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Interaction between the Oligomycin Sensitivity Conferring Protein and the F_o Sector of the Mitochondrial Adenosinetriphosphatase Complex: Cooperative Effect of the F_1 Sector†

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ABSTRACT: Beef heart mitochondrial oligomycin sensitivity conferring protein (OSCP) labeled with [^{14}C]-*N*-ethylmaleimide ([^{14}C]OSCP) at the only cysteine residue, Cys-118, present in the sequence [Ovchinnikov, Y. A., Modyanov, N. N., Grinkevich, V. A., Aldanova, N. A., Trubetskaya, O. E., Nazimov, I. V., Hundal, T., & Ernster, L. (1984) *FEBS Lett. 166*, 19–22] exhibits full biological activity in a reconstituted F_o–F_1 system [Dupuis, A., Issartel, J. P., Lunardi, J., Satre, M., & Vignais, P. V. (1985) *Biochemistry 24*, 728–733]. The binding parameters of [^{14}C]OSCP with respect to the F_o sector of submitochondrial particles largely depleted of F_1 and OSCP (AUA particles) have been explored. In the absence of added F_1, a limited number of high-affinity OSCP binding sites were detected in the AUA particles (20–40 pmol/mg of particles); under these conditions, the low-affinity binding sites for OSCP were essentially not saturable. Addition of F_1 to the particles promoted high-affinity binding for OSCP, with an apparent K_d of 5 nM, a value 16 times lower than the K_d relative to the binding of OSCP to F_1 in the absence of particles. Saturation of the F_1 and OSCP binding sites of AUA particles was attained with about 200 pmol of both F_1 and OSCP added per milligram of particles. The oligomycin-dependent inhibition of F_1-ATPase bound to AUA particles was assayed as a function of bound OSCP. At subsaturating concentrations of F_1, the dose–effect curves were rectilinear until inhibition of ATPase activity by oligomycin was virtually complete, and maximal inhibition was obtained for an OSCP to F_1 ratio of 1 (mol/mol). Experiments carried out with F_1 labeled in the β subunit by [^{14}C]dicyclohexylcarbodiimide ([^{14}C]F_1) indicated that [^{14}C]F_1, in spite of extensive inactivation [Pougeois, R., Satre, M., & Vignais, P. V. (1979) *Biochemistry 18*, 1408–1413], was able to bind to the AUA particles specifically, even in the absence of OSCP, as does native F_1, with a K_d value of 60 nM. OSCP enhanced by about 6 times the binding affinity of [^{14}C]F_1 for the AUA particles but not its binding capacity. The mutual promotion of OSCP and F_1 for binding to AUA particles was corroborated by a double-labeling experiment carried out with [^{3}H]OSCP and [^{14}C]F_1. Whereas the binary associations between OSCP and particles or between F_1 and particles were readily reversible, the ternary complex formed by particles, F_1, and OSCP was much more difficult to dissociate. These results make it likely that OSCP is a key component required for the stability of the mitochondrial F_1–F_o complex. Photoirradiation with an azido derivative of OSCP in the presence of AUA particles resulted in the photolabeling of mainly the β subunit of F_1, whereas, in the absence of particles, azido-OSCP reacted with both the α and β subunits of F_1, with strong preference for the α subunit, suggesting that F_1 undergoes conformational changes when it binds to the F_o sector of the ATPase complex.

The coupling between the catalytic sector F_1[1] and the proton channel F_o of the mitochondrial ATPase complex is central to the mechanism of ATP synthesis. The oligomycin sensitivity conferring protein (OSCP) (Mc Lennan & Tzagoloff, 1968) is thought to be one of the peptides that link the catalytic sector F_1 of the mitochondrial ATPase complex to the membrane sector F_o (Mc Lennan & Asai, 1968). F_1 per se is insensitive to oligomycin; however, in the reconstituted F_1–F_o complex, F_1 becomes sensitive to oligomycin, a ligand of F_o, provided that OSCP is added to the F_1–F_o complex [see Tyler (1984) for review]. Although OSCP is recognized as a key factor in oxidative phosphorylation, its exact function remains enigmatic, partly due to the lack of a precise assessment of the

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[1] Abbreviations: OSCP, oligomycin sensitivity conferring protein; [^{14}C]OSCP, OSCP labeled by [^{14}C]NEM; TPCK-trypsin, trypsin treated by L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; ATPase, adenosinetriphosphatase; F_1, catalytic sector of H^+-dependent ATPase; F_o, membrane sector of H^+-dependent ATPase; [^{14}C]F_1, F_1 labeled in the β subunit by [^{14}C]DCCD; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; DCCD, dicyclohexylcarbodiimide; AP-OSCP, azidophenacyl-OSCP; AUA particles, submitochondrial particles depleted of F_1 and OSCP after treatment with urea and ammonia; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; MES, 4-morpholineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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topographical relationship between OSCP, F_1, and F_o within this complex. For example, the same group who proposed OSCP as the stalk linking F_1 and F_o reported that added F_1 was able to bind to OSCP-depleted submitochondrial particles (Mc Lennan & Asai, 1968; Mc Lennan & Tzagoloff, 1968). Furthermore, it has been suggested that the β subunit of F_1 interacts directly with the F_o sector of the ATPase complex (Hoppe et al., 1984). Interest in the mechanism of action of OSCP was recently revived by the report of the whole sequence of this peptide (Ovchinnikov et al., 1984). Structural homologies have been suggested to exist between OSCP on the one hand and the δ subunit of the *Escherichia coli* F_1 (Walker et al., 1982; Ovchinnikov et al., 1984) and subunit b of *E. coli* F_0 (Ovchinnikov et al., 1984) on the other. These homologies have been discussed in terms of the possible function of OSCP as a link between F_1 and F_o. The availability of a radiolabeled derivative of OSCP, still fully active in reconstitution assays (Dupuis et al., 1985a,b), has led us to study the binding stoichiometry of OSCP with respect to isolated F_1 and the location of bound OSCP with respect to the F_1 subunits. It was concluded that there is one high-affinity OSCP binding site per F_1 and that OSCP interacts with both α and β subunits (Dupuis et al., 1985a,b). The aim of this work was the analysis of interactions of radiolabeled OSCP with the F_o sector of the ATPase complex. Reconstitution experiments with depleted particles supplemented with radiolabeled OSCP and F_1 were carried out to explore the relationships between the recovery of sensitivity of ATPase to oligomycin in a reconstituted system and the binding stoichiometry of OSCP with respect to F_o and F_1.

Experimental Procedures

Materials. [^{14}C]-*N*-Ethylmaleimide (30 mCi/mmol) and [^{14}C]dicyclohexylcarbodiimide (54 mCi/mmol) were from CEA (Saclay, France). Pyruvate kinase, ATP, and phosphoenolpyruvate were from Boehringer. Oligomycin (A + B + C mixture) and soybean trypsin inhibitor were from Sigma. TPCK-trypsin was from Worthington. Peroxidase-conjugated anti-IgG antibody was provided by Pasteur Production (France). All other chemicals were of the highest purity available. ^{125}I-Labeled protein A was from Amersham.

