The Oligomycin Sensitivity Conferring Protein of Rat Liver Mitochondrial ATP Synthase: Arginine 94 Is Important for the Binding of OSCP to F_1[†]

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ABSTRACT: The oligomycin sensitivity conferring protein (OSCP) is an essential subunit of the mitochondrial ATP synthase (F_0F_1) long regarded as being directly involved in the energetic coupling of proton transport to ATP synthesis. To gain insight into the function of OSCP, mutations were made in a highly conserved central region of the subunit, and the recombinant proteins were studied using several biochemical assays. Rat liver OSCP was expressed to high levels in *Escherichia coli*, solubilized from inclusion bodies, renatured, and purified to homogeneity. The recombinant protein was able to reconstitute oligomycin-sensitive ATPase activity to inner membrane vesicles depleted of F_1 and OSCP, and bound to F_1 with a stoichiometry of 1:1. A novel fluorescence anisotropy assay was developed to study the affinity of binding of F_1 to OSCP, providing a K_d value of 51 ± 11 nM. Two highly conserved, charged residues (E91 and R94) which lie within the central region of OSCP were mutated, and the recombinant proteins (E91Q, R94Q, and R94A) were purified to homogeneity and judged by CD spectroscopy to have structures similar to that of the wild-type protein. Both R94 mutants demonstrated little or no binding to F_1, while the E91Q bound in a manner identical to that of wild-type OSCP. Significantly, all three mutant proteins were able to reconstitute F_1 with membranes and to confer oligomycin sensitivity to the same extent as wild-type OSCP. These results demonstrate that a single tight binding site exists on isolated rat liver F_1 for OSCP, and implicate arginine 94 as playing a critical role in this site. In addition, these results indicate that this tight binding site is not required for conferral of oligomycin sensitivity to the reconstituted F_0F_1 complex.

ATP synthases (F_0F_1)[1] are found in the energy-transducing membranes of mitochondria, chloroplasts, and most bacteria, where they use the energy stored as an electrochemical proton gradient to drive the synthesis of ATP (for review, see refs *1*−*4*). The two activities of these enzymes can be separated physically and functionally into a proton channel (F_0) and a soluble catalytic domain (F_1) which is the site of ATP synthesis or hydrolysis. ATP hydrolysis and ATP synthesis can occur on isolated F_1, but net ATP synthesis (turnover) can occur only when these two parts are tightly coupled since proton translocation through F_0 is required for product (ATP) release from F_1 (*5*).

Although the structure of the F_1 portion of the mitochondrial enzyme is known to atomic resolution (*6*, *7*), little is known about the structure of F_0, or about how the F_1 and F_0 moieties interact. The oligomycin sensitivity conferring protein (OSCP) is a subunit of the mitochondrial enzyme which is known to be essential for ATP synthesis (*8*). OSCP remains with the inner mitochondrial membrane when F_1 is purified, and on the basis of its ability to bind independently to F_1 and to F_0, and of cross-linking (*9*) and trypsin sensitivity (*10*) data, OSCP has been pictured as physically located between F_1 and F_0. In this stalk region, OSCP could participate directly in the communication between the two moieties. OSCP has not been considered to be part of F_0 because it can be selectively removed by alkali, and because it is related to the F_1-δ subunit of *Escherichia coli*, which is an integral component of *E. coli* F_1. These two proteins exhibit 22% identity and 36% homology in their amino acid sequences, concentrated in the carboxy termini and the amino termini of each protein, and in a central region corresponding to residues 88−95 of rat liver OSCP. Mutagenesis studies have implicated that the conservation at the termini of the proteins is important for function (*11*−*16*). The significance of the conserved central region of OSCP has not been investigated previously.

OSCP and bacterial δ are known to be required for the coupling of proton translocation through F_0 to ATP synthesis or hydrolysis on F_1 (*17*), but their specific role in the coupling mechanism is unknown. As mentioned above, they have been traditionally modeled in the stalk region of the ATP synthase where they would play an active role in transmitting the energy of proton movement through F_0 to the catalytic sites on F_1. Recently, however, an alternative model based

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[1] Abbreviations: OSCP, oligomycin sensitivity conferring protein; F_1, catalytic moiety of ATP synthase; F_0F_1, the complete ATP synthase complex; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IPTG, isopropyl thiogalactopyranoside; SMPs, submitochondrial particles; AUPs, alkaline-urea particles; CPM, (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; PVDF, polyvinylidine difluoride; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; GuHCl, guanidine hydrochloride; MW, molecular weight; BSA, bovine albumin; DTT, dithiothreitol; CD, circular dichroism; psi, pounds per square inch.

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on experiments with the *E. coli* and chloroplast δ subunits has been proposed. According to this model, OSCP is located at the exterior of the complex where it may act as a clamp or "stator" to hold the $\alpha_3\beta_3$ hexamer stationary relative to the proton channel while the γ subunit rotates during ATP synthesis (*18*, *19*). Thus, OSCP is not considered to be a dynamic player in the coupling process, but merely serves a structural role. Whether either of these models is correct remains to be demonstrated.

We have examined the highly conserved region around amino acid residues 88−94 of OSCP to determine its importance to the coupling of F_0 to F_1 or to the interaction of OSCP with F_1. OSCP and mutants of OSCP were prepared in large amounts, and their interaction with the F_1 moiety was examined using biochemical, biophysical, and molecular biological approaches. The results of these studies, summarized below, indicate that the central region of OSCP is involved in tight binding to F_1, and provide insights into the relationship of this binding interaction to that required to confer oligomycin sensitivity to F_1 upon its binding to F_0. This new information is likely to be valuable in our eventual understanding of both the mechanism of energy coupling within ATP synthase and the mechanism by which the enzyme is assembled.

EXPERIMENTAL PROCEDURES

Materials. *N*-[ethyl-1,2-^{3}H]-maleimide was purchased from DuPont NEN. CM-sepharose CL-6B was purchased from Pharmacia. IPTG was purchased from Diagnostic Chemicals Limited. *N*-(1-pyrene) maleimide and coumarin maleimide (CPM) were purchased from Molecular Probes. Restriction enzymes were purchased from New England BioLabs. The pET-15b expression vector and BL21 (DE3) cells were purchased from Novagen, and the endonuclease-deficient SURE cells were purchased from Stratagene. The enhanced chemiluminescent kit (ECL kit) and the Sequence Version 2 DNA sequence kit were purchased from Amersham. The BioSpin 6 column and the Coomassie blue dye binding protein assay were purchased from BioRad. The centricon-10s and the microcon-100 concentration devices were purchased from Amicon. The Quiaprep Spin Miniprep kit was purchased from Qiagen. The Prosec program 2.1 was from Aviv Associates and the Enzfitter program from Biosoft. Anti-bovine OSCP antibodies were kindly provided by Dr. Youssef Hatefi, Scripps Research Institute, La Jolla, CA. All other materials were of the highest grade commercially available.

Cloning of Rat Liver OSCP. The cDNA encoding OSCP was amplified from rat liver mRNA by RT-PCR using primers based on the published sequence (*20*) and which incorporated restriction sites to facilitate cloning into expression vectors. The resulting DNA was cloned into the pET-15b expression vector, placing OSCP expression under the control of the T7 promoter. The gene was inserted at the plasmid *Nco*I and *Bam*HI sites, removing the His-Tag coding sequence from the final construct. Initial screening for the correct plasmid construct was carried out in restriction-negative and endonuclease-deficient SURE cells, which do not contain the T7 polymerase and are therefore unable to express the cloned gene. Competent cells were prepared by the method of Chung (*21*). Transformation of competent cells was carried out as described in Sambrook et al. (*22*). The coding sequence in the final construct was sequenced and found to be identical to that published previously (*20*).

Expression and Purification of OSCP. For OSCP expression, the vector was transformed into BL21(DE3) cells which contain the T7 RNA polymerase under control of the *lacUVB* promoter. Cultures of 0.5 or 1 L were grown to log phase ($OD_{600\ nm} = 0.5$) at which time OSCP expression was induced by IPTG addition. High levels of expression were seen after 2.5 h of induction with 0.1 mM IPTG.

