Oligomycin Sensitivity Conferring Protein (OSCP) of Bovine Heart Mitochondrial ATP Synthase: High-Affinity OSCP−F_o Interactions Require a Local α-Helix at the C-Terminal End of the Subunit†

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ABSTRACT: Earlier studies on oligomycin sensitivity conferring protein (OSCP) of bovine mitochondrial ATP synthase (F_1F_o) indicated that a deletion mutant form (CD-10), lacking the last 10 amino acid residues (K181−L190), was unable to bind to the F_o segment, or reconstitute energy-linked reactions in OSCP-depleted F_1F_o complexes [Joshi et al. (1996) *Biochemistry 35*, 12094−12103]. So far as known, the K181−L190 region of all mammalian species of OSCP harbors four charged residues at positions 181, 184, 187, and 188, while secondary structure predictions suggest that the K178−M186 region has a high propensity to form a helix [Ovchinnikov et al. (1984) *FEBS Lett. 166*, 19−22; Higuti et al. (1993) *Biochim. Biophys. Acta 1172*, 311−314; Grinkevich et al. (1994) *Biol. Membr. 11*, 310−323; Engelbrecht et al. (1991) *Z. Naturforsch., C: Biochem., Biophys., Biol., Virol. 46*, 759−764]. Present studies were undertaken to clarify the role of individual amino acids in the K181−L190 region in OSCP-stimulated energy coupling. Our data show that simultaneous replacements of all four charged residues by uncharged but polar glutamines, or of K181−R184 by apolar alanines, had no significant influence either on the total α-helix content of the mutant forms or on the ability of mutant OSCPs to couple energy-linked reactions. However, a substitution of the K181−M186 region by six proline residues led to complete loss in the coupling activity of the resultant mutant. A detailed analysis of the 6-proline mutant form revealed that the variant was indistinguishable from WT OSCP with respect to expression characteristics, affinity for S-Sepharose, and ability to interact with F_1, but was unable to complex with the F_o segment. These studies suggest that the global protein structure was not destabilized. The helix potential prediction analyses showed that the 6-proline OSCP displayed a marked decrease in the helix-forming propensity in the region corresponding to residues 175−190. Quantitative CD analyses to measure helical content demonstrated that both of the mutant forms 6-proline-OSCP and CD-10 had a somewhat lower α-helical content compared to WT protein, while synthetic peptides corresponding in sequence to the K178−L190 region displayed a high propensity to form a helix. Taken together, these results suggest that the C-terminal end of OSCP encompasses an α-helix which is crucial for high-affinity interactions of the C-terminal end of this subunit with F_o in the F_1F_o enzyme.

The F_1F_o-type[1] ATP synthase catalyzes the synthesis of ATP in response to an electron-transfer-generated proton electrochemical gradient ($\Delta\mu H^+$) and, in the reverse direction, generates an ATP-hydrolysis driven $\Delta\mu H^+$ for use in ion and substrate transport processes. It consists of a soluble segment, F_1, that contains the catalytic sites and a membrane-integrated segment, F_o, that contains a transmembrane channel for proton transport [for review, see Boyer (1993), Fillingame (1990), Pedersen and Amzel (1993), Penefsky and Cross (1991), and Senior (1990)]. Low-resolution electron microscopy studies reveal that the F_1 and F_o segments are linked by a slender stalk which is approximately 45 Å long and 25−30 Å in diameter (Fernandez-Moran, 1964; Gogol et al., 1987; Lücken et al., 1990). It is believed that the primary function of the stalk is to couple F_o and F_1 functions, by propagating energy-linked conformational changes produced at F_o to the catalytic sites at F_1. Hence, a knowledge of the structure/function relationships of stalk-forming subunits is essential for gaining insights on energy coupling mechanisms during ATP synthesis.

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[1] Abbreviations: F_1, membrane-extrinsic portion of the proton-translocating 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% PMSF; TFE, 2,2,2-trifluoroethanol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; 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, wild type form of recombinant OSCP; ECF_1-F_o, F_1F_o from *Escherichia coli*; CD-10, mutant form of OSCP with deletion of 10 residues from the C-terminus; K178−L190-GY-15, 15-mer synthetic peptide with the sequence NH_2-KIQKLSRAMREILGY-COOH; $(K178-L190)_2$-GY-28, 28-mer synthetic peptide with the sequence NH_2-KIQKLSRAMREILKIQKLSRAMREILGY-COOH.