Beef heart mitochondria were prepared as described by Smith (1967). The AUA particles used were submitochondrial particles depleted of the natural ATPase inhibitor as described by Klein et al. (1982); they were further extracted with urea and ammonia to remove F_1 and OSCP (Fessenden & Racker, 1966; Vadineanu et al., 1976). OSCP was isolated according to the procedure of Senior (1979) with a further step of purification consisting in Sephadex G-50 chromatography (Dupuis et al., 1983). Beef heart mitochondrial F_1 was purified as described by Knowles and Penefsky (1972). Its activity ranged from 80 to 90 μmol of ATP hydrolyzed per milligram per minute at 30 °C. Anti-OSCP and anti-β antibodies were raised in rabbits (Dupuis et al., 1985b).

Biological Assays. ATPase activity was measured in 0.5 mL of an ATP-regenerating system consisting of 5 mM ATP, 5 mM phosphoenolpyruvate, 0.04 mg/mL pyruvate kinase, 5 mM $MgSO_4$, 10 mM KCl, and 50 mM Tris–sulfate, pH 8.0. The ATPase reaction was initiated by addition of the particles and was carried out for 5 min at 30 °C; it was terminated by addition of 0.1 mL of 50% (w/v) trichloroacetic acid. After a 5-min centrifugation in an Eppendorf centrifuge, an aliquot of supernatant was withdrawn to measure P_i released by ATP hydrolysis (Fiske & SubbaRow, 1925). When Tween 20 was present in the experiment, $NaDodSO_4$ was added to the P_i assay medium to prevent Tween 20 from interfering with the molybdate reagent (Feng & McCarty, 1985). When ATPase oligomycin sensitivity was measured, 10 μg of oligomycin/mg of AUA particles was added to the F_1-reconstituted particles 10 min before the assay.

Soluble protein concentration was assayed by the coomassie G250 blue method (Bradford, 1976) with bovine albumin as standard. In the case of submitochondrial particles, protein was determined by the method of Folin, as modified by Dulley and Grieve (1975). The protein concentration determined by the two methods was in agreement with the amino acid contents for both OSCP and F_1.

Radioactivity was measured by liquid scintillation counting (Patterson & Green, 1965) in an Intertechnique SL 30 scintillation counter.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Gel electrophoresis on 12% polyacrylamide slab gels in 0.1% $NaDodSO_4$ was performed as described by Laemmli and Favre (1973).

Radiolabeling of OSCP by [^{14}C]NEM and of F_1 by [^{14}C]DCCD. ^{14}C-Labeled OSCP was obtained by incubation with [^{14}C]NEM (Dupuis et al., 1985a). Under these conditions, the single cysteinyl residue at position 118 (Ovchinnikov et al., 1984) is labeled. The [^{14}C]NEM-OSCP was separated from free [^{14}C]NEM by ammonium sulfate precipitation and dialysis against 50 mM Tris–SO_4, pH 7.5, at 0 °C. One mole of [^{14}C]NEM was bound per mole of OSCP, giving a specific activity for [^{14}C]OSCP of 70 dpm/pmol.

[^{14}C]F_1 was obtained by incubation with [^{14}C]DCCD in a buffer consisting of 50 mM MES (KOH), 4 mM ATP, and 2 mM EDTA, pH 6.5, in the presence of 0.5 mM [^{14}C]DCCD (Pougeois et al., 1979). Incubation lasted for 45 min at 25 °C. Free [^{14}C]DCCD was separated from [^{14}C]DCCD-F_1 by centrifugation filtration on a G-50 column (Penefsky, 1977). [^{14}C]DCCD-F_1 was prepared just before use in binding experiments. The [^{14}C]DCCD/F_1 ratio (mol/mol) was between 2 and 3, and the ATPase activity was inhibited to an extent of 80–90%. Routinely, the specific radioactivity of [^{14}C]F_1 amounted to 300 dpm/pmol of F_1. For convenience, [^{14}C]-NEM-OSCP and [^{14}C]DCCD-F_1 will be referred to as [^{14}-C]OSCP and [^{14}C]F_1.

Electroblotting, Immunocharacterization, and Immunotitration. After slab gel electrophoresis, electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose sheets (Schleicher & Schüll, 0.85 μm) was performed at 24 V for 1 h in 25 mM Tris, 33 mM glycine, 0.5% $NaDodSO_4$, and 20% methanol, final pH 8.3 (Towbin et al., 1979). The immunocharacterization of the proteins was then carried out with specific antibodies (anti-OSCP and anti-β) and peroxidase-conjugated antibody as described previously (Dupuis et al., 1985b).

For accurate immunological quantitation of OSCP or β subunits in submitochondrial preparations, a titration curve was performed with F_1 subunits and OSCP standards run on the same slab gel, as described by Penin et al. (1985), except that the ^{125}I-iodinated antibodies were replaced by ^{125}I-labeled protein A and peroxidase-conjugated anti-IgG antibody. It was checked with [^{14}C]OSCP and [^{14}C]F_1 that in the blotting procedure the amount of protein adsorbed on nitrocellulose was in proportion to the amount present in the gel and that addition of submitochondrial particles did not affect the amount of adsorbed radioactivity.

[^{14}C]OSCP and [^{14}C]F_1 Binding Experiments. AUA particles (0.5 mg/mL) were incubated with [^{14}C]OSCP or [^{14}C]F_1 for 1 h at 30 °C in 800 μL of buffer A consisting of 50 mM Tris–SO_4, 200 mM KCl, 1 mM ATP, 0.5 mM EDTA,

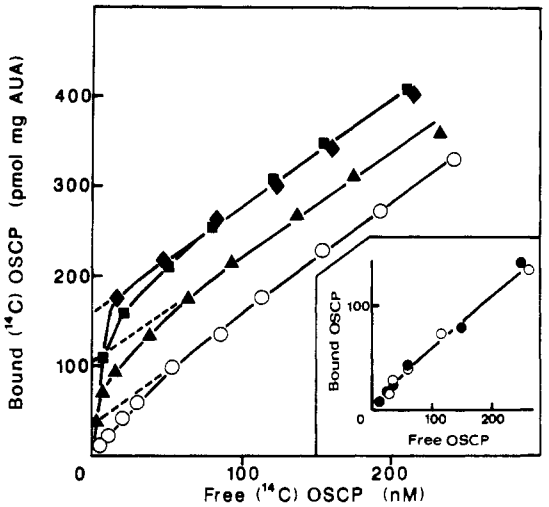

FIGURE 1: Effect of F_1 on binding of [^{14}C]OSCP to AUA particles. AUA particles (0.5 mg/mL) were incubated for 1 h at 30 °C with increasing concentrations of [^{14}C]OSCP in buffer A (cf. Experimental Procedures) in the absence of F_1 (○) or in the presence of 109 (▲), 218 (■), and 437 pmol of F_1/mg of particle protein (♦). Bound and free [^{14}C]OSCP were separated as described under Experimental Procedures. Extrapolation (dashed lines) to zero free [^{14}C]OSCP gives an estimate of the amount of high affinity bound [^{14}C]OSCP. The components were added every 30 s in the following order: AUA particles, F_1, and [^{14}C]OSCP. (Insert) Effect of heat treatment of AUA particles on binding affinity and capacity of particles. AUA particles at the concentration of 0.6 mg/mL in buffer A were heated at 56 °C for 15 min. Incubation with increasing concentrations of [^{14}C]OSCP was then carried out at 30 °C for 1 h without added F_1 (○) or with added F_1 (●), as above. F_1 was at the final concentration of 48 pmol/mg of AUA protein.