Cells were collected by centrifugation at 5000*g* for 5 min and then lysed as described in the pET system manual (Novagen). Briefly, pelleted cells were resuspended in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and then lysed by adding 0.1 mg/mL lysozyme and 0.1% Triton X-100 and incubating at 30 °C with shaking for 15 min. The lysate was sonicated for three 10 s pulses to shear DNA, and the insoluble protein (inclusion bodies) was sedimented by centrifugation at 12000*g* for 15 min. The protein pellet was washed with 0.5 M KCl, 2 mM EDTA and centrifuged as before. The final pellet was dissolved in 6 M GuHCl (guanidine HCl) containing 50 mM KPi (pH 8.0), 50 mM KCl, and 1 mM DTT. Any undissolved protein was removed by centrifugation and the GuHCl by dialysis against a buffer containing 50 mM KP_i (pH 8.0), 50 mM KCl, and 1 mM DTT.

The dialyzed protein was 85% pure as judged by SDS−PAGE and active in reconstitution assays. The protein was further purified by cation-exchange chromatography on a carboxymethyl-sepharose column, 1.5 cm diameter and 3 cm high. The column was equilibrated with 50 mM KPi (pH 8.0), 50 mM KCl, and 1 mM DTT. The dialysis supernatant was loaded, the column was washed with the same buffer containing 0.1 mM KCl, and the supernatant was eluted with a gradient of 0.1−0.5 M KCl. OSCP eluted at about 0.2 M KCl. Fractions containing OSCP were combined; Tween-20 was added to 0.01%, and the protein was concentrated in a centricon-10s device to 3−5 mg/mL. $(NH_4)_2SO_4$ was added to 30% saturation, and the protein was stored at 4 °C as an $(NH_4)_2SO_4$ precipitate.

Circular Dichroism. Circular dichroism (CD) spectra were collected on an Aviv Model DS CD spectropolarimeter. Protein was 0.1 mg/mL in 50 mM KP_i buffer (pH 8.0), 100 mM KCl, and 0.01% Tween-20. Five spectra were collected and averaged, corrected for protein concentration, and deconvoluted using the ProSec program 2.1 which employs the reference spectra and algorithm of Chang et al. (*23*).

Reconstitution Assay. The ability of OSCP to reconstitute F_1 with innermitochondrial membrane vesicles depleted in F_1 and OSCP (AUPs) was assayed using a method based on that previously described for the reconstitution of F_1 with F_1-depleted membrane vesicles (UPs) (*24*). Various concentrations of OSCP were incubated with 10 μg of F_1 and 100 μg of AUPs for 2 h at room temperature in a final volume of 50 μL. The membranes were then recovered by centrifugation for 20 min at 142000*g* (20 psi) in a Beckman airfuge and were resuspended in the same volume of H-medium. A 5 μL aliquot was assayed for oligomycin-sensitive ATPase activity using a spectrophotometric assay in which ADP released is coupled to the pyruvate kinase and lactic dehydrogenase reactions, and the decrease in optical density of NADH is monitored at 340 nm (*25*).

Rat liver F_1 was prepared as previously described (*25*) with the modification noted by Pedersen et al. (*26*). Urea particles (UPs) were obtained as previously described (*24*). Ammonia-urea particles (AUPs) were prepared by extracting urea particles (UPs) three times with NH_4OH. UPs (10 mg/mL) were treated with 0.34 N NH_4OH for 20 min on ice and centrifuged for 30 min at 120000*g* in a Beckman TY-65 rotor.

Binding of F_1 to F_0. F_1 was reconstituted with F_0 as previously described (*27*). Briefly, F_1 (0.67 μg) was incubated with F_0 (17 μg) for 1 h at room temperature, and an aliquot was assayed for ATPase activity and for sensitivity of that activity to oligomycin. F_0 was purified as previously described (*28*, *29*). To assay for peptide interference, 1 μg of the central-region peptide (sequence CNLMNLLAENGRL; Cys residue introduced at the N-terminus to facilitate labeling of the peptide for experiments not reported here) was preincubated with F_1 for 10 min before the addition of F_0 or added at the same time as the F_0.

Binding of OSCP to F_1. (A) Radiolabeled Assay. Pure OSCP was labeled with 5 μCi ^{3}H-NEM by incubation at room temperature for 30 min. Free ^{3}H-NEM was removed by passage through a gel filtration column (BioSpin) with a molecular weight cutoff of 6000 Da. Various concentrations of radiolabeled OSCP were incubated with 0.9 μM F_1 in 0.25 M KP_i (pH 7.5), 5 mM EDTA, and 0.01% Tween-20 in a final volume of 50 μL for 10 min at room temperature. Free OSCP was removed by centrifuging in a microcon-100 concentrator, followed by two brief washes to remove nonspecifically bound OSCP. The retained F_1−OSCP complex was collected by washing the membrane with 50 μL of the same buffer, and then assayed for protein concentration and for radioactivity. Radioactivity was measured using a LS6000IC liquid scintillation system (Beckman). To assay for interference by peptides, 500 μM central-region peptide or N-terminal peptide (sequence CEGRYATALYSAASKQKRLDQ) was included in the incubation mix and the complex was collected and measured as described above. As for the central-region peptide, a Cys residue was introduced at the N-terminus of the N-terminal peptide to facilitate labeling of the peptide for experiments not described here.

(B) Fluorescence Anisotropy Assay. Theory: The steady-state fluorescence anisotropy of a molecule is a measure of its rotational diffusion in solution. Under constant illumination, the intensity of fluorescence is measured twice, in planes perpendicular to ($I_\perp$) and parallel to ($I_{||}$) the plane of the polarized light used to excite the fluorophore. Anisotropy is defined as the following:

$$A = (I_{||} - I_\perp)/(I_{||} + 2I_\perp)$$

If a molecule is able to rotate between the time it is excited and the time that it emits, the emitted fluorescence will be random in orientation compared to the excitation beam. If, however, a molecule is relatively static, there will be little randomization, and the anisotropy value will increase. Upon binding to F_1 (370 kDa), the rotational diffusion of fluorescently labeled OSCP (21 kDa) is expected to decrease, and the anisotropy of the fluorescent probe attached to OSCP to increase accordingly.

The anisotropy of a mixture is described by the following equation:

$$A = \sum_{i=1}^{n} I_i\, c_i\, A_i$$

where I is the fluorescence intensity, c is the concentration, and A is the anisotropy of species i. Because incubation with F_1 does not change the fluorescence intensity of the labeled OSCP (Figure 4A), the anisotropy measured can be described as

$$A = \sum_{i=1}^{n} c_i\, A_i$$

The fraction of bound OSCP was taken to be (*29*)

$$F_b = \frac{(A_{meas} - A_{min})}{(A_{max} - A_{min})}$$

where A_{meas} is the value for anisotropy measured at a given F_1 concentration, A_{max} is the value of anisotropy measured when F_1 was present in a 20-fold molar excess over OSCP, and A_{min} was the anisotropy measure of the free labeled OSCP in solution. The moles of bound OSCP was then obtained by multiplying F_b by the OSCP concentration, and the resulting value was set equal to the moles of bound F_1 based on the one-to-one stoichiometry of F_1−OSCP binding observed under similar molar ratios in the experiments using radiolabeled OSCP. Free F_1 was calculated as total F_1 minus bound F_1. Binding constants were obtained by entering these values of free and bound F_1 into the following equation:

$$\text{bound} = \frac{(\text{capacity}\cdot\text{free})}{(K_d + \text{free})}$$

The equation was solved for capacity (total number of binding sites) and K_d using nonlinear regression analysis by the *Enzfitter* program.

Method. Pure OSCP was labeled with pyrene-maleimide by incubation with a 10:1 excess of probe for 15 min at room temperature. Free pyrene maleimide was removed by centrifugation through a gel filtration column (BioSpin 6). Fluorescence intensity and anisotropy measurements were made on a LS 50B luminescence spectrophotometer (Perkin-Elmer) with a thermostable cuvette-holder. All measurements were made at a temperature of 25 °C, with excitation and emission slit widths set at 5 nm, and using an excitation wavelength of 378 nm. For automatic anisotropy calculations, the default value of 1 for the G-factor was used.