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The oligomycin sensitivity conferring protein (OSCP), a major constituent of the stalk segment of mitochondrial ATP synthase, is presumably located in a "pit" next to a protrusion at the base of the F_1 particle (Abrahams et al., 1993; Kagawa & Racker, 1966; McLennan & Tzagoloff, 1968; Van de Stadt et al., 1972; Dupuis et al., 1985). Depletion/reconstitution studies of the ATP synthase show that OSCP can form independent complexes with both F_1 and F_o segments, although it is required neither for F_1-catalyzed inhibitor-insensitive ATP hydrolysis nor for F_o-mediated passive proton translocation (Dupuis et al., 1985; Dupuis & Vignais, 1987; Pringle et al., 1990). It is also clear that OSCP is not obligatory for binding of F_1 to F_o although it is absolutely essential for reassembly of oligomycin- and DCCD-sensitive ATPase and ATP synthesis activities in OSCP-depleted F_1F_o complexes (Kagawa & Racker, 1966; McLennan & Tzagoloff, 1968; Senior, 1971; Pringle et al., 1990; Joshi & Huang, 1991). Thus, the primary role of OSCP is possibly in the transmission of the energy of the proton electrochemical gradient between F_o and F_1, thereby facilitating the "coupling" of an "uncoupled" F_1-F_o complex. 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/structural importance using nested deletion strategy. Our preliminary studies showed that residues E188−L190 at the C-terminal end of OSCP could be deleted without compromising the energy coupling efficiency of this subunit but mutant forms lacking more than three amino acid residues from the C-terminus were neither able to bind to F_o nor restore ATP synthesis activity in OSCP-depleted F_1F_o complexes (Joshi et al., 1992. 1994, 1996). This indicated that amino acid residues K181−R187 (or some of the residues in this region) are critical for the coupling function of OSCP. Similar results were reported for yeast mitochondrial OSCP and the homologous subunit δ of *Escherichia coli* F_1F_o (Jounouchi et al., 1992; Hazard & Senior, 1994; Mao & Mueller, 1997). Therefore, it is clear that the C-terminal end of OSCP or OSCP-type subunits is important for coupling the energy of proton movements in F_o to the synthesis of ATP in F_1. Secondary structure predictions suggest that there are six consensus helical regions in the OSCPs of mammalian mitochondrial F_1F_o and in subunit δ of bacterial and chloroplast enzymes; one of these corresponds to residues 178−186 in OSCP (Engelbrecht et al., 1991). It is interesting that the C-terminal part of yeast mitochondrial OSCP is also predicted to be α-helical despite the fact that its predicted amino acid sequence is only 35% conserved with respect to the sequence for bovine OSCP (Uh et al., 1990; Mao & Mueller, 1997). From this, it would appear that the secondary structure elements of the C-terminal end of OSCP and its homologous subunit δ are conserved, and presumably highly helical. Furthermore, a multiple sequence alignment of mammalian mitochondrial OSCPs revealed the presence of four charged residues in the K181−L190 region, corresponding to positions 181, 184, 187, and 188 (Ovchinnikov et al., 1984; Higuti et al., 1993; Grinkevich et al., 1994). In order to investigate whether the inactivation of OSCP following deletion of the last 10 residues at the carboxyl end is due to loss of a putative α-helix and/or conserved charged residues, the region corresponding to residues K181−L190 was studied further by site-directed mutagenesis to alter its helical character or charge, followed by structural and functional characterization of the mutant forms. Furthermore, in order to know if in fact the 178−186 region of OSCP is α-helical to begin with, two oligopeptides with sequence corresponding to the 178−190 region of OSCP were synthesized. The CD spectroscopy data on these peptides suggest that the K178−L190 region of OSCP does display a propensity to form an α-helix. The significance of this α-helix at the C-terminus of OSCP for facilitating high-affinity interactions of the subunit with the proton translocating segment F_o of the F_1F_o enzyme is discussed.

EXPERIMENTAL PROCEDURES

DNA modifying enzymes and restriction endonucleases were obtained from New England Biolabs (Beverly, MA) and were used according to manufacturer's protocols unless otherwise stated. Klenow fragment of DNA polymerase and sequenase version II were obtained from U. S. Biochemicals (Cleveland, OH), and *Thermus aquaticus* DNA polymerase was from Perkin Elmer (Norwalk, CT). The nucleotides were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), [α-^{35}S]deoxyATP and L-[^{35}S]methionine were obtained from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively, and acrylamide gel reagents were from BioRad (Richmond, CA). All other chemicals were from Sigma (St. Louis, MO).

Nomenclature of OSCP Mutants and Construction of OSCP Expression Plasmids. In order to obtain a high-level expression of OSCP, the coding sequence corresponding to mature OSCP was isolated from the original OSCP expression plasmid pKOSCP WT and recloned into the pET-based expression vector pMW7 as described previously (Collinson et al., 1994; Joshi et al., 1996). The resultant construct, pRKOSCP WT, yielded 50−80 mg of purified WT form of OSCP per liter of cell culture using *E. coli* strain BL21(DE3) as the host. Amino acid replacements were carried out according to a PCR protocol (Joshi et al., 1992). The final plasmid constructs were characterized by sequencing the entire OSCP gene as well as subcloning sites. The mutant forms were named by single-letter symbol(s) of the residue(s) targeted for mutation, followed by its (their) position in the protein primary structure, followed by single-letter symbol(s) of the resultant residue(s). Thus, mutant form K181Q refers to mutation of a lysine residue at position 181 in the OSCP sequence to a glutamine; mutant form K181Q; R184Q; R187Q; E188Q represents a simultaneous mutation of amino acid residues at positions 181, 184, 187, and 188 to glutamine residues; and mutant form KLSR181−184AAAA signifies a simultaneous replacement of amino acids in positions 181−184 in the OSCP sequence to four consecutive alanines (see Table 1 for a list of OSCP mutants and their codons).

Transformation of E. coli KP3998 and BL21(DE3) Strains, Expression of the OSCP Gene, and in Vivo Radiolabeling and Purification of Recombinant OSCPs. OSCP expression plasmids were first amplified in *E. coli* strain KP3998, and transformants were grown overnight at 37 °C in LB agar containing 100 μg of ampicillin/mL. To obtain protein expression, *E. coli* strain BL21(DE3) was transformed and expression of recombinant OSCPs initiated according to published protocols (Collinson et al., 1994; Joshi et al., 1996). Harvesting of cells, cell lysis, and solubilization of recombinant OSCPs and their purification by S-Sepharose chromatography were carried out as described previously for the WT form of OSCP (Joshi et al., 1996).