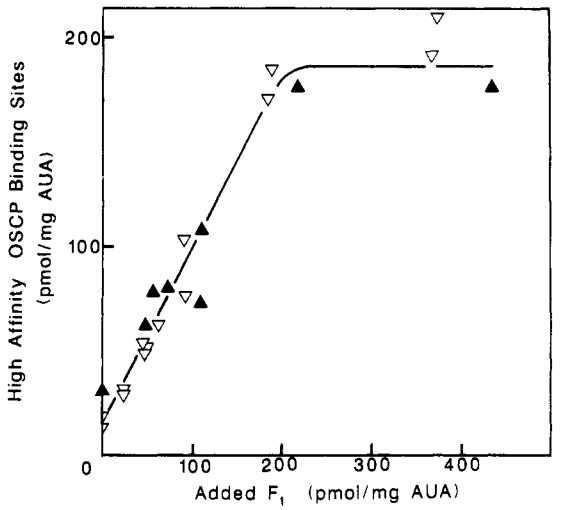

FIGURE 2: Relationship between the concentration of added F_1 and amount of [^{14}C]OSCP bound with high affinity to AUA particles. The amount of high affinity bound [^{14}C]OSCP was calculated as in Figure 1 and plotted against the F_1 concentration. (▽) and (▲) refer to two different preparations of AUA particles. The data points were fit by computer to a single titration curve.

and 0.02% Tween 20, pH 8.0. The particles were then sedimented by centrifugation for 2.5 min in an Eppendorf centrifuge. A 750-μL sample of the supernatant was withdrawn for measurement of free [^{14}C]OSCP or [^{14}C]F_1. Tubes were centrifuged once more, and any remaining liquid was carefully drained. Pellets were homogenized in 0.5 mL of buffer A, and radioactivity was counted for measurement of bound [^{14}C]OSCP or [^{14}C]F_1. Protein concentration was determined in aliquots of the resuspended pellets. Buffer A was chosen as it was previously used for equilibrium binding measurements of F_1 and [^{14}C]OSCP (Dupuis et al., 1985a). It was checked that the OSCP binding parameters were altered neither by a modification of the ionic strength of the medium between given limits nor by a modification of the ATP concentration.

Treatment of Biological Samples by Heat and Trypsin. Heat treatment of AUA particles in buffer A (0.8 mg of protein/mL) was carried out at 56 °C for 15 min. Trypsin treatment of AUA particles (0.8 mg/mL) or F_1 (2 mg/mL) in buffer A was performed by addition of TPCK-trypsin with a trypsin to particle or F_1 ratio of 1/100 (w/w) and incubation at 30 °C for 10 and 5 min, respectively. The reaction was stopped by addition of a 10-fold excess of soybean trypsin inhibitor.

RESULTS

Binding of [^{14}C]OSCP to AUA Particles. Incubation of [^{14}C]OSCP for 1 h with AUA particles in the absence of F_1 revealed mainly low-affinity OSCP binding sites (Figure 1). Saturation was not attained, even with as much as 2000 pmol of [^{14}C]OSCP bound per milligram of protein, which is far more than the amount of F_o sector of the ATPase complex present in the AUA particles. Besides the low-affinity OSCP binding sites, a small number of high-affinity sites were revealed in the absence of F_1 by the concavity of the binding curve at low concentrations of free OSCP; in the experiment illustrated in Figure 1, the high-affinity OSCP binding sites amounted to 40 pmol/mg of AUA particles, as calculated by extrapolating the linear portion of the binding curve (dashed line) to the ordinate. Depending on the preparation, this value ranged between 20 and 45 pmol of [^{14}C]OSCP/mg of protein.

When the AUA particles were supplemented with F_1 (109, 218, and 437 pmol of F_1/mg of particles), the shape of the binding curves was modified, with a marked enhancement of the number of high-affinity [^{14}C]OSCP binding sites. From the Scatchard plots (not shown), an apparent K_d value of 5 nM for the high-affinity OSCP binding sites was derived. Clearly, high-affinity OSCP binding is promoted by added F_1.

The plots of Figure 2 correspond to the titration of high-affinity OSCP binding sites performed with a number of fixed concentrations of F_1 and with two different preparations of AUA particles. The two titrations were in good agreement. The number of high-affinity [^{14}C]OSCP binding sites increased linearly as a function of added F_1 up to about 200 pmol of F_1/mg of AUA particles, a value that corresponded to the binding of 190 pmol of [^{14}C]OSCP/mg of particles. From the slope of the curve, a ratio of bound OSCP to added F_1 of 0.8 could be calculated; the correlation coefficient was 0.97.

It has been shown previously (Dupuis et al., 1985a) that isolated F_1 contains three OSCP binding sites, one of which differs from the others by a much higher affinity for OSCP (K_d = 80 nM vs. 3–4 μM). These data taken together with those of Figure 2 indicate that one high-affinity OSCP binding site is present in F_1, whether F_1 is isolated or bound to AUA particles to form a F_o–F_1 complex. The affinity of this site was, however, much lower when OSCP bound to isolated F_1 (K_d = 80 nM) than when it bound to F_1 in the presence of AUA particles (K_d = 5 nM).

Effect of Heat or Trypsin Treatment of AUA Particles on Binding Capacity of [^{14}C]OSCP in the Presence of F_1. Treatment of submitochondrial particles devoid of OSCP by heating at 56 °C for 15 min, or with trypsin, results in complete loss of oligomycin sensitivity of the reconstituted system consisting of particles, F_1, and OSCP (Bulos & Racker, 1968). As shown in the insert of Figure 1, heat treatment of AUA particles led to the virtual disappearance of the high-affinity [^{14}C]OSCP binding sites promoted by addition of F_1. A

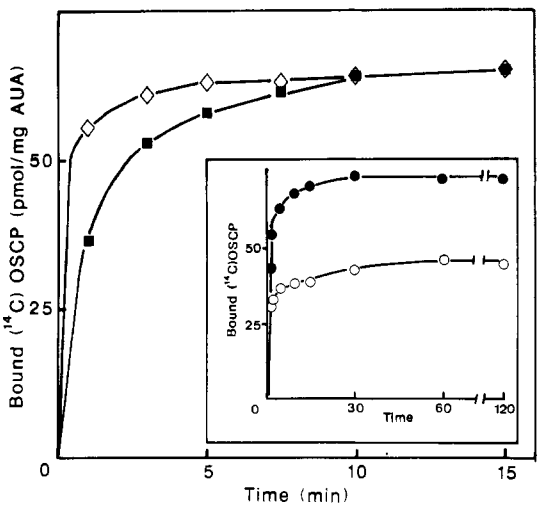

FIGURE 3: Effect of time of preincubation of AUA particles with F_1 on kinetics of binding of [^{14}C]OSCP to particles. AUA particles were preincubated with F_1 (109 pmol/mg of particles) in buffer A for 10 s (■) and 1 h (◊) at 30 °C. This was followed by addition of [^{14}C]OSCP (final concentration 42 nM). After incubation of the preincubated particles (0.5 mg/mL) with [^{14}C]OSCP for different periods of time at 30 °C, bound and free [^{14}C]OSCP were measured as described under Experimental Procedures. (Insert) Kinetics of binding of [^{14}C]OSCP to AUA particles. The particles (another preparation) were incubated at 30 °C with 42 nM [^{14}C]OSCP in the presence (●) or absence (○) of F_1 (109 pmol/mg of particles) for the periods of time indicated, and bound and free [^{14}C]OSCP were measured as described under Experimental Procedures.