To generate binding curves, various concentrations of F_1 were incubated with 50 nM labeled OSCP and the fluorescence anisotropy measured. Anisotropy values equilibrated within 1 min and were steady over at least 15 min.

Mutagenesis. Mutagenesis was carried out with the MutaGene phagemid in vitro mutagenesis kit, Version 2 (BioRad), using a method based on that developed by Kunkel (*30*, *31*). The primers used were the following: E91Q, 5′ACCATTTTGAGCAAG-3′; E91A, 5′-ACCATTTGCAGCAAG-3′; R94A, 5′-GTTGCCGAGCTCACCATTTTC-3′; R94Q, 5′GTTGCCGAGCTCACCATTTTC-3′. Each mutation was confirmed by DNA sequencing by the method of Sanger et al. (*32*). Automated sequencing of the entire OSCP coding

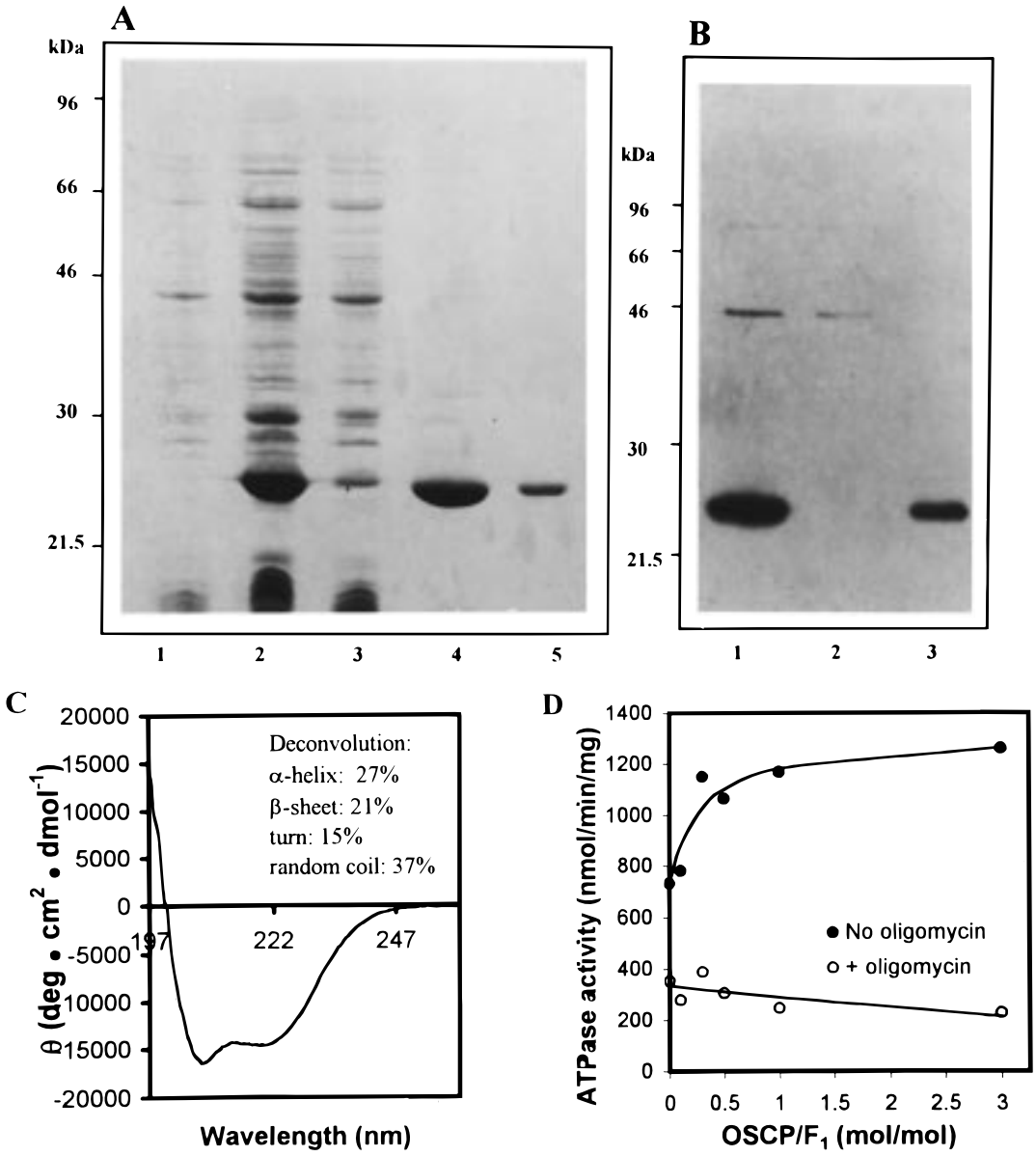

FIGURE 1: Expression, purification, and characterization of recombinant OSCP. (A) SDS−PAGE gel stained with Coomassie brilliant blue: lane 1, 100 μL of uninduced BL21 (DE3) cells transformed with the OSCP-containing pET vector, taken from culture immediately before IPTG addition; lane 2, 100 μL of the same culture after 2.5 h induction with 1 mM IPTG; lane 3, 10 μg of soluble protein after lysis of bacteria; lane 4, 4 μg of insoluble protein (inclusion bodies) after lysis of bacteria; lane 5, 0.4 μg of pure OSCP eluted from a CM-sepharose column. (B) Western blot probed with an antibody raised against the bovine heart OSCP (90% identical in amino acid sequence to rat liver OSCP): lane 1, 5 μL of induced BL21 (DE3) cells; lane 2, 10 μL of the same cells before induction; lane 3, 10 μg of mitochondrial protein. (C) Circular dichroism spectra of pure OSCP. Collection of spectra and deconvolution were as described under Methods. The inset shows the secondary structure approximation obtained by deconvolution. (D) Ability of OSCP to bind F_1 to F_1- and OSCP-depleted inner mitochondrial membranes (AUPs) in an oligomycin-sensitive manner. Total ATPase activity measured in the absence (filled symbols) and presence (open symbols) of 5 μg of oligomycin is shown for various molar ratios of OSCP and F_1. (See Methods.)

region confirmed that in each case the remaining sequence was wild-type.

Other Procedures. Rat liver mRNA was prepared using a single-step protocol involving guanidine thiocyanate (*33*). Bacterial DNA was purified from 5 mL cultures using the Qiaprep spin miniprep kit. Chain-termination DNA sequencing was carried out in the University Protein/Peptide/DNA facility on an ABI Prism 377 DNA Sequencer (Applied Biosystems). For N-terminal sequencing, protein bands were transferred from SDS−PAGE gels to PVDF membranes in CAPS buffer. Sequencing was carried out in the University Protein/Peptide/DNA facility located in the Department of Biological Chemistry. An ABI 492 Procise sequencer (Applied Biosystems), which uses the Edman degradation method (*34*), was employed. Peptides were synthesized in the Protein/Peptide/DNA facility. SDS−PAGE was carried out by the method of Laemmli (*35*). Western blotting was performed on nitrocellulose membranes with chemiluminescent band visualization using the ECL kit. Concentrations of soluble protein were routinely determined using the microassay protocol of Bio-Rad. For deconvolution of circular dichroism spectra, the protein concentration was confirmed by using the (BCA) assay from Pierce in addition to the Bio-Rad assay. Membrane protein concentration (SMPs, UPs, AUPs) was determined using the Biuret assay (*36*). BSA was used as the standard protein in all assays.

