380−430 mM NaCl on a linear salt gradient.

The CD mutant forms could be purified, similar to the WT form, by S-Sepharose column chromatography. However, the protein component for the ND mutant forms, especially the ones with deletion of more than 6 residues, did not adhere to the cation exchanger under the conditions used and collected unadsorbed together with the nonprotein contaminant in the flowthrough volume. This indicates that the ND mutant forms have a lower net positive charge in comparison to WT or CD mutant forms of OSCP. This is not surprising in view of the fact that the segment of OSCP being deleted contains no negative charges and up to four positively charged residues. Hence, the procedure for purifying ND mutant forms was slightly modified—the salt concentration in the dialysis buffer was dropped to 50 mM and the dialysate was subjected to DEAE-Sephadex chromatography prior to the S-Sepharose step. The contaminant with high absorbance at 260 nm was retained by the anion exchanger while the fractions corresponding to OSCP were not adsorbed and were collected in the void volume. Fractions enriched in OSCP were pooled and applied to an S-Sepharose column that was equilibrated with a buffer containing 50 mM NaCl. The ND mutant protein adhered to the cation exchanger at the lower salt concentration and could be eluted with a linear salt gradient containing 50−300 mM NaCl.

Circular Dichroism and Secondary Structure Analyses. The near-UV CD spectrum of WT OSCP showed negative minima at 220 and 208 nm, characteristic of a helical structure (data not shown). Secondary structure calculations indicated the composition to be 40.6% α-helix, 22.6% β-sheet, 10.8% β-turn, and 25.9% unordered structures for the WT form. CD spectra of OSCP at different temperatures indicated that the protein began to unfold at 45 °C and was completely denatured by 55 °C. However, upon lowering the temperature of the heated OSCP sample back to 5 °C, the original CD spectrum reappeared, suggesting that the temperature-induced unfolding transition of OSCP is reversible. Similar unfolding of OSCP was also observed upon addition of 6.0 M guanidine hydrochloride or 8.0 M urea to the protein solution, which could be reversed by subsequent removal of denaturing agents.

Table 1: Progressive Deletions in the N-Terminal End of OSCP Result in Increasing Losses in the Ability of Resultant Mutants to Reconstitute ATP−P_i Exchange Activity in OSCP-Depleted Complexes[a]

	P_i−ATP exchange [nmol min^{-1} (mg of AE-P)$^{-1}$]
AE-P + F_1	15
AE-P + F_1 + WT OSCP	170
AE-P + F_1 + mutant OSCP ND-7[b]	187
AE-P + F_1 + mutant OSCP ND-8	183
AE-P + F_1 + mutant OSCP ND-9	190
AE-P + F_1 + mutant OSCP ND-11	188
AE-P + F_1 + mutant OSCP ND-13	170
AE-P + F_1 + mutant OSCP ND-16	151
AE-P + F_1 + mutant OSCP ND-24	119
AE-P + F_1 + mutant OSCP ND-28	86

[a] Submitochondrial particles that were depleted of OSCP and F_1-ATPase (AE-P) were reconstituted with F_1 and various deletion mutant forms of OSCP and assayed for their ability to catalyze $^{32}P_i$−ATP exchange activity as described [Joshi et al., 1985]. The ATP−P_i exchange activity of AE particles is 8.5 nmol min^{-1} (mg of AE-P)$^{-1}$. [b] The numbers after ND (N terminal deletions) indicate the number of amino acid residues deleted starting from the N-terminus.

Table 2: Smaller Deletions within the K181−L190 Region Lead to a Progressive Loss in the Ability of Resultant Mutants to Reconstitute P_i−ATP Exchange Activity in OSCP-Depleted Complexes[a]

	P_i−ATP exchange [nmol min^{-1} (mg of AE-P)$^{-1}$]
AE-P + F_1	17
AE-P + F_1 + WT OSCP	166
AE-P + F_1 + mutant OSCP CD-1[b]	164
AE-P + F_1 + mutant OSCP CD-2	182
AE-P + F_1 + mutant OSCP CD-3	190
AE-P + F_1 + mutant OSCP CD-5	133
AE-P + F_1 + mutant OSCP CD-7	111
AE-P + F_1 + mutant OSCP CD-10	19

[a] Submitochondrial particles that were depleted of OSCP and F_1-ATPase (AE-P) were reconstituted with F_1 and various deletion mutant forms of OSCP and assayed for their ability to catalyze $^{32}P_i$−ATP exchange activity as described. [b] The numbers after CD (C-terminal deletions) indicate the number of amino acid residues deleted starting from the C-terminus.