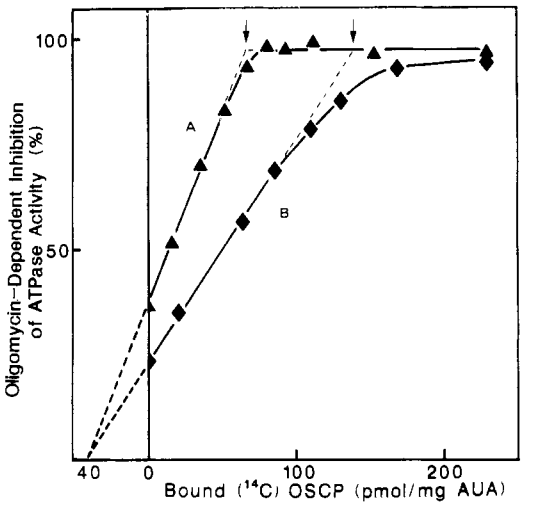

FIGURE 4: Correlation between the extent of [^{14}C]OSCP binding to AUA particles in the presence of added F_1 and appearance of oligomycin sensitivity of F_1. AUA particles (0.5 mg/mL) were incubated at 30 °C in buffer A with increasing concentrations of [^{14}C]OSCP in the presence of two fixed concentrations of F_1: 110 (▲) and 180 pmol/mg of particles (♦). After a 45-min incubation, two 100-μL aliquots were withdrawn for measurement of ATPase activity in the presence and absence of oligomycin. Fifteen minutes later, the remainder of the sample was centrifuged, and free and bound [^{14}C]OSCP were determined. By extrapolation (dashed lines), the plots for the two different concentrations of added F_1 intercept the negative region of the abscissa at the same value of 40 pmol of OSCP/mg of particles, which corresponds to the residual endogenous OSCP present in the AUA particle preparation. The end points of titration corresponded to 97% inhibition of ATPase activity by oligomycin (arrows). The specific activities of isolated and bound F_1 at 30 °C in the absence of oligomycin were 90 and 36 μmol of ATP hydrolyzed min^{-1} (mg of F_1 protein)$^{-1}$, respectively.

similar loss of binding affinity was observed with trypsinized AUA particles (not shown). This finding strengthens the view that the ability of OSCP to confer oligomycin sensitivity is related to the high-affinity binding to OSCP (Dupuis et al., 1985a).

Kinetics of [^{14}C]OSCP Binding to AUA Particles. The binding kinetics of [^{14}C]OSCP to AUA particles in the presence of F_1 depended on the period of preincubation of F_1 with the particles but not the equilibrium binding; whatever the preincubation period, the same binding plateau was attained. With a fixed concentration of 40 nM [^{14}C]OSCP corresponding for a large part to the high-affinity region of the OSCP binding curve (Figure 1), 90% of the plateau for OSCP binding was attained within 5 min when preincubation of particles with F_1 was for 10 s and in 1.5 min when preincubation was for 1 h (Figure 3). Full binding was achieved in 10 and 5 min, respectively. It is noteworthy that, in all experiments reported here concerning the binding of OSCP to AUA particles in the presence of added F_1, incubation lasted for 1 h, which corresponded to equilibrium conditions. In another experiment, the kinetics of [^{14}C]OSCP binding were assayed in the absence of added F_1 and compared to a control experiment carried out in the presence of F_1. In the absence of F_1, the full extent of [^{14}C]OSCP binding was lower than in its presence, and 90% of the binding plateau was attained only after 15 min (insert of Figure 3).

Following incubation of AUA particles with [^{14}C]OSCP and F_1, addition of a 20-fold excess of unlabeled OSCP resulted in a limited release of bound [^{14}C]OSCP (about 10% in 30 min), which was independent of the time of addition of the unlabeled OSCP. This contrasts with the reversible association of [^{14}C]OSCP with isolated F_1 in the absence of particles (Dupuis et al., 1985a).

Correlation between Binding of [^{14}C]OSCP to AUA Particles in the Presence of F_1 and Conferral of Oligomycin Sensitivity to Bound F_1. The dose–effect curves A and B shown in Figure 4 correspond to the oligomycin-dependent inhibition of ATPase activity in a reconstituted system consisting of AUA particles, F_1, and [^{14}C]OSCP, with increasing concentrations of [^{14}C]OSCP and two fixed concentrations of F_1, namely, 110 pmol of F_1/mg of particles (subsaturating concentration) and 180 pmol of F_1/mg of particles (nearly saturating concentration). It is noteworthy that F_1 incubated with the AUA particles in the absence of OSCP exhibited a significant sensitivity to oligomycin, which is the result of some residual OSCP bound to the particles. Residual OSCP was calculated by extrapolation of the linear portion of the dose-effect curve to the abscissa, by linear regression. In the experiment illustrated in Figure 4, residual OSCP was approximated to 40 pmol/mg of particles.

The titration curve A showed linearity (indicating high-affinity binding) nearly up to the end point of the titration and corresponded to about 78 pmol of bound [^{14}C]OSCP/mg of AUA particles. Taking into account the 40 pmol of residual OSCP/mg of particles, the total amount of OSCP was estimated to 110 pmol/mg of particles, i.e., the same value as that of added F_1 (110 pmol/mg of particles). As 97% of the F_1-ATPase activity is sensitive to oligomycin, it is clear that virtually all the added F_1 has bound to the particles. One can therefore safely conclude that the ratio of high affinity bound OSCP to bound F_1 is close to 1. This is in agreement with the direct binding data of Figure 2, bearing on the amount of OSCP bound with high affinity at different concentrations of added F_1. It must be stressed that, in a reconstituted system made of AUA particles, F_1, and OSCP, the conferral of oligomycin sensitivity to F_1 is linked to the high-affinity binding of OSCP to F_1.

In contrast to curve A, curve B departed from linearity in its final portion. Extrapolation of the initial linear portion of curve B to the maximal ATPase inhibition gave a value of 140

pmol of bound [^{14}C]OSCP/mg of particles. The amount of total bound OSCP (residual OSCP plus added [^{14}C]OSCP), i.e., 180 pmol/mg of particles, was the same as that of the added F_1 (180 pmol/mg of particles). However, the curvilinear portion of curve B attained maximal inhibition for about 215 pmol of bound [^{14}C]OSCP/mg of particles. Under these conditions, the total amount of bound OSCP was 255 pmol, and the ratio of bound OSCP to F_1 for maximal inhibition was 1.4. The curvilinearity of curve B found at high concentration of OSCP might result from a low-affinity binding of OSCP not effective in conferring oligomycin sensitivity to F_1. Four other titrations were performed with concentrations of F_1 ranging from 40 to 183 pmol/mg of AUA particles. By extrapolation of the linear portion of the curves, the mean ratio of bound OSCP (residual plus added OSCP) to F_1 was 1.1 ± 0.2 mol/mol of particles for maximal inhibition of F_1-ATPase by oligomycin; at nearly saturating concentrations of F_1, curvilinearity was observed as in curve B of Figure 4.