Synthetic Peptides. The oligopeptides were produced by the in-house protein facility of the Boston Biomedical Research Institute by solid state synthesis on an HMP resin, using Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] chemistry. The synthesized peptides were cleaved from the resin according to a trifluoroacetic acid-based protocol. In order to ascertain peptide purity and complete removal of protecting groups, the peptides after cleavage were analyzed by reversed-phase HPLC using an analytical C-8 column [Si−O−Si−$(CH_2)_7$−CH_3] from Phenomenex, as well as by MALDI-TOF mass spectrometry (PerSeptive Biosystems, Voyager RP). The salts were removed by molecular sieving on a Biogel P-2 (BioRad) column that was prequilibrated with distilled water.

The peptide concentrations were determined spectrophotometrically, using an extinction coefficient of $\epsilon_{275} = 1420 \cdot cm^{-1} \cdot M^{-1}$ (Bailey, 1966), or by amino acid analysis (Waters Picotag System). Both methods gave values that were in agreement within 10% of error.

Peptide K178−L190-GY-15 had its first 13 residues corresponding to OSCP amino acid residues K178−L190; the inclusion of a *tyr* at the C-terminus was for the convenience of spectrophotometric determination of peptide concentration, and of a *gly* (a helical breaker) upstream of the *tyr*, to minimize the structural effect of the added *tyr* on the rest of the peptide sequence (Chakrabartty et al., 1991). Peptide $(K178-L190)_2$-GY-28 had the same sequence as peptide K178−L190-GY-15 except that the sequence for residues 14−26 was a repeat of the sequence for residues 1−13. The increase in chain length was to provide additional stability to the putative α-helix (Scholtz et al., 1991).

Other Methods. All standard DNA manipulations were carried out according to protocols described elsewhere (Joshi et al., 1996). Bovine heart mitochondria (Joshi & Sanadi, 1979), OSCP-depleted submitochondrial particles (AE-P) at pH 9.8 (Fessenden & Racker, 1967), purified F_o fractions depleted of OSCP (UF_o) (Pringle et al., 1990), and soluble F_1-ATPase (Horstman & Racker, 1970) were isolated as described previously. SDS−polyacrylamide gel eletrophoresis, electrotransfer, gel staining, Western blotting, and ELISA assays were carried out according to previously published protocols (Joshi & Burrows, 1990).

Circular Dichroism (CD), Secondary Structure, and Helix Potential Analyses. Circular dichroism measurements were made at 5 °C in a 0.1 cm path length cell (Hellma 100-QS) on an Aviv Associates (Lakewood, NJ) Model 60 DS spectropolarimeter containing a thermoelectric temperature controller. All spectra were corrected against the spectrum of water alone unless specified otherwise. The helical contents were calculated according to Chen et al. (1972) from the relationship: $f_H = -([\Theta]_{222} + 2340)/30300$, where f_H is the fractional α-helical content and $[\Theta]_{222}$ is the mean molar residue ellipticity in degrees centimeter squared per decimole. The secondary structures were determined according to Mao and Wallace (1984) using the LINEQ program. An average helix length of 11 residues was used in the analysis. The helix potential profile was studied according to SEQ (Teeter & Whitlow, 1988) which is strictly based on Chou and Fasman's algorithm (1978).

Reconstitution of Oligomycin-Sensitive ATPase and [^{32}P]P_i−ATP Exchange. Oligomycin-sensitive ATPase complexes were reconstituted by adding soluble F_1 and OSCP to fractions of ATP synthase that had been depleted previously 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), OSCP (5 μg), 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 using a 10 μL aliquot (Pringle et al., 1990).

For the reconstitution of [^{32}P]P_i−ATP exchange activity, 200 μg aliquots of OSCP- and F_1-depleted submitochondrial particles prepared at pH 9.8 (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 [^{32}P]P_i−ATP exchange activity was initiated by adding 200 μL of a solution containing 7.5 μmol of ATP, 2.5 μmol of ADP, and 10 μmol of $MgCl_2$ (pH 7.5), and 50 μL of a solution containing 10 μmol potassium phosphate buffer (pH 8.0) and 500 000 cpm of [^{32}P]P_i. Samples were incubated at 37 °C. After 15 min, 0.25 mL of 20% trichloroacetic acid was added to terminate the exchange activity. [γ-^{32}P]ATP was separated from [^{32}P]P_i as described previously (Joshi et al., 1985).

Binding of ^{35}S-OSCP to Isolated F_1 or UF_o Segments. The binding of ^{35}S-OSCP to F_1-ATPase was determined by allowing incubation of soluble F_1 with the indicated concentrations of radiolabeled OSCP, and separating the F_1-bound OSCP from free OSCP by centrifugation through a spin column filled with the cation exchanger CM Sephadex-C25 (Dupuis et al., 1985). The binding of ^{35}S-OSCP to UF_o was performed essentially as described by Dupuis and Vignais (1987) and Joshi et al. (1996).

Presentation of Data. The data shown in various figures and tables are averages of five independent experiments.

RESULTS

As pointed out in an earlier section, a mutant form of bovine OSCP lacking the last 10 residues (K181−L190) from the carboxyl end has been shown to be incompetent in its ability to couple proton movements in F_o with the catalytic site events at F_1 in the reconstituted F_1F_o ATP synthase preparations (Joshi et al., 1992, 1996). Since this region of OSCP is predicted to have a high propensity to form a helix and since it is also known to harbor four conserved charged residues, selected residues in the K181−L190 region of OSCP were replaced by helix-breaking proline(s) or by uncharged but polar glutamine(s) or apolar alanines (see Table 1 for a list of the mutants). The recombinant OSCPs were evaluated for structural integrity, by analyzing expression and secondary structure characteristics, and for functional competence, by testing their ability to reconstitute the terminal step in ATP synthesis in OSCP-depleted complexes. Those mutant forms that retained global structural characteristics but lost energy coupling activity were analyzed further for their ability/inability to reconstitute individual steps in the synthesis of ATP that require OSCP. Furthermore, to ascertain whether or not the K178−M186 region of OSCP is involved in the formation of a helix, helical contents of recombinant OSCPs as well as of two synthetic oligopeptides with sequences corresponding essentially to the K178−L190 region of OSCP were measured by quantitative CD spectroscopy.