Mutants forms CD-7, CD-10, ND-13, and ND-28 were also analyzed by CD in order to detect any putative alterations and/or defects in protein folding. The spectrum for each mutant form was found to be essentially similar to that of the WT OSCP except for ND-28, in which the α-helix contribution dropped from 40.6% to 32%. This drop in α-helix character is consistent with the deletion of a putative α-helix that has been proposed for the region corresponding to residues 14-24 in OSCP sequence [Engelbrecht et al., 1991].

Reconstitution of $^{32}P_i$-ATP Exchange. In order to determine the ability of various mutant forms of OSCP to restore the P_i−ATP exchange activity in OSCP-depleted preparations, submitochondrial particles were prepared by extraction of mitochondria with ammonia and EDTA at pH 9.8. These particles have a low exchange activity [8−10 nmol min^{-1} (mg of AE-P)$^{-1}$], which is maximally stimulated by a factor of 2 following supplementation with F_1-ATPase (Tables 1 and 2, row 1). However, addition of the WT form of recombinant OSCP gave rise to greater than 10-fold enhancement of exchange activity rates (compare row 2 to row

1 in Tables 1 and 2). This demonstrates that the AE particles obtained at pH 9.8 are also deficient with respect to OSCP. The amount of OSCP required for the restoration of 50% of maximum stimulation ranged between 0.3 and 0.4 μg of OSCP/mg of AE-P. These results show that a test for the stimulation of P_i−ATP exchange activity of pH 9.8 AE particles by OSCP constitutes a sensitive assay for measuring the energy coupling efficiency of this coupling factor. In independent experiments the radiolabeled ^{35}S-OSCP was found to be just as efficient a coupling factor as unlabeled OSCP, as established by the titration curves of radiolabeled versus unlabeled OSCP for stimulating exchange activity rates of reconstituted AE particles (data not presented).

Data in rows 3−7 in Table 1 demonstrate that deletion of the first 13 amino acid residues had no significant effect on the ability of resultant OSCPs to promote energy coupling. If anything, the improvement in exchange rates provided by ND-7 to ND-11 mutant forms was slightly higher compared to the one provided by the WT form of OSCP (Table 1, compare rows 3−6 with row 2). However, the removal of 16 or more residues led to progressively increasing losses in the ability of truncated OSCPs to restore ATP−P_i exchange activity in reconstituted particles (Table 1, compare rows 8−10 with row 2). Parallel studies involving deletions at the C-terminal end of OSCP revealed that up to three amino acid residues could be deleted without inactivating OSCP (Table 2, compare rows 3−5 with row 2). However, the elimination of five or more residues gave rise to an increasing impairment in the ability of truncated OSCPs to enhance energy coupling (Table 2, compare rows 6−8 with row 2). These data suggest that the border for functional OSCP lies 13−15 residues away from its N-terminus and 3 or 4 residues away from its C-terminus.

In order to understand whether the deletion-associated loss in OSCP coupling function is due to inability of the altered subunit(s) to confer inhibitor sensitivity to reconstituted ATPase or confer cold-stability to reconstituted F_1F_o enzyme, or to defects in their interactions with F_1, F_o, or both F_1 and F_o segments, mutant forms ND-28 and CD-10 were selected for a detailed analysis of their ability to reconstitute partial reactions of ATP synthesis in OSCP-depleted preparations.

Reconstitution of Inhibitor-Sensitive ATPase. In order to study the effect of deleting the terminal ends of OSCP on the ability of resultant mutants to restore inhibitor-sensitive ATPase, OSCP-depleted membrane fractions (UF_o) of purified F_1F_o ATPase were recombined with soluble F_1 and various recombinant forms of OSCP. As reported previously, these membrane fractions have no OSCP as detected by SDS−PAGE and Western blot analyses against anti-OSCP antiserum and are completely dependent on additional OSCP for reassembly of oligomycin-sensitive ATPase [Pringle et al., 1990]. Data presented in Table 3 demonstrate that the ATPase activity of reconstituted UF_o·F_1 complex in the absence of exogenous OSCP had very low (<10%) sensitivity to F_o inhibitory ligands oligomycin, DCCD, TBT·Cl, or venturicidin (Table 3, column 2). However, the inhibitor sensitivity was increased sharply to >84% following the addition of the WT form of OSCP (Table 3, column 3). When mutant form ND-28 was used during reconstitution, the results obtained were essentially similar to the ones observed for complexes containing WT form of OSCP (Table 3, column 4). On the other hand, the ATPase activity of complexes containing mutant form CD-10 was inhibited partially by oligomycin or DCCD and only marginally by TBT·Cl or venturicidin (Table 3, column 5). These studies suggest that amino acids K181−L190 of OSCP are necessary for inhibition of coupling between proton translocation in F_o and catalytic site events in F_1 in reconstituted F_1·F_o complexes while residues F1−R27 are not critical for this inhibition. Thus, it would appear that the observed defects in the coupling efficiency of deletion mutant ND-28 could not be due to its inability to reassemble inhibitor-sensitive ATPase activity.