The inverse relationship between the amount of added F_1 and the sensitivity to oligomycin in the absence of added OSCP (curves A and B of Figure 4), confirmed in the four other titrations, deserves some comment. One might imagine that added F_1 could distribute at random in AUA particles between the F_o sites devoid of OSCP and those filled with residual OSCP (F_o–OSCP complexes). Alternatively, added F_1 might bind preferentially to the F_o sites filled with OSCP. If added F_1 distributed randomly, the oligomycin-dependent inhibition of the F_1-ATPase in the reconstituted system would be independent of the amount of added F_1, which is not the case. On the other hand, if F_1 binds preferentially to the F_o–OSCP complex, the extent of the oligomycin-dependent inhibition of F_1-ATPase in the reconstituted system in the absence of OSCP will be inversely proportional to the amount of added F_1. This prediction fits well with the experimental data in Figure 4. It is confirmed by the finding of heterogeneous F_1 binding sites in AUA particles in the absence of added OSCP (see below).

When the F_1 concentration was in excess of the saturating concentration of 200 pmol/mg of particles, a fraction of F_1 remained free; this fraction was therefore no longer susceptible to inhibition by oligomycin (not shown).

Immunochemical Determination of Endogenous OSCP. The amount of residual OSCP found in AUA particles by enzymatic titration of the oligomycin-conferring sensitivity to F_1-ATPase; as described in the above section, was compared to that determined by an immunochemical assay (see Experimental Procedures). The latter assay, conducted with an anti-OSCP antiserum on four different preparations of AUA particles, gave a value of 156 ± 38 pmol of endogenous OSCP/mg of AUA particles, which is 4–5 times higher than the value derived by enzymatic titration. Thus, a large proportion of the residual OSCP in the AUA particles is apparently nonfunctional. A similar immunochemical assay with an anti-β antiserum indicated that the AUA particles, which are expected to be depleted in bound F_1, still contained an average of 68 ± 32 pmol of β subunits/mg of particles. Although the presence of residual α subunits in the AUA particles was ascertained by qualitative blotting assay, the titer of our anti-α antiserum was too low for accurate immunotitration of these α subunits. The limited ability of AUA particles to bind [^{14}C]OSCP with high affinity in the absence of added F_1 (see Figure 1) is readily explained by available OSCP binding sites in residual α and β subunits present in AUA particles. The large excess of nonfunctional OSCP in the AUA particles revealed by immunochemical assays might be due to structural alteration of these OSCP molecules or

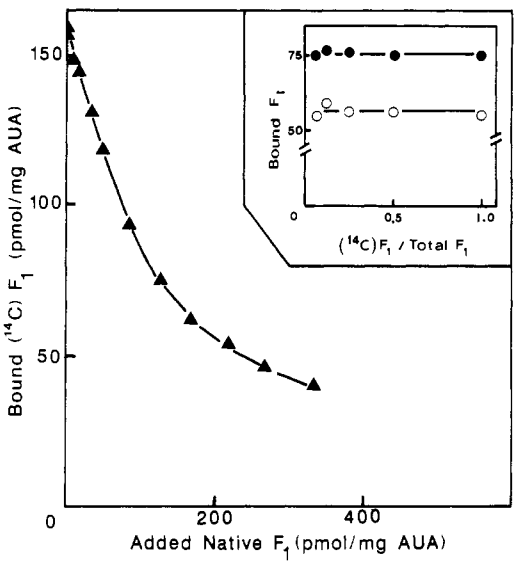

FIGURE 5: Sequential titration of F_1 binding sites on AUA particles with native F_1 and [^{14}C]F_1. F_1 samples at different concentrations were incubated with AUA particles in buffer A for 1 h at 30 °C. After centrifugation, the pellets were resuspended in buffer A, and the particles were supplemented with 90 nM [^{14}C]F_1 and 480 nM OSCP. After a 45-min incubation at 30 °C, the particles were again sedimented, and free and bound [^{14}C]F_1 were determined. (Insert) Isotopic dilution experiment. [^{14}C]F_1 mixed in different proportions with native F_1 in buffer A was tested for binding to AUA particles. F_1 was added at the final concentration of 100 nM to 0.5 mg of AUA particles/mL in the absence (○) or presence (●) of 420 nM OSCP. After a 1-h incubation at 30 °C, the particles were sedimented by centrifugation, and bound [^{14}C]F_1 was measured.

to their entrapment in right-side-out particles, i.e., particles with no F_o sites available for F_1 binding. Additional immunochemical titrations carried out with beef heart mitochondria (four experiments) gave values of 1.6 ± 0.5 (three preparations) for the molar ratio of OSCP to F_1, which is in agreement with the overstoichiometry found by Penin et al. (1985).

Binding of [^{14}C]F_1 to AUA Particles. As shown above, added F_1 promotes high-affinity binding of [^{14}C]OSCP in the presence of AUA particles. The converse effect of OSCP on the binding of F_1 to AUA particles will now be described. F_1 was labeled with [^{14}C]DCCD (Pougeois et al., 1979). Although [^{14}C]F_1 had lost most of its ATPase activity (Pougeois et al., 1979) due to the modification of a strategic carboxylic group by [^{14}C]DCCD (Esch et al., 1981), it remained able to recombine to AUA particles with the same affinity as native F_1 did, as demonstrated by isotopic dilution experiments in which [^{14}C]F_1 was mixed in different proportions with native F_1 before addition to the particles (insert in Figure 5). In addition, the binding specificity of [^{14}C]F_1 to the AUA particles was ascertained by the sequential titration illustrated in Figure 5, in which the particles were incubated first with different concentrations of F_1 followed by addition of [^{14}C]F_1 and OSCP. Binding of [^{14}C]F_1 to the particles was decreased in proportion to the amount of native F_1 preincubated with these particles. These results suggested firm binding of added F_1; only the sites not occupied by native F_1 were available for [^{14}C]F_1 binding.

The plateau of binding of [^{14}C]F_1 to the AUA particles in the presence of OSCP was attained in 10–15 min, independent of the duration of the preincubation of OSCP with the particles. In the absence of OSCP, the rate of binding of [^{14}C]F_1 to the particles was much slower; the plateau was barely attained after 60-min incubation (Figure 6). In the presence of added OSCP, the bound [^{14}C]F_1 was not displaced upon addition of a 10-times excess of unlabeled F_1, which is con-

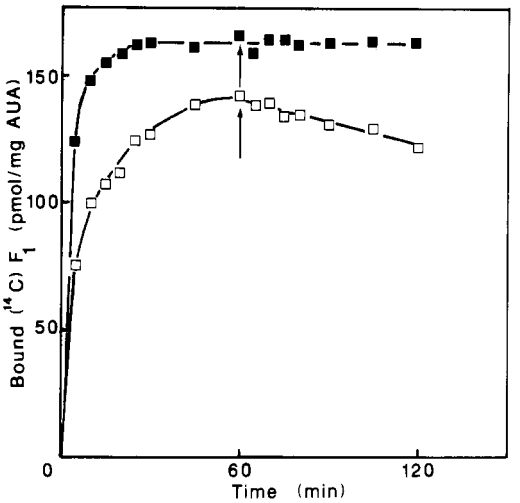

FIGURE 6: Effect of OSCP on kinetics of binding of [^{14}C]F_1 to AUA particles. [^{14}C]DCCD–F_1 was incubated at 30 °C at the final concentration of 90 nM with AUA particles (0.5 mg/mL) in buffer A, in the absence (□) or presence (■) of 406 nM OSCP. At given intervals of time, 0.4-mL fractions were withdrawn for determination of bound and free [^{14}C]F_1 as described under Experimental Procedures. After a 1-h incubation, a 10 times excess of native F_1 was added (arrow).