Table 1: Mutant Codons and α-Helix Content of Resultant OSCPs

OSCP	codons (WT/mutant)	% α-helix[a]
WT		40.60
CD-10		38.50
K181Q	AAG/CAG	40.93
R184Q	AGA/CAA	41.21
R187Q	CGG/CAG	40.72
E188Q	GAG/CAG	41.04
K181Q; R184Q; R187Q; E188Q	AAG/CAG;AGA/CAA; CGG/CAG;GAG/CAG	41.10
KLSR181−184AAAA	AAGCTGAGCAGA/GCGGCGGCCGCA	40.85
KLSRAM181−186PPPPPP	AAGCTGAGCAGAGCAATG/ CCACCTCCACCACCACCT	36.50
I179P	ATT/CCT	41.14
L182P	CTG/CCG	40.65
M186P	ATG/CCG	40.89

[a] Helical contents were estimated from CD spectra according to Chen et al. (1972), as described under Experimental Procedures.

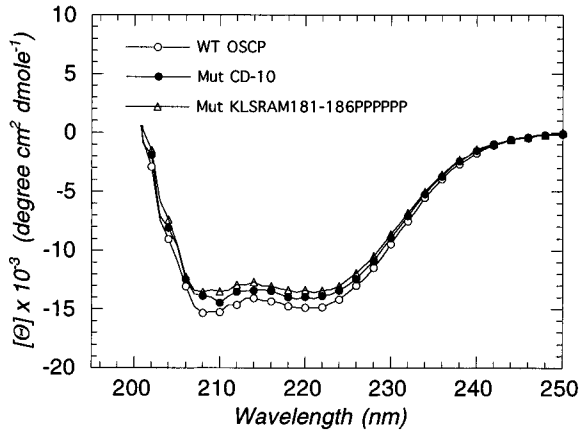

FIGURE 1: Circular dichroism spectra of mutant forms CD-10 and KLSRAM181−186PPPPPP show a small decrease in their α-helix content with respect to WT OSCP. Circular dichroism spectra of various OSCPs were measured in 20 mM Tris·HCl, pH 7.5, buffer at 25 °C as described under Experimental Procedures. Data were collected at 0.5 nm intervals although only 25% of the data points are shown in the figure for the purposes of enhancing clarity.

(I) Evaluation of Mutant Forms of OSCP for Structural Integrity

Expression, Isolation, and Purification of Recombinant OSCPs. Similar to WT OSCP, all of the mutant forms investigated in the present studies could be expressed in *E. coli*, and in each instance, the recombinant subunit accumulated in the inclusion body fraction. Furthermore, Western blots of total cell homogenates containing recombinant OSCPs revealed a single band whose staining intensity against a polyclonal anti-OSCP serum and relative mobility in SDS−PAGE were comparable to the pattern obtained for the WT form of OSCP (data not presented). This suggests that every mutant form investigated was of correct molecular mass, and was indistinguishable from the WT form of the subunit with respect to its subcellular location, yield, and stability *in vivo* to host proteases.

Another common feature observed between the mutant forms studied and WT OSCP was that their protein moiety could be readily solubilized by resuspension of the inclusion body pellet in buffers containing 6.0 M guanidine hydrochloride, and be purified further by S-Sepharose column chromatography (Collinson et al., 1994; Joshi et al., 1996).

Circular Dichroism, Secondary Structure, and Helix Potential Analyses of Recombinant OSCPs. (a) CD Measurements of WT and Mutant Forms of OSCP. Data in Figure 1 show that the WT OSCP has a typical α-helical character as judged from two negative minima at 222 and 208 nm seen in its far-UV CD spectrum [see also Dupuis et al. (1983), Engelbrecht et al. (1991), and Joshi et al. (1996)]. The secondary structure calculations indicated the composition to be 40.6% α-helix, 22.6% β-sheet, 10.8% β-turn, and 25.9% unordered. Most of the mutant forms analyzed turned out to be very similar to WT OSCP both with respect to the shape of their CD spectrum as well as with respect to their α-helical content except for mutant forms KLSRAM181−186PPPPPP and CD-10 (Table 1). Both of these displayed a drop in their α-helical content, from 40.6 ± 0.5% for the WT form to 36.5 ± 0.5% for the 6-proline mutant form, and to 38.5 ± 0.5% for the deletion mutant CD-10 (Table 1; Figure 1). The experimentally determined decreases in the helical content corresponded to a value of 4.1 ± 0.5% compared to a predicted value of 4.5% for the 6-proline mutant form, and 2.1 ± 0.5% in comparison to a predicted value of 2.5% for the deletion mutant CD-10. These decreases, although small, were reproducible as judged by careful quantitative analyses of CD spectra of six independent samples for each of the WT, deletion mutant CD-10, and mutant form KLSRAM181−186PPPPPP OSCPs. The magnitude of the observed drop in the α-helix content was suggestive of a deletion/disruption of a localized helical segment.