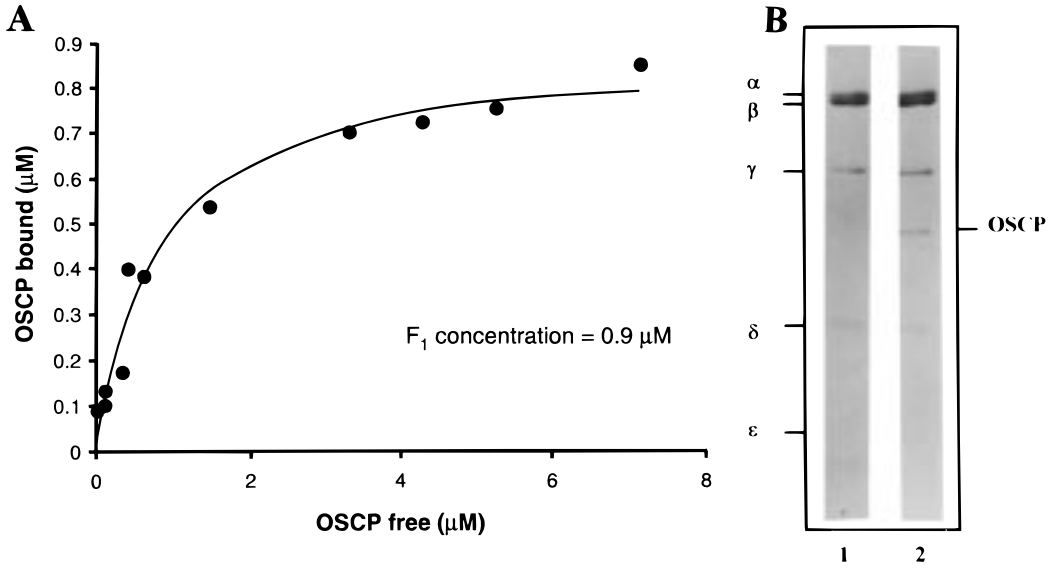

FIGURE 2: Determination of the stoichiometry of the rat liver F_1−OSCP complex. (A) OSCP was labeled with tritiated NEM and incubated with 0.9 μM F_1 for 10 min. (See Methods.) Free OSCP was removed by filtration through a 100-kDa MW cutoff membrane. The retained F_1−OSCP complex was recovered, the protein concentration was assayed, and the radioactivity was counted. Data shown represent the average of 3 independent experiments conducted with three different OSCP preparations. (B) Coomassie-stained SDS−PAGE gels showing the complex obtained when unlabeled OSCP is incubated with F_1 in a 5:1 molar ratio and the complex purified as described in A. The amount of control F_1 (lane 1) and F_1−OSCP complex (lane 2) loaded on the gels was 7.5 μg. At this protein load all subunits are visualized except the ϵ subunit.

RESULTS

Cloning and Expression of Rat Liver OSCP. The sequence of the OSCP cDNA obtained by RT-PCR amplification from rat liver mRNA (see Methods) was determined and found to be identical to that reported previously (*20*). The amplified DNA was cloned into a pET vector, which allowed a high level of expression of the recombinant OSCP in *E. coli*. OSCP protein was obtained (40−50 mg/L of cell culture), nearly all of which was found in inclusion bodies (Figure 1A, compare lanes 3 and 4). The identity of this protein was confirmed by Western blotting with an antibody raised against the bovine OSCP protein (Figure 1B) and by N-terminal sequencing of the first 10 amino acids (MFSKLVRPPV). The initiating methionine is not cleaved by the bacteria, resulting in a recombinant protein one amino acid longer than the protein found in tissue. Otherwise, the sequence obtained is identical to that reported (*20*).

Solubilization of Inclusion Bodies, Renaturation, and Purification of OSCP. The accumulation of most of the recombinant OSCP in inclusion bodies allowed for an efficient first purification step. GuHCl was found to be much more efficient at solubilizing the inclusion bodies than ammonium hydroxide, which was used previously to solubilize bovine OSCP expressed in *E. coli* (*13*). Significantly, after renaturation of the protein by slow removal of the GuHCl by dialysis, the protein is already about 85% pure as judged by SDS−PAGE (Figure 1A, lane 4).

The recombinant OSCP was further purified to homogeneity by ion-exchange chromatography on a carboxymethyl-sepharose column (see Methods). OSCP bound to the column at pH 8 and 9, consistent with a predicted pI of 10.4. The protein eluted at approximately 0.2 M KCl. The resulting OSCP was at least 99% pure as judged by SDS−PAGE (Figure 1A, lane 5). Routinely, 75% of the protein was recovered after solublization with GuHCl and subsequent renaturation of the inclusion body protein by dialysis. Of this, 60−75% was recovered from the ion-exchange column, for a final yield of 20−30 mg of pure OSCP/L of cell culture.

Spectroscopic Characterization of Renatured Recombinant OSCP. Two methods were used to characterize the structure of the renatured OSCP: circular dichroism (CD) and fluorescence spectroscopy. Both indicate that the protein adopts a nativelike structure. The CD spectrum of the pure recombinant OSCP (Figure 1C) is highly reproducible, being nearly identical for various protein preparations, and for protein analyzed directly after dialysis versus protein resuspended from an ammonium sulfate precipitate. Deconvolution of the spectrum indicates that OSCP consists of 27%, 21%, 15%, and 37% α-helix, β-sheet, turns, and random coil, respectively. Although the helical content is somewhat less than that reported for the CD spectra of bovine and porcine OSCP purified from heart tissue (*37*, *38*), this may be the result of using different deconvolution programs, or due to the different buffers used.

The probe coumarin maleimide (CPM) becomes fluorescent only after reaction with a sulfhydryl group as is found on cysteine residues, and is a sensitive reporter of the polar nature of its environment. OSCP contains only one cysteine, Cys118, which has been previously demonstrated to be easily labeled by a number of cysteine-reactive probes without an affect on activity (*39*). When CPM was reacted with recombinant OSCP, a blue shift of its fluorescence maximum was seen (data not shown), indicating that it is located within a hydrophobic area, as implicated in an earlier report (*39*).

Characterization of the Activity of Renatured, Recombinant OSCP. A reconstitution assay was developed to demonstrate that the recombinant OSCP was functionally active. The protein was found to reconstitute F_1 with F_1- and OSCP-depleted membranes (AUPs) in an oligomycin-sensitive manner (Figure 1D). The maximum effect on binding and on conferral of oligomycin sensitivity is seen at a ratio of approximately 1 mol of OSCP/mol of F_1.

Binding of OSCP to F_1. Both the stoichiometry and the affinity of binding of recombinant OSCP to rat liver F_1 were studied. To determine the stoichiometry of OSCP in the F_1−OSCP complex, a simple filtration assay was developed using OSCP radiolabeled at Cys118 with ^{3}H-NEM. Free OSCP was separated from bound F_1−OSCP complex by spinning through a membrane with a molecular weight cutoff of 100 kDa. Binding was found to saturate at a near one-to-one complex (Figure 2), indicating the existence of only one tight binding site for OSCP on F_1. A small amount of tween-20 was found to minimize nonspecific binding of OSCP to the reaction tubes (as in ref *40*) and was therefore included in all binding assays.

Although the above radioactive binding assay provided information about the OSCP−F_1 stoichiometry, it could not be used to study the affinity of the OSCP−F_1 interaction due to nonspecific binding of OSCP to the membrane. Therefore, a fluorescence anisotropy assay was developed to obtain this information. OSCP was labeled on Cys118 with the fluorescent probe pyrene-maleimide. Subsequent incubation of labeled OSCP with F_1 did not result in a change of either the maximum or the intensity of the emission spectrum of the labeled OSCP (Figure 3A). However, incubation with F_1 did cause an increase of the fluorescence anisotropy of the labeled protein (Figure 3B). From this anisotropy change a dissociation constant, K_d, could be calculated (Figure 3C) as described in detail under Methods. Although some variability was observed among different OSCP preparations, the K_d obtained was always in the range 30−120 nM (Figure 3C), with an average for all experiments of 51 ± 11 nM.