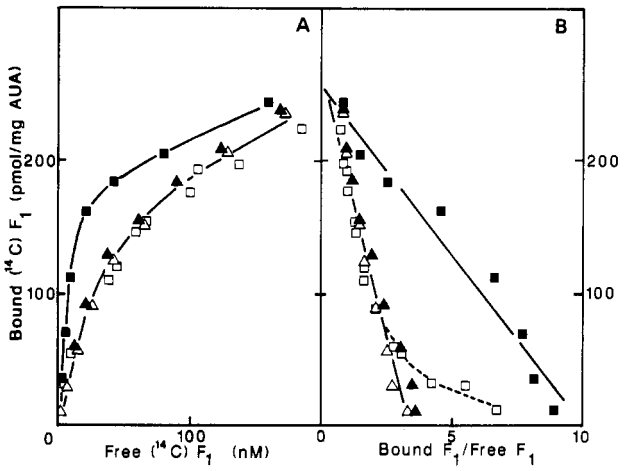

FIGURE 7: Equilibrium binding of [^{14}C]F_1 to AUA particles in the presence or absence of OSCP; effect of mild trypsin treatment of F_1. Trypsinization of [^{14}C]F_1 was carried out as follows. [^{14}C]F_1 (1.7 mg/mL) in buffer A was incubated for 5 min at 30 °C with 1% (w/w) TPCK-trypsin. The reaction was terminated by addition of a 10-fold excess of soybean trypsin inhibitor. Control [^{14}C]F_1 was incubated under the same conditions, except that soybean inhibitor was added prior to trypsin. The trypsin-treated (△, ▲) and control [^{14}C]F_1 (□, ■) were then incubated for 1 h at 30 °C with AUA particles (0.5 mg/mL) in buffer A in the absence (△, □) or presence (▲, ■) of 640 pmol of OSCP/mg of particles. It is noteworthy that a similar control curve was obtained when trypsin inhibitor and trypsin were omitted. (Panel A) Direct binding curves; (panel B) Scatchard plots.

sistent with the results of the sequential titration of Figure 5. In contrast, a significant chase of bound [^{14}C]F_1 was found in the absence of OSCP.

Under equilibrium conditions (incubation for 1 h at 30 °C), in the presence of an excess of OSCP, the Scatchard plots revealed, for the range of F_1 concentrations used, essentially one class of F_1 binding sites in the AUA particles with a total number of sites of 240 pmol/mg of particles and an apparent K_d value close to 10 nM (Figure 7). This number of sites might be slightly overestimated due to a minor fraction of low-affinity sites suggested by the Scatchard plots. When OSCP was omitted, the K_d value increased up to 60 nM, but the total number of F_1 binding sites in the particles did not

Table I: Mutual Promotion of Binding of [^{14}C]F_1 and [^{3}H]OSCP to AUA Particles: Effect of Heating[a]

duration of heating of AUA particles at 56 °C	added [^{14}C]F_1 (nM)	added [^{3}H]OSCP (nM)	bound [^{14}C]F_1 (pmol/mg of particles)	bound [^{3}H]OSCP (pmol/mg of particles)
0 min (specific + nonspecific binding)	77	0	92	0
	0	139	0	94
	77	139	120	174
15 min (nonspecific binding)	77	0	46	0
	0	139	0	60
	77	139	46	68
Δ (0 min − 15 min) (specific)	77	139	74	106

[a] [^{3}H]OSCP was prepared with [^{3}H]NEM (cf. Experimental Procedures). Incubation of AUA particles with [^{14}C]F_1 and [^{3}H]OSCP was carried out under the same conditions as in Figure 1. The values correspond to the means of duplicate determinations; these determinations differed by less than 5%. The specific binding was obtained by difference between the binding values with native AUA particles and those with heated particles.

change. This is at variance with the results of Vadineanu et al. (1976) on F_1 binding to depleted submitochondrial particles, on the basis of enzymatic assay of free and bound F_1-ATPase, where it was shown that added OSCP resulted in the increase of F_1 binding sites in the particles. In a number of assays bearing on F_1 binding in the absence of OSCP, the Scatchard plots were biphasic, as typically shown in Figure 7B. Under these conditions the small fraction of higher affinity [^{14}C]F_1 sites (K_d 5–6 nM) could be approximated to 35–40 pmol of [^{14}C]DCCD–F_1 bound per milligram of particles; it is explained by residual OSCP attached to the particles as already discussed in this paper (cf. Figure 4).

Upon mild trypsinization of [^{14}C]F_1, which is known to suppress oligomycin sensitivity of bound F_1 without altering ATPase activity (Hundal & Ernster, 1981), the [^{14}C]F_1 binding curves in the presence and the absence of OSCP were superimposable (Figure 7). Trypsinization even abolished the small amount of [^{14}C]F_1 high-affinity binding sites revealed in the absence of added OSCP and depending on residual OSCP.

The conclusions arrived at with [^{14}C]F_1 in the presence of unlabeled OSCP, or with [^{14}C]OSCP in the presence of unlabeled F_1, were corroborated by a double-labeling experiment (Table I) conducted with [^{14}C]F_1 and [^{3}H]OSCP. When both ligands were present, each of them promoted the binding of the other. This promoting effect was abolished by heat treatment of the AUA particles at 56 °C for 15 min. The specifically bound [^{14}C]F_1 and [^{3}H]OSCP calculated from the difference of binding data before and after heating amounted to 74 and 106 pmol/mg of AUA particles, respectively, leading to an OSCP to F_1 ratio of 1.4.

[^{14}C]F_1 Binds Specifically to F_o Sector of AUA Particles Even in the Absence of OSCP. To ascertain whether the binding of [^{14}C]F_1 with respect to the F_o sector of the AUA particles was specific, even in the absence of OSCP, the following experiment was carried out in two steps. In the first step, aliquots of AUA particles were incubated in separate tubes with increasing amounts of [^{14}C]F_1 (inactivated to 84% by derivatization with [^{14}C]DCCD), without added OSCP, for 1 h at 30 °C. The particles were pelleted by centrifugation and resuspended in buffer A (see Experimental Procedures). The second step consisted in adding to the particle suspension in each tube 160 pmol of nonmodified F_1/mg of AUA particles, followed by an excess of OSCP (0.5 μM). After a 45-min incubation at 30 °C, aliquots were withdrawn to assay