(b) CD Meaurements of Synthetic Peptides. In order to find out whether the K178−M186 region of OSCP has a tendency to form a helix, two oligopeptides with sequences corresponding to the K178−L190 region of this protein were synthesized, and their propensity to form a helix was examined by CD spectroscopy. In some of the experiments, trifluoroethanol (TFE), a solvent that is known to stabilize synthetic peptide structures in solution, was also included (Moroder et al., 1975) (see Experimental Procedures for additional details).

The near-UV CD spectrum of the smaller peptide K178−L190-GY-15 indicated that the peptide chain had very little (<5%) intrinsic helicity in aqueous solution as measured by Θ_{222} (Figure 2 panel A, ●). However, the inclusion of increasing concentrations of TFE in the peptide suspension medium promoted a steady increase in the peptide helical content (compare △ with ●). Similar series of experiments on the larger peptide $(K178-L190)_2$-GY-28 showed that it had a higher intrinsic helicity (13.7%) and clearer negative minima at 222 and 208 nm, compared to the smaller peptide (panel B, ●); the observed increment in the intrinsic helicity of the larger peptide was presumably due to additional stability of the helical structure arising from an increase in the peptide length from 15 to 28 residues. Furthermore, similar to the 15-mer peptide, the $(K178-L190)_2$-GY-28

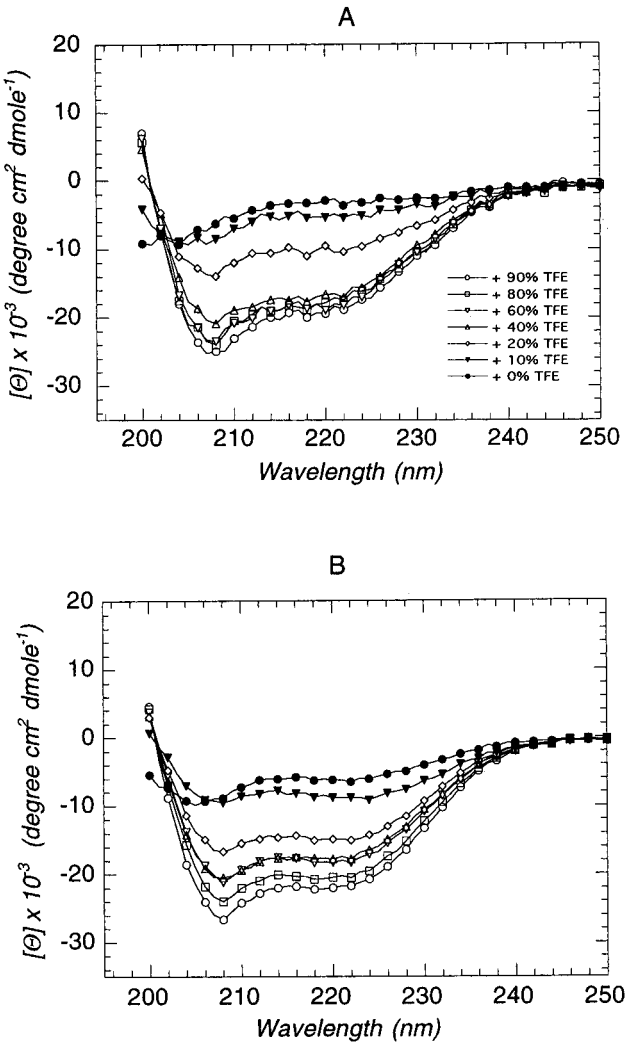

FIGURE 2: Circular dichroism spectra of synthetic peptides corresponding to the K181−L190 region of OSCP reveal a propensity to form an α-helical structure. Samples of peptides for measuring circular dichroism were diluted to 50 μM concentration in 5 mM potassium phosphate buffer, pH 7.2; where indicated, TFE was included in the dilution buffer. Data were collected at 0.5 nm intervals although only 25% of the data points are shown in the figure for the purposes of enhancing clarity. Panel A, peptide K178−L190-GY-15; panel B, peptide $(K178-L190)_2$-GY-28.

peptide also displayed consistent increases in helicity with increasing concentrations of TFE. These data demonstrate that the peptide sequence KIQKLSRAMREIL (or some part of it) does have an inherent propensity to fold into a helix; TFE when included simply provides a stabilization to preformed secondary structures that are innate to the peptide amino acid sequence [for further information on the mode of action of TFE, see Lu et al. (1984), Jimenez et al. (1987), Dyson et al. (1988), Pena et al. (1989), and Sonnichsen et al. (1992)].

(c) Helix Potential Analyses of WT and Mutant Forms of OSCP. Studies based on helix potential prediction algorithms indicated that none of the OSCP mutant forms with replacement of residues in the K181−L190 region displayed any change in its potential to form helices except for mutant form KLSRAM181−186PPPPPP. This particular mutant showed a pronounced drop in its helix-forming propensity; the decrease in helix potentail was in one specific region corresponding to residues A175−L190; no change was evident in the rest of the protein sequence (data not presented). This suggests that the substitution of residues K181−M186 by six proline residues resulted in disruption of a specific helix which is located in the A175−L190 region of OSCP.

(II) Evaluation of Mutant Forms of OSCP for Functional Competence

Reconstitution of [^{32}P]P_i−ATP Exchange. Table 2 presents data on the ability of WT and various mutant forms of OSCP to reconstitute P_i−ATP exchange activity in OSCP-depleted AE-particles. It is clear from data in rows 1 and 2 that the AE particles prepared at pH 9.8 have quite low exchange activity even upon supplementation with F_1-ATPase (row 1), but get stimulated by a factor of at least 10 upon addition of OSCP (row 2). This demonstrates that pH 9.8 particles are highly suitable for assaying the biological activity of OSCP [see also Joshi et al. (1992, 1996)].