Table 3: Deletions at the N-Terminal End Do Not Affect but Deletions at the C-Terminal End Result in a Decrease in the Inhibitor Sensitivity of Reconstituted ATPase in OSCP-Depleted Complexes[a]

	% inhibitor sensitivity addition of OSCP			
addition of inhibitor	− OSCP	WT OSCP	ND-28 OSCP	CD-10 OSCP
+ 0.5 μg of oligomycin	8	99	95	40
+ 50 μM DCCD	2	99	93	40
+ 50 μM TBT·Cl	0	97	68	20
+ 10 μM venturicidin	6	84	75	8

[a] Aliquots (50 μg) of UF_o were reconstituted with 12.5 μg of F_1 and 5 μg of OSCP and assayed for ATPase activity as described under Experimental Procedures. In order to determine the inhibitor sensitivity, aliquots of reconstituted ATPase complexes were incubated with 1 μg of oligomycin (10 min at room temperature), 50 μM DCCD (30 min at room temperature), 50 μM TBT·Cl (10 min at room temperature), or 10 μM venturicidin (75 min on ice) prior to assay for ATPase activity. The specific ATPase activity of reconstituted UF_o·F_1·OSCP complex without any inhibitor was 8.5 μmol min^{-1} (mg of UF_o)$^{-1}$.

Table 4: Deletions at the C-Terminal End Do Not Affect but Deletions at the N-Terminal End Lead to a Decrease in the Cold Stability of Reconstituted ATPase Complexes[a]

	ATPase activity (μmol min^{-1} mg^{-1})		
reconstituted complex	17 h at 23 °C	17 h at 0 °C	% cold stability
UF_o·F_1	7.3	0.6	8.2
UF_o·F_1·WT OSCP	8.8	6.5	73.8
UF_o·F_1·ND-28	7.2	1.9	26.4
UF_o·F_1·CD-10	9.0	6.2	68.9

[a] Reconstitution of membrane fraction with F_1 and OSCP was carried out as described under Table 3. In order to test the stability of these complexes to low temperature, small aliquots were incubated on ice for 17 h prior to assay for catalytic activity.

Reconstitution of Cold-Stable ATPase. In order to find out whether deletions at the ends of OSCP have any impact on the ability of resultant recombinants to reconstitute cold-stable ATPase, samples of OSCP-depleted membrane fraction of F_1·F_o ATPase were combined with soluble F_1 and various OSCPs and incubated for 17 h at 0 °C prior to assay for catalytic activity. Data in Table 4 demonstrate that the ATPase activity of reconstituted UF_o·F_1 with no OSCP (row 1), or of complexes containing mutant form ND-28 (row 3), was >70% inactivated upon incubation of the samples at low temperature (rows 1 and 3; compare column 3 with column 2 in corresponding rows). On the other hand, complexes containing either the WT (row 2) or mutant form CD-10 (row 4) retained nearly 70% of their activity under similar conditions (rows 2 and 4, compare column 3 with column 2). However, the residual ATPase activity of complexes containing WT or ND-28 mutant form could still be inhibited up to 95% by oligomycin, suggesting that the F_1 in these complexes must be membrane-bound. Since preservation of cold-stable ATPase is primarily a function of F_1−OSCP interactions, it would appear that the region of

Table 5: Deletions at the N-Terminal End Do Not Affect but the Ones at the C-Terminal End Result in Decreased Binding of the Subunit to F_1-ATPase[a]

addition of OSCP (μM)	OSCP bound (mol of OSCP/mol of F_1)		
	WT	ND-28	CD-10
3.0	0.63	0.03	0.81
4.5		0.03	
6.0	0.75	0.03	0.89

[a] Aliquots (50 μg) of F_1-ATPase were incubated for 10 min at 30 °C with indicated concentrations of ^{35}S-labeled WT or mutant forms of OSCP in a 115-μL volume of a buffer consisting of 50 mM Tris·SO_4, 1 mM ATP, 0.5 mM EDTA, 50 mM NaCl, and 0.05% Tween 20. In order to separate the F_1−OSCP complex from free OSCP, the incubation mixture was centrifuged through a spun column containing CM-Sephadex C25 as described under Experimental Procedures. Controls for elution of free ^{35}S-OSCP gave values lower than 5% of bound ^{35}S-OSCP.