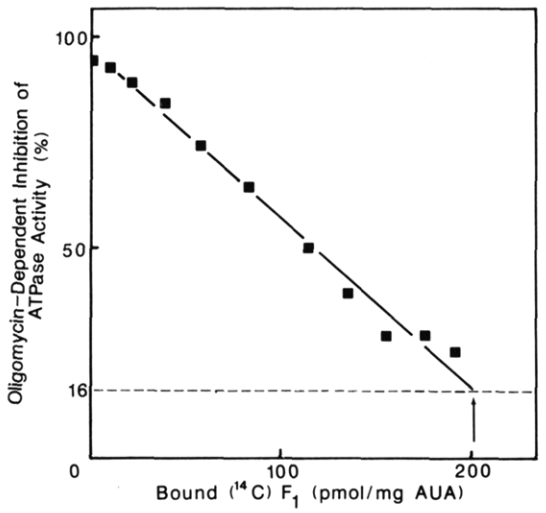

FIGURE 8: Inverse relationship between occupancy of F_1 binding sites on AUA particles by inactivated [^{14}C]F_1 and ability of AUA particles to bind native F_1 and to confer to it sensitivity to oligomycin. Increasing concentrations of [^{14}C]F_1 were incubated for 1 h at 30 °C with AUA particles (0.5 mg/mL) in buffer A in the absence of added OSCP. The particles were then sedimented by centrifugation, and the pellets consisting of [^{14}C]F_1 bound to particles were resuspended in 0.4 mL of buffer A. Native F_1 (160 pmol/mg of particles) was then added, followed after 1 min by OSCP (400 pmol/mg of particles). After 45 min of incubation at 30 °C, 100-μL aliquots were withdrawn for measurement of ATPase activity in the presence and absence of oligomycin. Fifteen minutes later, the remainder of the samples was centrifuged, and free and bound [^{14}C]F_1 were determined. The broken line, parallel to the abscissa, corresponds to 16% oligomycin-sensitive ATPase activity, a situation that occurs when all F_o sites in the AUA particles are occupied by [^{14}C]F_1. In fact, the [^{14}C]F_1 used was inactivated to 84%, and the 16% remaining ATPase activity was supposed to be fully inhibited by oligomycin when [^{14}C]F_1 has bound to AUA particles. The arrow indicates the end point of the titration of the F_1 binding sites in the particles.

the amount of particle-bound [^{14}C]F_1 and the oligomycin-sensitive ATPase activity. The oligomycin sensitive ATPase activity vs. particle-bound [^{14}C]F_1 decreased linearly with the amount of bound F_1 (Figure 8), indicating that even in the absence of OSCP [^{14}C]F_1 was able to bind specifically to the F_o sector of the AUA particles (first step) and therefore to prevent the binding of native F_1 (second step). Conversely, the sequential addition of F_1 followed by [^{14}C]F_1 did not suppress the oligomycin ATPase sensitivity.

It should be recalled that the [^{14}C]F_1 preparation used was inhibited to 84% due to the bound [^{14}C]DCCD. By extrapolation of the linear plots to a base line (dashed line in Figure 8) corresponding to the 16% remaining ATPase activity of the [^{14}C]F_1 preparation, 200 pmol of [^{14}C]F_1 binding sites/mg of AUA particles could be calculated. The small difference between this value and that obtained in direct binding assays (240 pmol) is due to the fact that a small amount of unspecific binding sites are revealed at high concentrations of [^{14}C]F_1, which are ineffective in competing with native F_1 in the enzymatic assay illustrated in Figure 8.

Effect of AUA Particles on Covalent Photolabeling of F_1 by Azidophenacyl-OSCP. Azidophenacyl-OSCP, a photoactivable derivative of OSCP with full functional activity in the dark, is able to bind covalently after photoirradiation to both the α and β subunits of isolated F_1, with some preference for the α subunit (Dupuis & Vignais, 1985). When F_1 and azidophenacyl-OSCP were supplemented with AUA particles, the covalent linkage resulting from photoirradiation occurred mainly at the level of the β subunit, as shown by immunoreaction after electrotransfer to a nitrocellulose sheet (Figure 9). This shift in the specificity of photolabeling upon

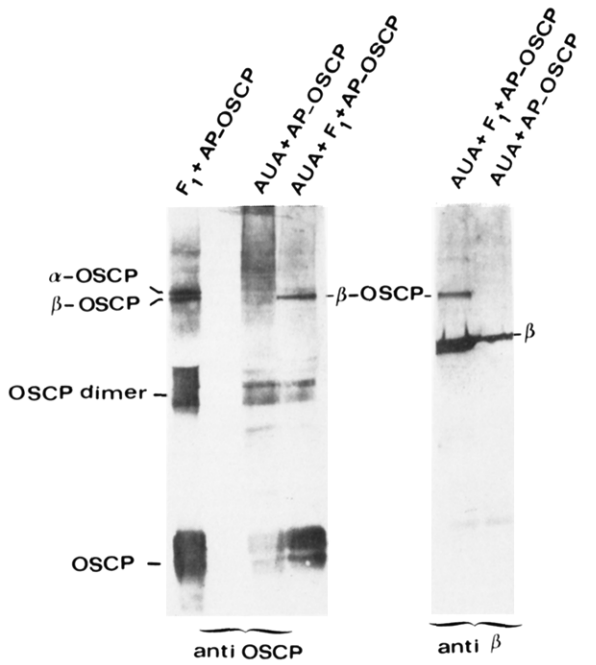

FIGURE 9: Effect of incubation of AUA particles with F_1 on covalent photolabeling of major F_1 subunits by azidophenacyl-OSCP (AP-OSCP). F_1 (170 μg) was incubated for 15 min at 30 °C with AUA particles (410 μg) and 7 μg of AP-OSCP in 70 μL of buffer A. The mixture was then photoirradiated for 10 s with a xenon lamp XB10, 1000-W HS, equipped with a KG3 filter. Photoirradiation in the presence of AP-OSCP was also performed with F_1 alone or AUA particles alone. The photoirradiated samples were then subjected to $NaDodSO_4$–sulfate polyacrylamide gel electrophoresis, followed by electrotransfer and immunoreaction with anti-OSCP antiserum and anti-β antiserum. The immunoreactive peptides were revealed by a peroxidase-conjugated antibody followed by reaction with diaminobenzidine. AP-OSCP covalently binds to both the α and β subunits of isolated F_1 (lane 1) and essentially to the β subunit of F_1 bound to AUA particles (lane 3 and 4). The reaction of the anti-β antiserum with the AUA particles in the absence of F_1 corroborates the presence of β subunits on the particles (lane 5).

addition of AUA particles is probably the result of the association of F_1 with the F_o sector of the particles and suggests the occurrence of some conformational changes (see Discussion).

Discussion

The main conclusions to be drawn from the experimental results are as follows. (1) In the absence of F_1, OSCP exhibits a nonsaturable binding to the AUA particles. (2) In the absence of OSCP, F_1 exhibits a specific and saturable binding to the AUA particles. (3) In the presence of OSCP, the binding affinity of F_1 to the particles is increased; the number of binding sites however remains constant. (4) Conversely, F_1 bound to the AUA particles promotes high-affinity binding to OSCP. (5) Full oligomycin sensitivity for F_1-ATPase in a reconstituted F_1–F_o complex at nonsaturating concentrations of F_1 requires the binding of 1 mol of OSCP/mol of F_1. (6) Upon binding of F_1 to the AUA particles, conformational changes occur in F_1, which are reflected by a different interaction of OSCP with the major subunits of F_1. The physiological significance of these data will now be discussed.

Interactions of OSCP with F_1 and F_o Sector of ATPase Complex in AUA Particles. The idea that OSCP is a stalk joining the F_1 and F_o sectors of the ATPase complex stemmed from resolution experiments on inside-out submitochondrial particles, indicating that upon appropriate treatments F_1 is removed first, followed by OSCP (Tzagoloff et al., 1968), and also from electromicrographs showing an intermediary piece

between F_1 and the mitochondrial membrane (McLennan & Asai, 1968). This idea is, however, difficult to reconcile with a number of results reported here, namely, that F_1 is able to bind specifically to the F_o sector of the ATPase complex in the AUA particles and that, whereas OSCP binds with high affinity to F_1, it binds loosely to F_o. In other words, OSCP appears to secure the link that probably exists already between F_1 and F_o and to act as a clip to maintain the F_1–F_o association.