The data on replacement of charged residues in the K181−L190 region of OSCP by polar but uncharged glutamines indicated that the mutations to eliminate charged character had no significant influence on the ability of resultant OSCPs to reconstitute energy-linked reactions in AE particles (Table 2, compare rows 3−6 with row 2). In order to inquire if polar residues are the critical ones for OSCP function, residues K181−R184 were replaced simultaneously by apolar alanines. The rationale for replacing only the first 4 out of 10 residues in the K181−K190 region was that residues A185 and M186 are nonpolar already, and residues E188−L190 are known not to be necessary for energy coupling activity as established previously (Joshi et al., 1994, 1996). The results clearly indicated that similar to mutant form K181Q;R184Q;R187Q;E188Q, the 4-alanine mutant form also experienced no negative impact in its ability to promote energy coupling (compare row 7 with row 2 in Table 2). These data show that neither charged residues nor residues with uncharged side chains in the K181−L190 region of OSCP are critical to mitochondrial energy coupling. The replacement of residues K181−M186 by six proline residues, however, led to a substantial inactivation of the coupling activity of OSCP (row 8 in Table 2). The inability of mutant form KLSRAM181−186 PPPPPP to enhance energy coupling was found to be coincident with the predicted decrease in the potential of the mutant subunit to form a specific helix in the A175−L190 region (see also previous section). Subsequent experiments designed to identify specific residue(s) that might be critical for the helical conformation of the 175−190 region of OSCP showed that the replacement of residues I179, L182, or M186 by a proline caused no significant impact on the predicted helix potential profile of the single proline mutant forms, and resulted in only a 15−20% reduction in the ability of mutant OSCPs to stimulate P_i−ATP exchange activity (Table 2, rows 9−11). This, however, was not surprising since it has been reported previously that single proline residues may only cause a kink in the helix and the mutant protein may still fold and behave essentially normally (Schulz et al., 1974). In fact, it has been shown that a single proline can be substituted at several positions including the center of a helix, with none or only modest affects in the catalytic activity, indicating that protein structures are adaptable and can compensate for amino acid substitutions at many sites (Suh et al., 1996). In essence, our functional analysis of targeted mutagenesis studies encompassing residues K181−L190 of OSCP, in conjunction with an analysis of predicted helix potential profiles of the resultant mutants, shows that the energy coupling function

Table 2: Amino Acid Substitutions in the K181−L190 Region of OSCP Affect the Ability of Resultant Mutants to Reconstitute ATP−P_i Exchange Activity in OSCP-Depleted Complexes[a,b]

	P_i−ATP exchange [nmol min^{-1} (mg of AE-P)$^{-1}$]
AE-P + F_1	13
AE-P + F_1 + WT OSCP	160
AE-P + F_1 + mutant OSCP K181Q	144
AE-P + F_1 + mutant OSCP R184Q	168
AE-P + F_1 + mutant OSCP R187Q	190
AE-P + F_1 + mutant OSCP K181Q; R184Q; R187Q; E188Q	159
AE-P + F_1 + mutant OSCP (KLSR181−184AAAA)	164
AE-P + F_1 + mutant OSCP (KLSRAM181−186PPPPPP)	15
AE-P + F_1 + mutant OSCP I179P	141
AE-P + F_1 + mutant OSCP L182P	130
AE-P + F_1 + mutant OSCP M186P	135

[a] The reconstitution of depleted submitochondrial particles with F_1−ATPase and the WT and various mutant OSCPs and the measurement of P_i−ATP exchange activity in the reconstituted complexes were performed as described under Experimental Procedures. [b] The P_i−ATP exchange activity of AE particles supplemented with F_1−ATPase and the mutant form of OSCP with deletion of residues K181−L190 was 16 ± 4 nmol min (mg of AE-P)$^{-1}$.

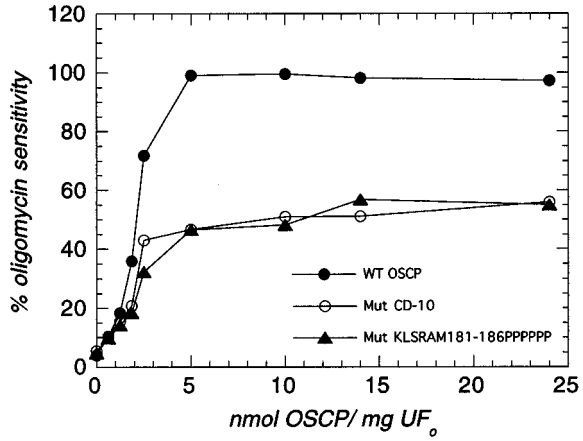

FIGURE 3: Site-directed mutation of OSCP residues K181−M186 by prolines leads to a decreased oligomycin sensitivity of reconstituted ATPase. Samples containing 50 μg of UF_o and 12.5 μg of F_1 were incubated for 30 min at 30 °C with the indicated concentrations of WT or mutant forms of OSCP in a 50 μL volume of a buffer consisting of 50 mM Tris·HCl, pH 7.5, 250 mM sucrose, 10 mM DTT, and 0.02% Tween 20. Aliquots (5.0 μL) of reconstitution mixture were assayed for ATPase activity in the absence or presence of 0.5 μg of oligomycin as described under Experimental Procedures.

provided by this subunit has no specific requirements for basic or acidic residues, or residues with uncharged side chains, but may involve an α-helix; breaking of this putative α-helix and consequent inactivation of the subunit coupling function require insertion of more than one proline. In order to assess if such a mutant retains any of the partial reactions of ATP synthesis normally ascribed to OSCP, the mutant form KLSRAM181−186PPPPPP was subjected to the following analyses, and the results were compared with those obtained for the WT and the deletion mutant form CD-10.