Significance of the Centrally Conserved Region of OSCP for its Capacity to Interact with F_1 and OSCP-Deficient Membranes (AUPS). Most of the sequence identity and homology between rat OSCP and *E. coli* δ is found in the C-terminal half of the proteins (Figure 4), but another stretch of eight amino acids in the interior of the sequence, LLAENGRL, is almost absolutely conserved in all known OSCP and δ subunits. A peptide consisting of the sequence CNLMNLLAENGRL was synthesized and found to interfere in the interaction of F_1 with F_0 and in the interaction of F_1 with OSCP. In an assay for the reconstitution of purified F_0 with F_1 (see Methods), preincubation of F_1 with this peptide or co-incubation of the peptide with F_1 and F_0 caused a reduction of the ATPase activity of the resulting F_0F_1 complex (265 ± 24 and 338 ± 97 nmol/(min mg) respectively) compared to the value observed in the absence of peptide (551 ± 24 nmol/(min mg)). The peptide did not inhibit the ATPase activity of F_1 in the absence of F_0. The peptide was also found to prevent the binding of radiolabeled, recombinant OSCP to soluble F_1 (see Methods). When OSCP was incubated with F_1 in a molar ratio of 1:1 (0.30 ± 0.03 pmol of OSCP bound), the peptide blocked the binding (0.16 ± 0.09 pmol of OSCP bound). A peptide homologous to the N-terminus of OSCP had no effect on binding at the same concentration (0.33 ± 0.06 pmol of OSCP bound).

To analyze the significance of this conserved region for OSCP function in detail, the two charged residues in this stretch, Glu91 and Arg94 (indicated by arrows in Figure 4), were each mutated to glutamine and alanine. The mutant proteins were expressed and purified exactly as wild-type OSCP. One of the mutants, E91A, was unstable and was poorly expressed in *E. coli* and degraded during purification (data not shown). The other three were expressed to levels similar to that of wild-type OSCP, and were purified and assayed for their ability to bind F_1 and to reconstitute oligomycin-sensitive ATPase activity. The migration on SDS−PAGE for each of the three mutants is identical to that of the wild-type OSCP (Figure 5A), and the CD spectra are almost identical to that of wild-type OSCP (Figure 5B).

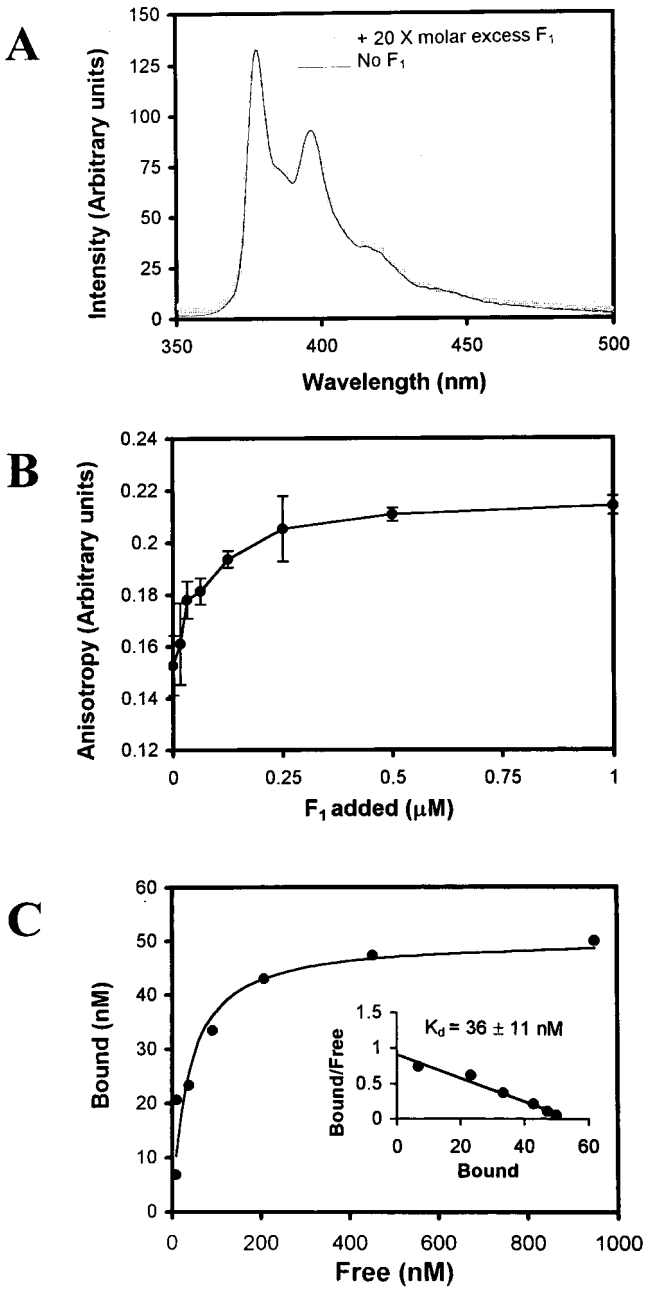

FIGURE 3: Determination of the affinity of binding of OSCP to F_1 using fluorescence anisotropy. Fluorescence anisotropy measurements and the labeling of OSCP with pyrene maleimide were carried out exactly as described under Methods. (A) Emission spectra of pyrene-labeled OSCP in the presence and absence of a 20-fold molar excess of F_1. (B) Increase in the fluorescence anisotropy of the labeled OSCP with increasing concentrations of F_1. The data are the average of the 3 different experiments carried out on one OSCP preparation. Error bars are equal to one standard deviation. The data are representative of those obtained with 6 different OSCP preparations. (C) Determination of binding constants from the data in B. The average K_d value for all 6 OSCP preparations was 51 ± 11 nM.

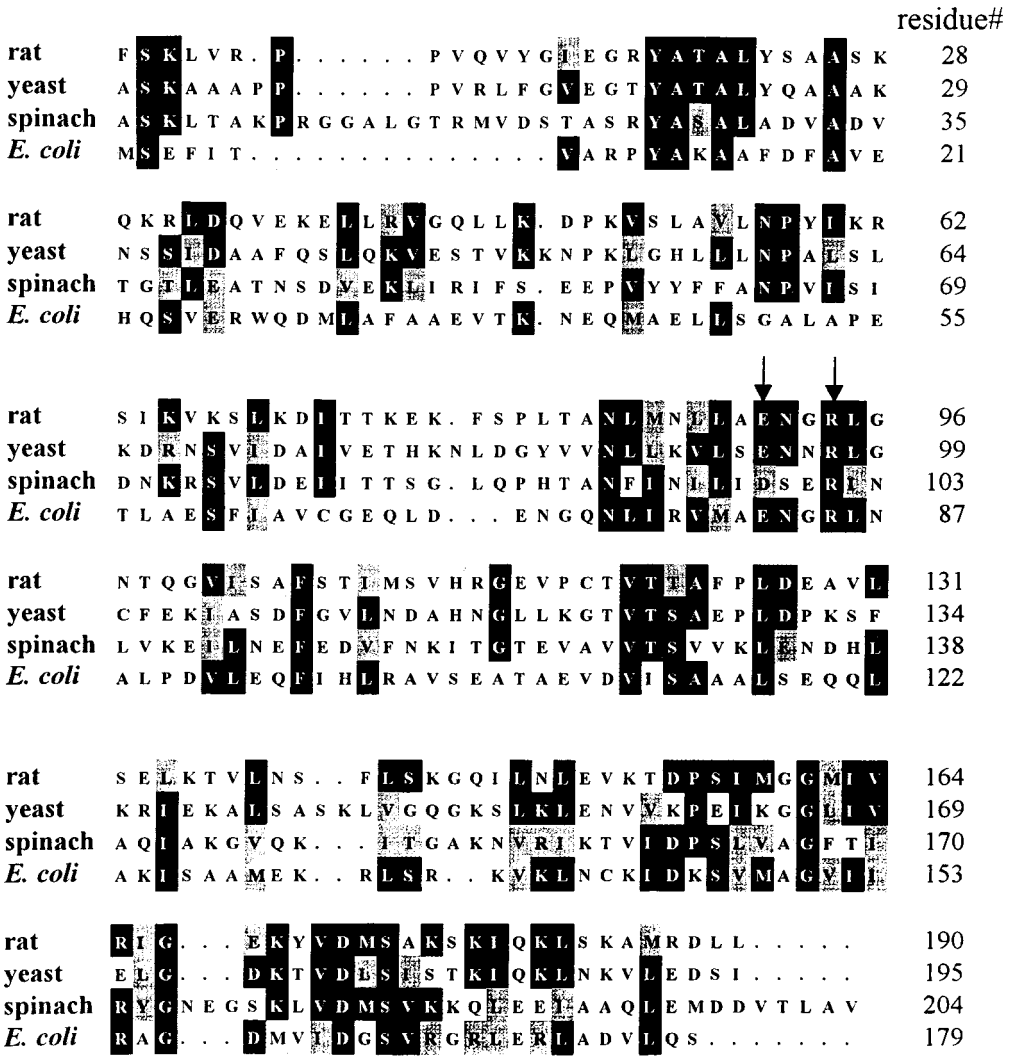

FIGURE 4: Alignment of amino acid sequences of OSCP and its δ subunit analogues from four different sources. The sources were rat (OSCP), yeast (OSCP), spinach (δ subunit), and *E. coli* (δ subunit). Identical amino acids are shaded black. Similar amino acids are shaded gray. Only those amino acids which are similar in at least 3 of the 4 sequences have been highlighted. The 2 residues which were mutated, Glu91 and Arg94, are indicated with arrows.