OSCP corresponding to residues K181−L190 is not as critical while the one corresponding to residues F1−R27 is important for interactions of the protein with the F_1 part in intact F_1F_o enzyme.

Binding of OSCP to Soluble F_1 or OSCP-Depleted Membrane Segment of F_1F_o ATPase. The binding parameters of WT and mutant forms of OSCP to F_1 and F_o segments of the enzyme were studied using ^{35}S-OSCP essentially according to Dupuis et al. (1985) and Dupuis and Vignais (1987).

The titration curves for the binding of WT OSCP and of mutant form CD-10 to the F_1 segment of F_1F_o ATPase revealed a very similar saturation plateau. As shown in Table 5, the values corresponded to the binding of 0.75 mol of the WT form (column 2) or 0.89 mol of the mutant form CD-10 (column 4) per mole of soluble F_1-ATPase. Mutant form ND-28, on the other hand, did not show any significant binding to F_1 at any concentration studied (column 3). These results on direct binding of mutant forms of OSCP with soluble F_1 are consistent with the observations made on the ability of mutant forms to confer cold stability to reconstituted UF_1·F_o ATPase activity discussed earlier.

Experiments on the binding of OSCP to the membrane fraction showed that the binding of the WT form increased linearly as a function of added subunit (Figure 1, inset, ●). The binding sites in the membrane were not saturated even after binding of 900 pmol of OSCP/mg of UF_o. Inclusion of F_1-ATPase in the experiment resulted in a slight (20%) increase in total binding (Figures 1 and 2; compare ● in the two insets) but in a significantly bigger increase in the slope of the binding curve (compare ● in Figures 1 and 2). This suggests that binding of the WT form of OSCP to reconstituted UF_o·F_1 as compared to membrane alone is at the high-affinity sites. These results on the binding of WT form of OSCP to OSCP-depleted complexes (±F_1 ATPase) are essentially consistent with the observations of Dupuis and Vignais (1987) with the exception that we did not find any high-affinity sites in UF_o for OSCP unless F_1-ATPase was included during reconstitution.

Studies involving binding of mutant forms of OSCP to depleted membrane fraction showed that, as compared to the WT form, the total binding of mutant form ND-28 was significant (Figure 1, inset, compare △ with ●), although with somewhat lower affinity as suggested by the slope of the binding curve (Figure 1, compared △ with ●). When F_1 was included in the binding experiments there was a small increase in the total binding (Figures 1 and 2, compare △ in

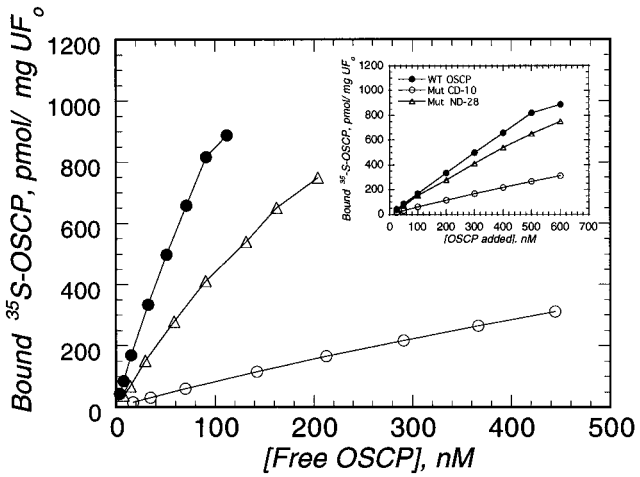

FIGURE 1: Deletions at the C-terminal end of OSCP result in decreased binding of the resultant subunit to UF_o. Aliquots of UF_o (0.5 mg/mL) were incubated for 1 h at 30 °C with indicated concentrations of ^{35}S-labeled WT (●) or mutant forms ND-28 (△) or CD-10 (○) of OSCP in a 100-μL volume of buffer A as described under Experimental Procedures. Free ^{35}S-OSCP was separated from UF_o-bound ^{35}S-OSCP by centrifugation of reconstitution mixture, and suitable aliquots of supernatant and resuspended sediment were counted as described under Experimental Procedures. The inset represents the same data as in the main figure except that the *x*-axis indicates total OSCP concentration rather than free OSCP concentration.