Reconstitution of Oligomycin-Sensitive ATPase. In order to test the ability of mutant form KLSRAM181−186PPPPPP to confer oligomycin sensitivity to membrane-bound ATPase, aliquots of an OSCP depleted membrane fraction of F_1F_o (UF_o) were reconstituted with soluble F_1 and the mutant form of OSCP, and assayed for ATP hydrolytic activity in the presence of 0.5 μg of inhibitor. Data presented in Figure 3 demonstrate that the ATPase activity of such complexes in the absence of added OSCP was only marginally (<10%) sensitive to oligomycin (see the first data point), although the inhibitor sensitivity could be restored to >90% by adding approximately 5.0 nmol of WT subunit/mg of UF_o (●). In contrast, mutant form KLSRAM181−186PPPPPP could only provide a partial enhancement of the oligomycin sensitivity to reconstituted ATPase complexes (▲). Increasing the amount of mutant subunit or oligomycin up to a factor of 10 did not improve the inhibitor sensitivity any further. Essentially similar results were obtained when mutant form CD-10, with deletion of residues K181−L190, was used (○). It is curious that neither of these two mutant forms was effective in enhancing the energy-linked activity of OSCP-depleted complexes (compare line 8 with line 1 in Table 2) despite their ability to confer nearly 50% inhibitor sensitivity to reconstituted ATPase. Evidently, the complexes so formed are not well coupled; presumably the secondary structure elements that get deleted or disrupted are critical for interactions of this subunit with one or both segments of the enzyme.

Binding of Mutant Form KLSRAM181−186PPPPPP to Soluble F_1 and Reconstitution of Cold-Stable ATPase. In order to investigate whether the inability of mutant form KLSRAM181−186PPPPPP to restore high inhibitor sensitivity to the reconstituted ATPase is related to defects in the ability of the altered subunit to bind to the F_1 segment, the binding characteristics of the mutant form to soluble F_1 were compared with the ones for WT OSCP. The titration curves obtained for binding of these OSCPs to F_1 displayed very similar saturation plateaus; the values corresponded to the binding of 0.85 ± 0.04 mol of OSCP/mol of F_1 for the mutant form KLSRAM181−186PPPPPP, compared to a value of 0.75 mol of OSCP/mol of F_1 for WT OSCP (data not presented). To further test if the loss of coupling function in the 6-proline mutant form was associated with defects in its ability to confer cold stability to the F_1−OSCP complex, aliquots of soluble F_1 were combined with the mutant form, and the mixture was incubated for 17 h at 0 °C prior to assay. The ATPase analysis data on these samples showed that the catalytic activity of soluble F_1 was reduced to <30% as expected, while samples of F_1 containing either of the two OSCPs retained nearly 70% of the original activity (data not presented). Therefore, positive data on the reconstitution of cold-stable F_1−OSCP complex by mutant form KLSRAM181−186PPPPPP indicate that the mutations introduced did not cause perturbations in proper OSCP−F_1 interactions.

Binding of Mutant Form KLSRAM181−186PPPPPP to OSCP-Depleted Membrane Segment (UF_o). Experiments to

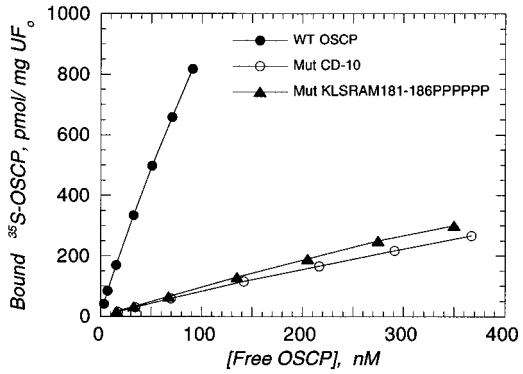

FIGURE 4: Site-directed mutation of OSCP residues K181−M186 by prolines results in decreased binding of the subunit to UF_o. Aliquots of UF_o (0.5 mg/mL) were incubated for 1 h at 30 °C with the indicated concentrations of ^{35}S-labeled WT, or mutant forms KLSRAM181−186PPPPP or CD-10 OSCP in a 100 μL volume of a buffer consisting of 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 as described in Joshi et al. (1996). Free ^{35}S-OSCP was separated from UF_o-bound ^{35}S-OSCP by centrifugation, and suitable aliquots of supernatant and resuspended sediments were counted as described under Experimental Procedures.

measure the binding of mutant form KLSRAM181−186PPPPPP to the F_o segment indicate that there was a concentration-dependent increase in the binding of the mutant to the membranous segment of the F_1F_o enzyme. However, the total binding of the mutant subunit as well as its binding affinity was substantially reduced (Figure 4, ▲) as compared to the corresponding values for the WT form of OSCP (Figure 4, ●). Essentially similar results were obtained for the deletion mutant form CD-10 (Figure 4, ○). These data suggest that the replacement of residues K181−M186 by prolines, similar to the deletion of residues K181−L190, does cause a disruption in high-affinity interactions of the subunit with the F_o segment [see also Joshi et al. (1996)].