As described for the wild-type OSCP, each mutant was labeled with ^{3}H-NEM and its ability to bind to F_1 was measured (Figure 6). E91Q was found to bind in a manner very similar to that of wild-type OSCP, while R94Q and R94A demonstrated little or no binding to F_1 in this assay. To determine how this reflected a change in binding affinity, each mutant was labeled with pyrene maleimide and assayed in the fluorescence anisotropy assay. Again, E91Q was found to bind with an affinity very similar to that of wild-type OSCP (Figure 7A,B) with a K_d near 77 nM. R94Q, however, showed very little change in anisotropy when incubated with F_1, and no saturation (Figure 7C).

The mutant proteins were then assayed for their ability to bind F_1 to mitochondrial inner membrane vesicles deficient in OSCP (AUPs), and to confer oligomycin sensitivity to the ATPase activity. All three mutants were perfectly capable of reconstituting F_1 with membranes and conferred the same extent of oligomycin sensitivity (Figure 8A,B). In addition, no change in the stability of the reconstituted complex was observed. When AUPs were kept at room temperature, the ATPase activity of reconstituted AUPs decreases over time, with no associated change in the oligomycin sensitivity of the remaining activity. Reconstituted particles with wild-type or mutant OSCPs lost activity at the same rate (Figure 8C).

DISCUSSION

The data presented here indicate that the conserved region consisting of OSCP residues 88−95, and especially Arg94, is involved in the binding of OSCP to isolated F_1 in solution. Characterization of this binding provides evidence that there is only one binding site for OSCP on F_1, and that this site is tight, with a K_d of about 50 nM. However, removal of this binding site by mutagenesis does not affect the capacity of OSCP to reconstitute F_1 with membranes and restore oligomycin sensitivity. Thus, other interactions in the complete F_0F_1 complex appear important for OSCP function beyond those involved in the formation of the F_1−OSCP complex per se. Implications of these findings are discussed below.

The sequence LLAENGRL is almost absolutely conserved in all OSCP and δ subunits sequenced to date. Initial experiments conducted with a peptide containing this sequence indicated that this region might interact with F_1, since

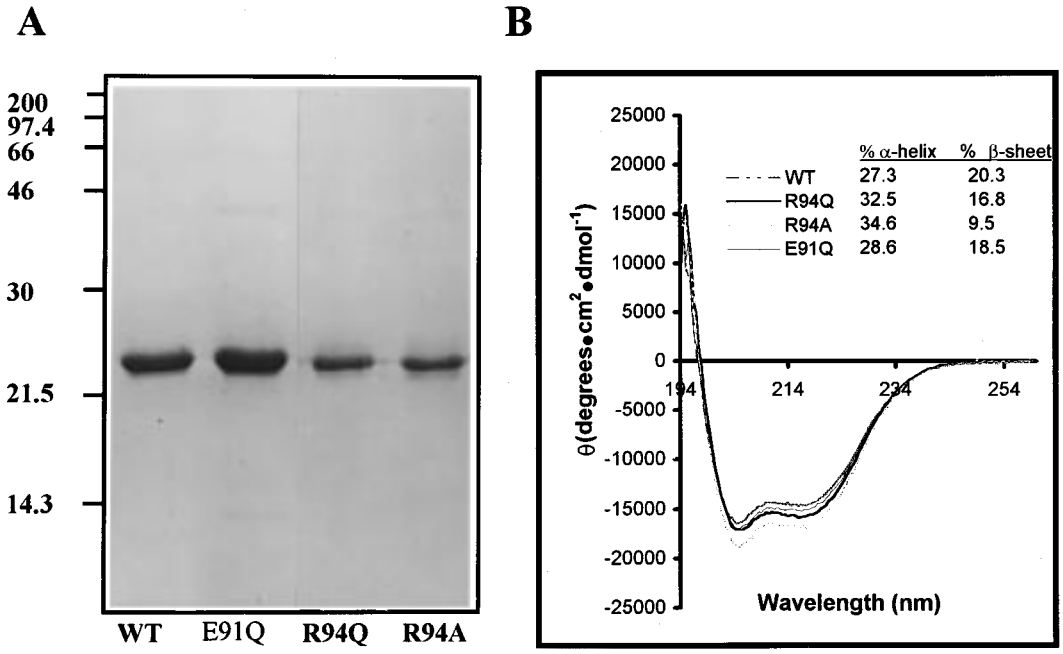

FIGURE 5: Purification and structure of the OSCP mutants. (A) SDS−PAGE gel stained with Coomassie brilliant blue. Each lane contains 1 μg of the purified protein. (B) CD spectra of each of the mutants and wild-type OSCP preparations. The inset shows the α-helix and β-sheet composition obtained by deconvolution of the CD spectra. β-turn and random coil composition did not change.

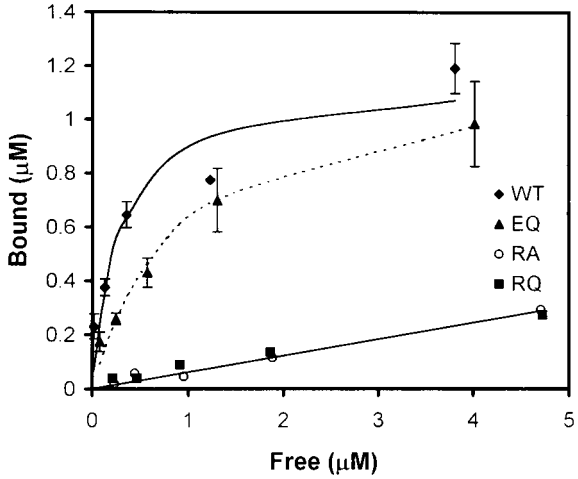

FIGURE 6: Assay of the ability of the mutant forms of OSCP to bind to F_1. The binding of radiolabeled OSCP, either wild type or mutant, to F_1 was measured exactly as described under Methods. The averages of 2 independent experiments are shown for WT, E91Q, and R94Q. Data are from one experiment for R94A. All OSCP proteins were assayed simultaneously. Error bars represent average deviation.

preincubation of F_1 with the peptide before addition of F_0, or co-incubation of the peptide with F_1 and F_0, resulted in a reduction of the ATPase activity recovered from an $F_0 + F_1$ reconstitution experiment (see Results). The peptide did not inhibit the ATPase activity of F_1 alone, and the ATPase activity of the reconstituted mixture was sensitive to oligomycin, indicating that the activity inhibited by the peptide was not due to F_1 which was unable to complex with F_0. Instead, the peptide may disturb the proper interaction between F_1 and F_0, blocking the ATPase activity of the complex. The peptide was found to compete for the binding of radiolabeled OSCP to F_1, reducing binding by about 50% while a peptide of the N-terminal sequence of OSCP had no effect on F_1−OSCP binding at the identical concentration (see Results).