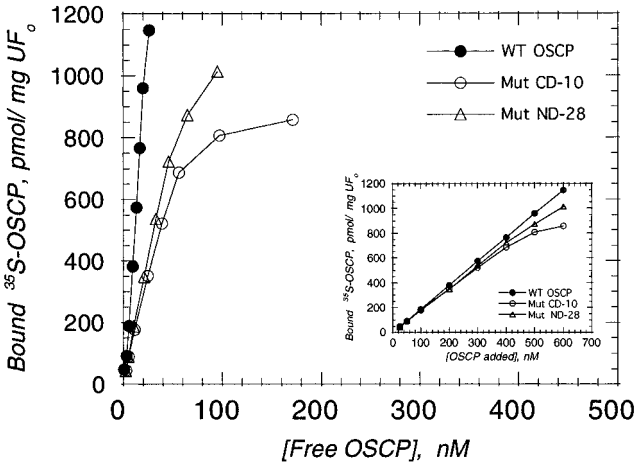

FIGURE 2: Deletions at the C-terminal end lead to decreases in the binding affinity of the subunit to UF_o·F_1 complex. Experimental details and presentation of data in the inset and in the main figure were essentially as described for Figure 1 except that the reconstitution mix also included 12.5 μg of F_1/50 μg of UF_o.

the two insets) but a big increase in the binding affinity of the mutant (compare △ in Figures 1 and 2) just as was observed for the WT form of OSCP, although the values obtained for both the binding and binding affinity for the N-deletion mutant continued to remain lower compared to the corresponding values for the WT OSCP (Figure 2, compared △ with ●). Parallel studies on mutant form CD-10 showed that the binding of the mutant to the membrane (Figure 1, compare ○ with ● and △ in the inset) as well as its affinity for binding were substantially reduced compared to both the WT (Figure 1, compare ○ with ●) and ND mutant forms (Figure 1, compare ○ with △). In the presence of F_1-ATPase the binding of the altered subunit to UF_o increased significantly (compare ○ in the insets of Figures 1 and 2), but the binding affinity of the CD-10 mutant compared to the binding affinity of the WT or ND-28 mutant form still remained substantially lower (Figure 2, compare ○ with ●

Table 6: Deletions at the N-Terminal End Do Not Affect but Deletions at the C-Terminal End Do Affect the High-Affinity Binding of OSCP to OSCP-Depleted Complexes[a]

expt	addition of OSCP (nmol/mg of UF_o)	bound ^{35}S-OSCP (nmol/mg of UF_o)	
		− F_1-ATPase	+ F_1-ATPase
(1)	^{35}S-WT, 2.4 nmol	1.57	1.91
(2)	^{35}S-WT, 2.4 nmol, followed by cold WT, 24.0 nmol	0.45	1.80
(3)	^{35}S-ND-28, 2.4 nmol	1.18	1.37
(4)	^{35}S-ND-28, 2.4 nmol, followed by cold ND-28, 24.0 nmol	0.32	1.02
(5)	^{35}S-CD-10, 2.4 nmol	0.38	1.17
(6)	^{35}S-CD-10, 2.4 nmol, followed by cold CD-10, 24.0 nmol	0.29	0.36

[a] Aliquots of UF_o complexes were incubated with 0.48 nmol of ^{35}S-OSCP and 0.5 nmol of F_1-ATPase (where indicated) at 30 °C in buffer A as described in Experimental Procedures. After 1 h, a 10-fold excess of unlabeled OSCP was added in experiments 2, 4, and 6 and incubation continued for another 30 min at 30 °C. Bound and free ^{35}S-OSCP were separated as described under Figure 1 and Experimental Procedures.

and △). Thus, it would appear that the reduction in the ability of mutant form ND-28 to improve the rate of $^{32}P_i$−ATP exchange in OSCP-depleted UF_o·F_1 complex is primarily due to decreased binding of the mutant to the complex, while the inability of mutant form CD-10 to enhance coupling activity is due not only to a decrease in total binding but also to the inability of the altered subunit to bind at high-affinity sites as suggested by the slope of the binding curve.