Through immunochemical determinations (Gautheron et al., 1985; this paper), it has been shown that urea treatment of submitochondrial particles results only in partial release of the dissociated F_1 subunits with nearly half of the α subunit and one-fifth of the β subunit remaining associated with the membrane. Residual OSCP is also found in AUA particles; this is not astonishing, due to the tendancy of OSCP to adhere firmly to membranes (Dupuis et al., 1985a). As previously stressed, only one-fifth of this residual OSCP was functional in conferring oligomycin sensitivity to added F_1. Our results are consistent with the contention that, in a reconstituted F_1–F_o–OSCP system, the binding of OSCP to the high-affinity OSCP binding site of F_1 is sufficient to provide the ATPase activity of the membrane-bound F_1 with maximal sensitivity to oligomycin. This result contrasts with the fact that, in heart mitochondria, the molar OSCP to F_1 ratio is close to 1.8 (Gautheron et al., 1985; Penin et al., 1985) in agreement with personal observations. Two explanations can be offered for this apparent contradiction. (1) It has been reported (Gautheron et al., 1985) that the maximal rate of ATP synthesis by bound F_1 in a reconstituted system requires twice as much OSCP than full sensitivity of F_1-ATPase to oligomycin does. It is therefore possible that, besides the high-affinity OSCP binding site responsible for conferring oligomycin sensitivity to F_1-ATPase, low-affinity OSCP binding site(s) might play a role in ATP synthesis. (2) As an alternative explanation, the excess of OSCP might serve to displace the binding equilibrium between OSCP, F_1, and F_o toward the accumulation of the F_1–F_o–OSCP complex, thus playing the role of a safety device to prevent the presence of free F_1 in the mitochondrion.

Mild trypsinolysis of F_1 at the α and β subunits (Hundal & Ernster, 1981; di Pietro et al., 1983; Walker et al., 1985) results in the loss of specific contacts between F_1 and OSCP (Hundal et al., 1983; Dupuis et al., 1985; this paper). Trypsin has been shown to remove a segment of 14 or 15 amino acids from the N terminus of the α subunit and a section of five, six, or seven amino acids from the N terminus of the β subunit (Walker et al., 1985). These segments of the α and β subunits might correspond to parts of the specific binding sites for OSCP; it is also possible that cleavage of α and β subunits results in a change in the conformation of F_1, which thereby becomes unable to bind OSCP.

Binding of OSCP to Isolated F_1 vs. Binding of OSCP to Membrane-Bound F_1. The binding of OSCP to isolated F_1 resembles or differs from the binding of OSCP to the membrane-bound F_1 (F_o–F_1 complex) by the following features: (1) A single high-affinity binding site on F_1 is titrated with [^{14}C]OSCP in both cases; (2) azido-OSCP photolabels the α and β subunits of isolated F_1, but only the β subunit of the F_o–F_1 complex; (3) the binding affinity of OSCP for the F_o–F_1 complex is much higher than that of OSCP for the isolated F_1 (K_d = 5 vs. 80 nM). Furthermore, binding of OSCP to isolated F_1 is fully reversible, whereas removal of bound OSCP from the F_o–F_1 complex is limited. These different points deserve the following comments: (Point 1). Given that there is only one high-affinity OSCP binding site per F_1 in spite of the presence of three α and three β subunits per F_1, it may be concluded that mitochondrial F_1-ATPase (either isolated or bound F_1) exhibits a $^1/_3$ site reactivity with respect to OSCP. A similar partial site reactivity was previously reported for reversible binding ligands, namely, the natural ATPase inhibitor (Klein et al., 1980) aurovertin (Lunardi et al., 1986), and for irreversible chemical modifiers and photolabels [for review, see Vignais and Lunardi (1985)]. (Point 2). The contrast between the photolabeling of the α and β subunits in isolated F_1 by azido-OSCP and the photolabeling of only the β subunit of F_1 bound to particles must be interpreted with caution; it does not mean that the presence of AUA particles induces total loss of contact between the whole molecule of OSCP and the α subunit of F_1. In fact, as the phenacyl residue bearing the azido group is linked to Cys-118 in OSCP, the azido group maps a restricted area in the vicinity of Cys-118. A minimal explanation for this is that, upon binding of added F_1 to the F_o sector of the AUA particles, F_1 undergoes conformational changes, resulting in a displacement of its interaction with OSCP. (Point 3). The tight binding of OSCP to the F_o–F_1 complex probably reflects a "locked" ternary complex, OSCP–F_1–F_o, in contrast to the reversible binary associations between F_1 and F_o (this paper) and between F_1 and OSCP (Dupuis et al., 1985a). It is suggested that the locked association OSCP–F_1–F_o takes place through some conformational changes in the components of this complex, as discussed under point 2.

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Properties of a Water-Soluble, Yellow Protein Isolated from a Halophilic Phototrophic Bacterium That Has Photochemical Activity Analogous to Sensory Rhodopsin†

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ABSTRACT: A water-soluble yellow protein, previously discovered in the purple photosynthetic bacterium *Ectothiorhodospira halophila*, contains a chromophore which has an absorbance maximum at 446 nm. The protein is now shown to be photoactive. A pulse of 445-nm laser light caused the 446-nm peak to be partially bleached and red-shifted in a time less than 1 μs. The intermediate thus formed was subsequently further bleached in the dark in a biphasic process occurring in approximately 20 ms. Finally, the absorbance of native protein was restored in a first-order process occurring over several seconds. These kinetic processes are remarkably similar to those of sensory rhodopsin from *Halobacterium*, and to a lesser extent bacteriorhodopsin and halorhodopsin; although these proteins are membrane-bound, they have absorbance maxima at about 570 nm, and they cycle more rapidly. In attempts to remove the chromophore for identification, it was found that a variety of methods of denaturation of the protein caused transient or permanent conversion to a form which has an absorbance maximum near 340 nm. Thus, by analogy to the rhodopsins, the absorption at 446 nm in the native protein appears to result from a 106-nm red shift of the chromophore induced by the protein. Acid denaturation followed by extraction with organic solvents established that the chromophore could be removed from the protein. It is not identical with *all-trans*-retinal and remains to be identified, although it could still be a related pigment. The *E. halophila* yellow protein has a circular dichroism spectrum which indicates little α-helical secondary structure (19%). Although the yellow protein is unique in its properties, it has characteristics of both bacterial rhodopsins (in terms of photochemistry) and the animal retinol binding proteins (in terms of solubility).

A water-soluble, yellow protein was previously isolated from the extremely halophilic purple phototrophic bacterium *Ectothiorhodospira halophila* (Meyer, 1985). This protein was found to have a relatively low molecular weight (15 000), and the chromophore has a strong absorbance peak at 446 nm (ϵ = 48 mM^{-1} cm^{-1}). The chromophore was not released by a variety of methods of denaturation. This combination of properties is unlike those of any previously characterized protein. Although the spectral properties of the chromophore were reported to have a superficial resemblance to flavins with O, N, or S substituents at the 8-position of the aromatic ring, we now present evidence that the yellow protein has photo-

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