DISCUSSION

Subunit OSCP of mitochondrial F_1F_o and subunit δ of ECF_1F_o and CF_1F_o enzymes are considered to be equivalent since all three are thought to be necessary for coupling proton translocation in F_o to the catalytic site events in F_1, and since all three have been reported to be highly α-helical. According to secondary structure prediction algorithms, there are six consensus helical segments in the sequences of bovine OSCP as well as subunit δ which correspond to residues 14−24, 26−51, 61−71, 100−113, 125−140, and 178−186 in bovine OSCP (Engelbrecht et al., 1991). The last segment corresponding to positions 178−186 in bovine OSCP and positions 170−178 in *E. coli* δ is of interest in the context of oxidative phosphorylation since mutant forms harboring a deletion of this particular segment were found to be impaired with respect to their energy coupling function (Jounouchi et al., 1992; Joshi et al., 1992, 1996). In addition, multiple sequence alignment of OSCP from various sources revealed that there are four charged residues within the K181−L190 region which correspond to positions 181, 184, 187, and 188 in all sequences. In order to assess if the conserved charged character and/or predicted helical nature of the C-terminal residues of OSCP was critical for energy coupling, mutations were targeted to the K181−L190 region of recombinant OSCP and mutant forms analyzed for their structural integrity and functional competence.

(a) Structural and Functional Studies on Mutant Forms of OSCP Are Suggestive of the Presence of an a-Helix at the C-Terminal End of the Subunit. The data on structural and functional characterization of mutant forms of OSCP, arising from mutagenesis targeted to the K181−L190 region, demonstrate that none of the mutant forms, except for KLSRAM181−186PPPPPP, was functionally or structurally different from WT OSCP (Tables 1, 2; Figure 1). Similar results were previously obtained for deletion mutant CD-10 (Joshi et al., 1992, 1996). The functional analysis of mutant OSCP with six proline residues revealed that the mutant subunit was unable to bind with high affinity to the membranous fraction of the F_1-F_o enzyme (Figure 4) or reconstitute high inhibitor sensitivity (Figure 3) or P_i−ATP exchange activity in OSCP-depleted complexes (Table 2), although it retained the ablitity to maintain normal interactions with the soluble segment F_1. Regarding structural characterization, the 6-proline mutant form was indistinguishable from WT OSCP with respect to general characteristics relating its overexpression in *E. coli* (viz., subcellular location, molecular mass in SDS−PAGE, yield, stability in the host to proteases), gross secondary structure characteristics as judged by CD spectroscopy and helix-forming propensity, and three-dimensional structure as judged by affinity to S-Sepharose. However, quantitative CD analyses of the mutant forms CD-10 and KLSRAM181−186PPPPPP demonstrated a distinct decrease in the α-helical content whose magnitude was consistent with deletion/disruption of a localized helical segment (Figure 1). Parallel CD studies on the synthetic peptide K178−L190-GY, displaying a propensity to form an α-helix, provided support for the hypothesis that the observed drop in the helical content of mutant forms CD-10 and KLSRAM181−186PPPPPPP is due to the deletion/disruption of a specific helical segment encompassing residues K178−L190 (Figure 2). The predicted helix potential analyses of various OSCPs also indicated that only mutant form KLSRAM181−186 PPPPPP displayed a drop in its helix potential profile, and this drop was observed only in one specific region corresponding to residues A175−L190. Thus, the data presented strongly suggest that there is an α-helix at the C-terminal end of OSCP. These findings are consistent with previously reported secondary structure analyses of OSCPs from bovine and yeast mitochondria, and homologous subunit δ of ECF_1 and CF_1 enzymes, which indicated the possibility of a consensus helical segment at the C-terminal end of these subunits. Therefore, the inability of six-proline-mutant form to interact with the proton-pumping segment F_o, or to reconstitute the terminal step in ATP synthesis in the present experiments, does not appear to be primarily due to a global effect, arising from gross structural destabilization, but to a local effect from loss of a consensus helical element in the K178−M186 region. Based on the data presented, we suggest that (i) the K181−L190 region at the C-terminal end of OSCP encompasses an α-helix or part of an α-helix, and (ii) interactions between the C-terminal end of OSCP and subunit(s) of F_o in the F_1-F_o enzyme do not specifically require acidic or basic residues, or residues with uncharged side chain, but do involve an α-helix.

(b) High-Affinity OSCP−F_o Interactions May Involve Subunit b of F_o. Several laboratories have reported that OSCP interacts with subunit *b* of F_o. These reports are based on evidence derived from experiments involving *in vitro* reconstitution of the stalk segment from isolated subunits of

F_1F_o, as well as cross-linking of intact F_1F_o enzyme using cross-linking reagents of different chemical reactivities and effective distances (0−12 Å) (Archinard et al., 1986; Joshi & Burrows, 1990; Belogrudov et al., 1995). Beckers et al. (1992) showed formation of a cross-link between a basic amino acid residue of CF_1 *δ* and an acidic residue at the C-terminal end of CF_o *b*. Cox et al. (1984) proposed that the ECF_o subunit *b* interacts with the C-terminal part of ECF_1 *δ* which is an extended α-helix. So far no evidence has emerged to indicate any interactions of OSCP or subunit *δ* of ECF_1 or CF_1 with any other subunit of F_o except *b*. It is of interest to add that interactions between helical segments of subunit *δ* of ECF_1, and hydrophilic, helical projections of subunit *b* of ECF_o, have been implicated in the formation of the stalk that connects the F_1 and F_o segments of the F_1F_o enzyme (Hazard & Senior, 1994). Therefore, in light of available evidence and our present data, we propose that the region corresponding to residues K181−L190 of OSCP constitutes a site or part of a site for interactions with subunit *b* of F_o, although this suggestion should be treated as tentative until OSCP binding sites on F_o are completely mapped.

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