Binding Affinity of UF_o or Reconstituted UF_o·F_1 for Deletion Mutant Forms ND-28 and CD-10. In order to get a better understanding regarding the ineffectiveness of deletion mutants in improving the P_i−ATP exchange activity of OSCP-depleted complexes, the relative binding affinities of UF_o and reconstituted UF_o·F_1 complexes for the WT versus mutant forms of OSCP were examined directly. Aliquots of UF_o or UF_o·F_1 complexes were incubated first with radiolabeled OSCP to allow binding of labeled protein, and next with a 10-fold excess of unlabeled subunit, in order to allow exchange (if any) of membrane-bound radiolabeled OSCP with unlabeled OSCP in solution. Samples were next subjected to centrifugation in order to separate membrane-bound OSCP from OSCP in the medium. It was argued that higher the binding affinity, the lower would be the exchangeability of bound OSCP. Data in Table 6 demonstrate that incubation of UF_o with radiolabeled WT OSCP resulted in significant binding to the membrane (1.57 nmol of OSCP/mg of UF_o), as expected. However, 68% of the bound radiolabeled OSCP could be dissociated following addition of excess unlabeled subunit prior to centrifugation (Table 6, column 3, compare row 2 to row 1). When similar experiments were carried out in the presence of F_1, the binding of radiolabeled WT OSCP to reconstituted UF_o·F_1 compared to UF_o alone increased only slightly (20%; Table 6, row 1, compare column 4 with column 3), but the majority of OSCP that was bound initially continued to remain associated with the membrane even after addition of excess unlabeled subunit (Table 6, column 4, compare row 2 with row 1). These data suggest that OSCP by itself binds at the low-affinity sites in UF_o, but in the presence of F_1, binding at high-affinity sites is promoted. This is in agreement with the previously published observations of Dupuis and Vignais (1987).

Experiments done in parallel to examine the binding affinity of UF_o or reconstituted UF_o·F_1 complex for mutant form ND-28 demonstrate that the binding of ND-28 to UF_o (Table 6, column 3, compare row 3 with row 1) or to reconstituted UF_o·F_1 (Table 6, column 4, compare row 3 with row 1) was somewhat lower compared to corresponding values obtained for the binding of WT subunit. However, similar to WT OSCP, the UF_o·F_1-bound N-terminal deletion mutant did not readily exchange with the ND-28 protein in solution (Table 6, column 4, compare row 4 with row 3), indicating that the binding of deletion mutant in UF_o·F_1 complex was at high-affinity sites at UF_o. These studies suggest that (i) F_o−OSCP interactions rather than F_1−OSCP interactions are crucial for binding of the protein at high-affinity sites in F_1·F_o−OSCP complexes and (ii) while amino acids 14−28 (or some of the residues in this region) of OSCP are critical for F_1−OSCP interactions, these are not critical for the F_1-induced increase in the binding affinity of the subunit toward reconstituted UF_o·F_1 complex.

In parallel studies involving deletion mutant CD-10, the radiolabeled subunit was found to bind poorly to UF_o alone (Table 6, row 5, column 3) but the binding increased substantially in the presence of F_1 (Table 6, row 5, column 4) as expected from the data in Figures 1 and 2. However, in contrast to results obtained for the WT or mutant form ND-28, the F_1-stimulated increment in the binding of radiolabeled mutant form CD-10 to the membrane disappeared following the addition of excess unlabeled subunit (Table 6, compare row 6 with row 5 in column 4). This suggests that in experiments involving mutant form CD-10, no high-affinity binding sites in the membrane are revealed by association of the latter with F_1. This is in disagreement with the observations of Dupuis and Vignais, which suggest that the binding affinity of OSCP is higher for F_oF_1 ($K_d = 5$ nM) compared to its affinities for isolated F_1 (the two K_d for the high- and low-affinity binding sites are 80 nM and 6−8 μM, respectively) or F_o complex. It would appear, therefore, that the observed increase in the binding of CD-10 mutant to UF_o following the addition of F_1 is a direct consequence of the binding of the mutant subunit to F_1, and of association of the resultant F_1−OSCP complex to UF_o via F_1. Since the increased binding of OSCP is essentially via F_1, the additional OSCP presumably never enters into any stable interactions with UF_o and consequently exchanges readily with the free subunit in the medium. These results indicate that F_o−OSCP interactions rather than F_1−OSCP interactions effect the high-affinity binding of the subunit in reconstituted F_1F_o complex, and residues K181−M186 of OSCP are critical for high-affinity interactions of the subunit with the membrane.