To study the importance of the central region of OSCP more closely, the charged residues in the stretch (E91 and R94) were mutated. The resultant mutant OSCP proteins E91Q, R94Q, and R94A were purified (Figure 5A) and shown to have similar secondary structures (Figure 5B) to that of wild-type OSCP. Significantly, both the R94Q and R94A mutants displayed little capacity for binding to F_1 (Figures 6 and 7), though they were completely normal in their capacity to confer oligomycin sensitivity on F_1 in a membrane assay (Figure 8). The E91Q mutant was unimpaired in its capacity to bind to F_1 (Figures 6 and 7) and to confer oligomycin sensitivity on F_1 in a membrane assay (Figure 8). These results indicate that a rather limited region including Arg94 but excluding Glu91 is involved in the interaction between soluble F_1 and OSCP, though it is not the only interaction important for conferring oligomycin sensitivity on F_1. Experiments to determine whether the mutations have an effect on the ATP synthase activity or proton pumping ability of the reconstituted F_0F_1 complex were attempted, but the process of stripping F_1 and OSCP from AUPs also removed other components, leaving the membranes leaky. It would be interesting to see whether the mutations have an affect on these other assays of coupling when appropriate reconstitution systems become available.

Interestingly, in the recently deduced three-dimensional structure of the N-terminal half of the *E. coli* δ subunit (*41*), Arg85, the residue analogous to rat liver OSCP Arg94, is seen to be in the center of a loop (Figure 9A) residing in space near the N-terminus. A mutagenesis study conducted in *E. coli* found that changing Arg85 to glutamine resulted in a 50% decrease in the amount of ATPase activity bound to bacterial membranes in vivo (*42*). Taken with the results reported here, this argues that OSCP is a structural as well as a functional homologue of the δ subunit, and that the Arg residue may reside in a similar fold and play a similar role in OSCP as in bacterial δ, but be required to a greater extent in the binding to mitochondrial F_1. Significantly, deletion

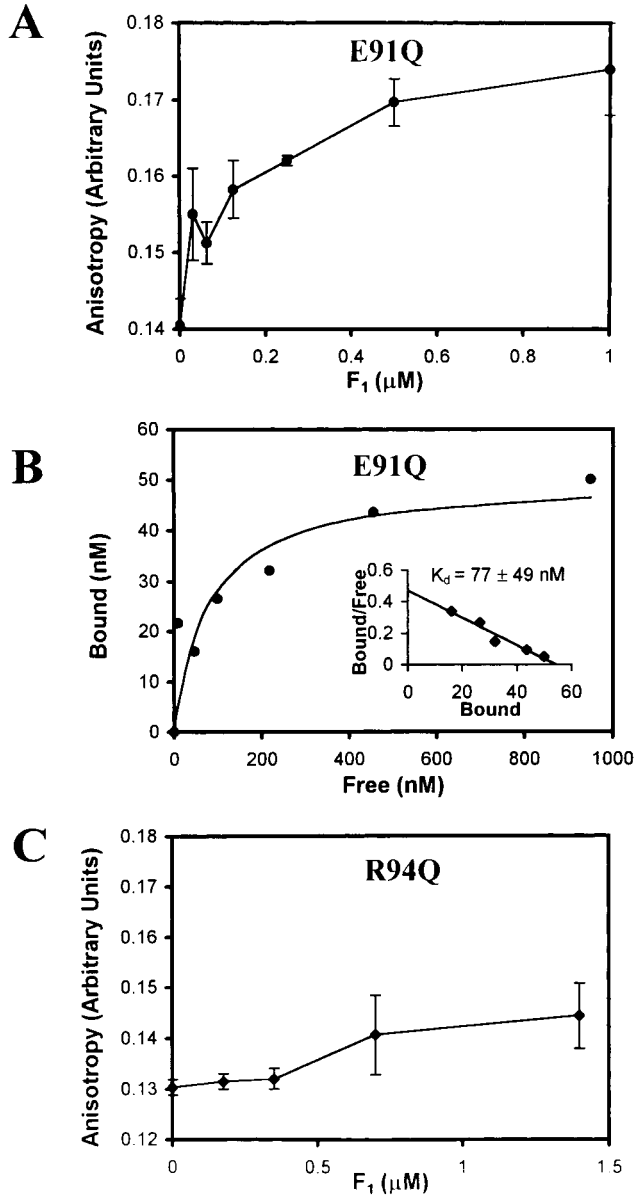

FIGURE 7: Determination of the binding constants for OSCP mutants using fluorescence anisotropy. Fluorescence anisotropy measurements and labeling of OSCP with pyrene maleimide were carried exactly as described under Methods. (A) Increase in anisotropy of labeled E91Q upon addition of F_1. Data shown are the average of four experiments. (B) K_d determination by Enzfitter, using the data shown in A. (C) Binding of R94Q to F_1. The averages of 3 experiments are shown. Because saturation is not reached, a K_d value cannot be calculated from these data.

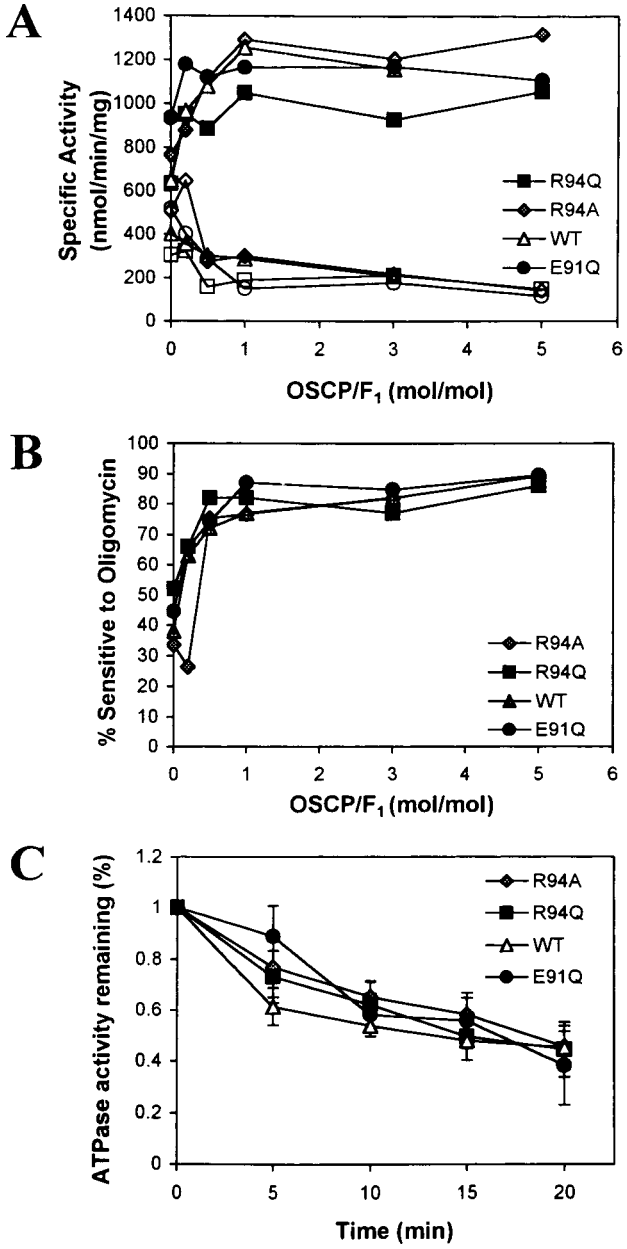

FIGURE 8: Capacity of mutant forms of OSCP to reconstitute oligomycin-sensitive ATPase activity. (A) OSCP-dependent binding of F_1 to F_1- and OSCP-depleted AUPs. Total ATPase activity measured in the presence (lower curves) and absence (upper curves) of 5 μg of oligomycin is shown for increasing amounts of mutant or wild-type OSCP added to AUPs. (See Methods.) (B) The percent of the ATPase activity sensitive to oligomycin, calculated from the data shown in A. (See Methods.) (C) Decrease in activity of AUPs reconstituted with F_1 and OSCP.

of the N-terminal 28 amino acids of bovine OSCP (*12*) results in a very similar phenotype to that of the Arg94 mutants, disrupting binding to F_1 but having little effect on the capacity of OSCP to confer oligomycin sensitivity on F_1 in a membrane assay. Therefore, the N-terminus and that part of the central loop including Arg94 may define a face of OSCP which interacts with F_1.