DISCUSSION

As a result of previously published studies on elucidation of role of OSCP in facilitating interactions of the catalytic (F_1) with the proton-transporting membrane segment (F_o) of the F_1F_o enzyme, it is clear that (i) both OSCP and F_1 bind independently to F_o to form a binary complex but with a low binding affinity, (ii) when OSCP and F_1 are added together the total binding of OSCP to F_1F_o compared to F_o alone is increased only slightly, but the binding affinity is increased by a factor of 10 or more; likewise, the total binding of F_1 to OSCP−F_o relative to the membrane segment alone is not affected but the affinity is enhanced by a factor of 6, and (iii) the binary complexes OSCP−F_o and F_1F_o are

more readily dissociable compared to the ternary complex $F_1-OSCP-F_o$ [Dupuis & Vignais, 1987]. These results clearly establish that OSCP is central to stable interactions of the catalytic (F_1) and proton-pumping (F_o) segments of the enzyme (Dupuis & Vignais, 1987). Present studies have been focused on identifying the site(s) of interaction of OSCP with the F_1 and F_o segments and on determining the topology of this important subunit in the three-dimensional structure of the F_1F_o enzyme.

Involvement of the N-Terminal end of OSCP in F_1−OSCP Interactions. Studies involving deletions at the N-terminal end of OSCP show that up to 13 residues can be eliminated without affecting significantly the ability of the mutant form of the subunit to reassemble ATP-linked proton pumping in OSCP-depleted complexes (Table 1, rows 2−7). Deletions of 16 or more residues led to inability of the subunit to form a complex with soluble F_1 (Table 5, compare column 3 with column 2) and to confer cold stability to its ATPase activity (Table 4, row 3). Detailed analysis of these mutant forms for their ability (or inability) to restore partial reactions of ATP synthesis in OSCP-depleted complexes showed that these variants retained the ability to complex with F_o, although to a somewhat lesser extent and with a slightly reduced affinity compared to the WT form of OSCP (Figure 1, compare △ with ●). The addition of F_1 gave rise to a small increase in the total binding but to a substantially higher increase in the binding affinity (Figure 2, compare △ with ●). This was accompanied by restoration of inhibitor-sensitive ATPase activity (Table 3, compare column 4 with column 3) and P_i−ATP exchange activities (Table 1, rows 8−10) to OSCP-depleted complexes, although the maximum specific activity achieved for both reactions was lower than the corresponding values obtained for the WT form of OSCP. In brief the ND mutant forms of OSCP are similar to the WT form of the subunit with respect to their overall ability to interact with F_o or F_1F_o complexes. These results demonstrate that the N-terminal segment of OSCP is not essential for the restoration of OSCP-induced high affinity, stable interactions of F_1 and F_o but is necessary for F_1−OSCP interactions. This implies that the N-terminal end of OSCP corresponding to the region containing amino acids 16−28 (or some of the residues in this region) constitutes one of the binding site(s) for its interaction with the soluble F_1. It is conceivable that deletions at the N-terminus can cause structural changes elsewhere in the protein resulting in disruption of F_1−OSCP interactions. However, it does not seem likely in view of the fact that the truncated forms are able to restore energy-linked function in OSCP-depleted complexes. In fact, since the ND mutant forms of OSCP are able to reconstitute ATP-linked proton pumping in OSCP-depleted F_1F_o complexes, OSCP must harbor at least one more site that mediates interactions of the deletion mutant forms of OSCP with the catalytic segment of the enzyme. These observations regarding involvement of the N-terminal region of OSCP in F_1−OSCP interactions are consistent with the proposal of Mendel-Hartvig and Capaldi (1991), which implies that the N-terminal end of analogous subunit ECF_1 δ contacts the F_1 part and forms one site of binding. The latter proposal is based on the evidence that limited trypsin treatment of ECF_1 resulted in proteolytic degradation of C-terminal end of δ subunit, without causing a dissociation of the remainder of the subunit from intact ECF_1.

Involvement of the C-Terminal End of OSCP in F_o−OSCP Interactions. Our previously published studies showed that the deletion of even 10 amino acid residues (K181−L190) from the C-terminal end of OSCP led to a complete loss in the ability of truncated subunit to reassemble proton pumping in OSCP-depleted F_1F_o complexes [Joshi et al., 1992]. The present study, aimed at the elucidation of structural and functional consequences of deleting smaller segments in the K181−L190 region of OSCP, demonstrate that up to three residues from the carboxy terminus may be deleted without incurring a significant effect on the function of OSCP (Table 2, rows 2−5). Removal of five or more residues, however, results in increasing losses in the ability of the resultant mutants to reconstitute ATP−P_i exchange (Table 2, rows 6−8) or inhibitor-sensitive ATPase activity (Table 3, compare column 5 with column 3) in OSCP-depleted complexes. Thus, the border for functional OSCP lies 3 or 4 residues away from the C-terminal end of the protein. The inactivation of OSCP following deletion of five or more residues from the C-terminal end is presumably not due to structural destabilization or misfolding of OSCP as judged by overall expression characteristics, CD spectroscopy, and Western analyses of deletion mutants (data not presented). Binding studies demonstrate that loss of OSCP coupling function is not due to inability of the mutant to complex with F_1 (Table 5, compare column 4 with column 2) or F_o (Figures 1 and 2, ○) but is presumably related to inability of the resultant mutant to interact with F_o with high affinity in reconstituted $OSCP-F_1-F_o$ complexes (Table 6, compare rows 5 and 6 with rows 1 and 2, respectively; Figure 2, compare ○ with ●). The data suggest that the region of OSCP corresponding to residues K181−M186 contributes to one of the binding sites for interaction of the subunit with the proton-transporting segment (F_o) of ATP synthase.

The results described above are consistent with the *in vivo* C-terminal deletion studies of δ subunit in ECF_1F_o, wherein removal of three or more residues resulted in a protein that is unable to bind F_o [Jounouchi et al., 1992]. It is interesting to note that deletion of three residues at the C-terminal end of δ turns out to be equivalent to a five-residue deletion in mitochondrial OSCP as judged by an alignment of their deduced amino acid sequences [Hoesche & Berzborn, 1993]. The results obtained from deletion mutagenesis studies at the C-terminal end of OSCP are also in agreement with the studies of Mendel-Hartvig and Capaldi (1991), who reported that trypsin-treated ECF_1 that had lost about 20 residues from the C-terminus of δ was still able to bind to ECF_o but failed to regain sensitivity to DCCD. These studies indicate that the C-terminus of OSCP and bacterial δ are not necessary for interactions with soluble F_1 but are crucial for establishing stable communication between F_1 and F_o segments and for coupling the energy of $\Delta\mu H^+$ through F_o to the events at the catalytic sites.

Secondary and Tertiary Structural Features of OSCP. OSCP and its homologous subunit δ from ECF_1 and CF_1 are part of the stalk between F_1 and F_o and are buried within the F_1F_o complex as suggested by their inaccessibility to proteases and immunological probes [Joshi et al., 1986; Berzborn & Finke, 1989]. Gel filtration, sedimentation velocity, small-angle X-ray diffraction, and circular dichroism measurements suggest that these subunits are elongated in shape and highly α-helical in structure [Engelbrecht et al., 1991; Sternweis & Smith, 1977]. Based on secondary structure predictions, there are six consensus helical regions in OSCP and ECF_1-δ and CF_1-δ. These regions correspond to residues 14−24, 26−51, 61−71, 100−113, 125−140, and

178−186 in OSCP and to residues 7−17, 19−44, 53−63, 91−104, 116−131, and 165−175 in *E. coli* δ subunit [Engelbrecht et al., 1991]. On the basis of limited proteinase digestion and chemical labeling of ECF_1, Mendel-Hartvig and Capaldi (1991) have proposed a model for the bacterial δ wherein both the N- and C-terminii of the subunit are associated with F_1 while the central part is extended as a two-arm helical hairpin constituting the stalk. Since none of the predicted consensus helices is long enough by itself to span through the entire length of the stalk (45 Å), Hazard and Senior (1994) proposed a modification of the previously proposed hairpin model for ECF_1-δ. Accordingly the N- and C-terminii would still remain associated with F_1, but the central part of the polypeptide that forms two continuous α-helices constituting part of the stalk will contain residues 7−63 and 91−145, and the region corresponding to residues 64−90 will form the bend of the hairpin that interacts with F_o. However, neither of the two models proposed above is supported by deletion mutagenesis studies of ECF_1-δ (Jounouchi et al., 1992) or mitochondrial OSCP (present observations), both of which demonstrate that the carboxy-terminal end of the subunit is associated with F_o, not with the F_1 segment. In this context, Ziegler et al. (1994) recently found that amino acids C64 and C140 of δ subunit of ECF_1 are very close to each other *in situ* in F_1 ATPase. If this is correct, and if in fact residue C64 constitutes part of the δ-F_o interaction site as proposed by Hazard and Senior (1994), then it is hard to visualize association of the C-terminal end of the protein harboring residue C140 with the F_1 part of the enzyme. In view of this and our present findings on the ND mutant forms, which demonstrate that the N-terminal region of OSCP is necessary for OSCP−F_1 interactions but not for OSCP−F_o interactions, we propose that the N- and C-terminii of OSCP are at opposite ends of the stalk; the N-terminus is associated with F_1, the C-terminus is associated with F_o, and the central part of the protein forms three or more helices constituting the stalk. Presumably, there is more than one binding site in OSCP for both F_1 and F_o segments.

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