The mutagenesis study reported here identifies the critical importance of Arg94 to the binding of OSCP to isolated F_1, yet also indicates that the tight site detected between OSCP and F_1 in solution may not be the only interaction required for OSCP function in the reconstituted system, as assayed by oligomycin sensitivity of ATPase activity. Rather, another site of interaction between OSCP and F_1 may be involved in the conferral of oligomycin sensitivity. This other site might result from a conformational change in OSCP induced when OSCP interacts with one or more F_o components. Here, it is important to note that previous studies on ATP synthases from bovine heart (*43*), *E. coli* (*44*, *45*), and chloroplasts (*46*) show that OSCP (bovine heart) and the δ subunit (*E. coli*, chloroplast) interact with the F_o subunit "b". Therefore, although a mutation within the face defined by the loop containing Arg94 and the N-terminal helix of OSCP may dramatically affect its binding to isolated F_1 (Figure 9B), in reconstitution assays an unimpaired region of OSCP may still interact with the b subunit forming a b-OSCP binding site for F_1 (Figure 9C). It is this interaction

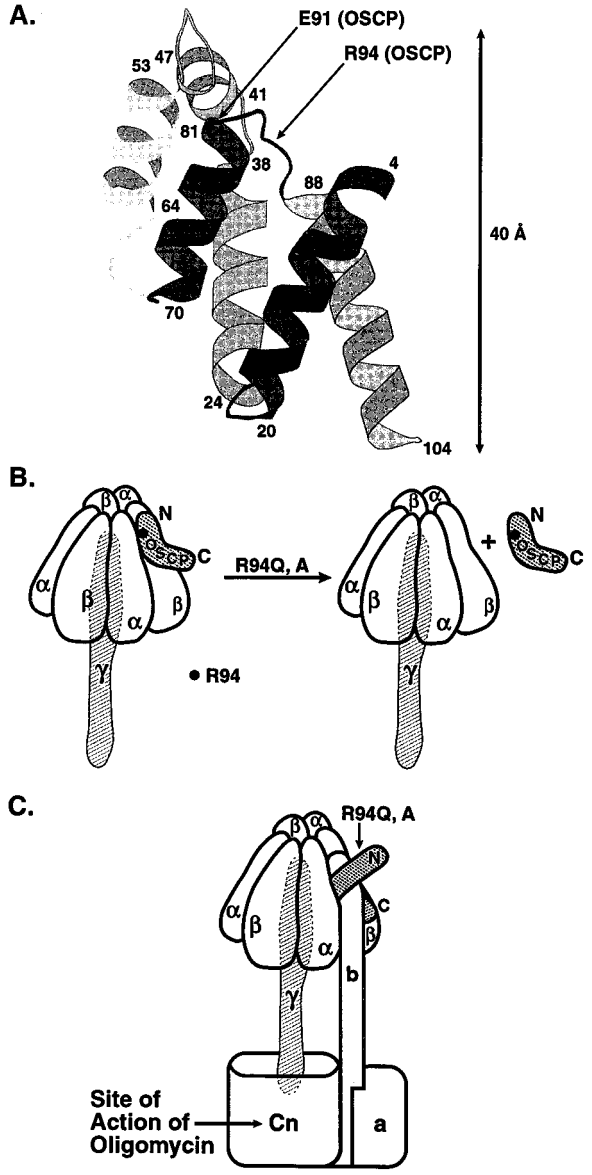

FIGURE 9: Models accounting for experimental observations obtained with wild-type and mutant forms of OSCP. (A) Location of the mutated residues (Glu91 and Arg94) of rat liver mitochondrial OSCP within the homologous region of the δ subunit of *E. coli* ATP synthase. The three-dimensional structure from Wilkens, et al. (*41*) of 105 N-terminal amino acid residues of the *E. coli* δ subunit is shown (reproduced with permission of the authors and the journal). The positions where Glu91 and Arg94 of OSCP are predicted to lie within this structure are indicated. These residues correspond respectively to Glu82 and Arg85 of *E. coli* δ. Both residues lie within a loop connecting two α-helical regions. (B) Illustration depicting how OSCP in analogy to the homologous *E. coli* or chloroplast δ subunits (*18*, *54*) may bind to the upper outer edge of the isolated F_1 molecule, and how mutagenesis of R94 in the N-terminal domain of the OSCP may disrupt this binding. (C) Illustration depicting how a second site derived from the interaction of OSCP with another subunit, most likely the b subunit (*43*−*46*), may account for the conferral of oligomycin sensitivity to F_1, even when the R94-dependent site is impaired. Whether the b subunit is a monomer (*50*) or a dimer (*9*) in mammalian ATP synthase remains unresolved.

that may be responsible, at least in part, for both oligomycin inhibition of ATPase activity and a stator function of OSCP.

The stoichiometry of binding of F_1 and OSCP has been debated, with 3 (*47*), 2 (*48*), and 1 (*49*, *50*) OSCP(s)/F_1 being proposed. A stoichiometry of 1:1 has become accepted, and is consistent with a stator function, though it is also consistent with a model in which OSCP moves between the three F_1 active sites. The radiolabeled-OSCP experiments reported here clearly support the existence of only one site (Figure 2), and of a reproducibly obtained 1:1 F_1−OSCP complex. The novel fluorescence anisotropy experiments (Figure 3) indicate that the interaction between rat liver OSCP and F_1 is very tight, with a dissociation constant in the range of 50 nM. The existence of one tight binding site for OSCP on F_1 opens speculation about the role of OSCP in the coupling mechanism, as F_1 has three equivalent active sites, one on each β subunit (*7*). The earlier discovery in this laboratory (*25*, *51*) that the small subunits (γ, δ, and ϵ) exist in single copies raised the question of how these subunits might interact equivalently with each of the β subunits. The suggestion that the γ and possibly additional subunits rotate relative to the β subunits (*52*, *53*) arose in part from this dilemma. Since OSCP is present in a single copy, it could be argued that it too may move between β's, in which case one might expect to find evidence for three OSCP binding sites on F_1. However, as only one tight binding site is detected, OSCP may serve a role that requires it to be static rather than dynamic during catalysis, as in the stator model.

Identification of the location of this one OSCP binding site on F_1 will be important for understanding the role of OSCP in coupling. Recent findings (*18*, *54*) have indicated that the *E. coli* and chloroplast δ subunits appear to reside at the upper outer edge of the $\alpha_3\beta_3$ hexamer (*7*) rather than in the stalk region. This, taken with the finding that the γ subunit does rotate a full 360° during catalysis (*55*), has been the basis for the stator model of δ (and OSCP) function in which δ is modeled into the cleft between an $\alpha\beta$ pair. If this is the location of OSCP in the F_1−OSCP complex, then it is unclear why there should be only one tight binding site rather than three. There is no evidence from cross-linking studies for an interaction of OSCP with any of the single-copy subunits of F_1, and the crystal structures of F_1 depict no difference between the clefts of the different $\alpha\beta$ pairs in this upper part of the enzyme. Interestingly, the δ subunit of the thermophilic bacterium PS3 binds to an $\alpha_3\beta_3$ complex, in the absence of nucleotide and of the γ and ϵ subunits, with a stoichiometry of 1:1 (*56*). The crystal structure of this $\alpha_3\beta_3$ complex (*57*) depicts a perfectly symmetrical hexamer, indicating that the binding of the δ subunit (or of OSCP) may be enough to induce an asymmetry in the enzyme. Alternatively, OSCP may span the top of the F_1 molecule, sterically blocking the binding of a second molecule of OSCP. Experiments are currently in progress to identify the precise location of OSCP within the F_1 complex.

In conclusion, it has been demonstrated that Arg94 is important for the binding of OSCP to a single, tight site on isolated F_1, but that this site alone is not essential for the coupling function of OSCP. Interactions between OSCP and subunits of F_0 are expected to modify the interaction of OSCP with F_1, creating a new or stronger binding interaction (Figure 9). In the context of the partial NMR structure of the *E. coli* δ subunit, and of mutagenesis studies conducted in the *E. coli* and bovine systems, the identification of the significance of Arg94 helps to define a face of OSCP involved in the binding to F_1. The finding of only one tight site on F_1 is curious in the absence of an interaction between

OSCP and the other single-copy subunits, and implies structural differences between $\alpha\beta$ pairs, or the binding of one OSCP to more than one $\alpha\beta$ pair. The identification of the binding site on F_1 will be the next step in understanding the function of this essential subunit.

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