Interactions between the Oligomycin Sensitivity Conferring Protein (OSCP) and Beef Heart Mitochondrial F_1-ATPase. 1. Study of the Binding Parameters with a Chemically Radiolabeled OSCP

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ABSTRACT: Upon treatment of beef heart mitochondrial oligomycin sensitivity conferring protein (OSCP) with [^{14}C]-*N*-ethylmaleimide ([^{14}C]NEM) or dithiobis(nitro[^{14}C]benzoate), 1 mol of either SH reagent was incorporated per mol of OSCP. Radiolabeling occurred at the level of the only cysteine residue, Cys-118, present in the OSCP sequence reported by Ovchinnikov et al. [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]; it did not alter the biological activity of OSCP tested in a reconstituted F_0–F_1 system that catalyzed oligomycin-sensitive ATPase activity or ATP–P_i exchange. The parameters of [^{14}C]NEM–OSCP binding to isolated beef heart mitochondrial F_1 were assessed by equilibrium dialysis. Addition of trace amounts of Tween 20 prevented unspecific adsorption of OSCP. The binding curves showed that each F_1 possesses a high-affinity OSCP binding site (K_d = 0.08 μM) and two low-affinity OSCP binding sites (K_d = 6–8 μM). Binding of OSCP to the high-affinity site on F_1 is probably responsible for the ability of OSCP to confer oligomycin sensitivity to F_1 in the ATPase complex.

The oligomycin sensitivity conferring protein (OSCP)[1] is a small component ($M_r \simeq 21\,000$) of the mitochondrial ATPase complex that is considered to act as a link between the catalytic sector, F_1, and the membrane sector, F_0. It possibly corresponds to the stalk observed in electron micrographs (MacLennan & Asai, 1968). OSCP makes the F_1 sector sensitive to oligomycin, an inhibitor which specifically binds to the F_0 sector. Although isolated F_1 can bind to F_0 in the absence of OSCP, F_1 in a reconstituted F_0–F_1 complex devoid of OSCP is insensitive to oligomycin, and furthermore, it loses its activity in the cold, as does isolated F_1 (Tzagoloff et al., 1968; MacLennan & Tzagoloff, 1968; Vadineanu et al., 1976). Rebinding of OSCP to the F_0–F_1 complex restitutes oligomycin sensitivity and cold stability to F_1-ATPase; it also stimulates ATP–P_i exchange and the ATP-driven reversal of electron transport in a reconstituted membranous system. Almost all reported OSCP titration assays are based on the ability of OSCP to confer oligomycin sensitivity to F_1 rebound to F_1-depleted submitochondrial particles. By this means, binding stoichiometries of 2–3 (Hundal & Ernster, 1979) or 1 mol of OSCP/mol of F_1 (Dupuis et al., 1983a) have been found. Interactions between beef heart OSCP and F_1 have been revealed by characterization of the OSCP–F_1 complex after centrifugation on sucrose gradient (Van de Stadt et al., 1972). More recently Liang & Fisher (1983) separated a rat liver F_1–OSCP complex by nondenaturing gel electrophoresis and reported 1 mol of OSCP binding site/mol of F_1 with a K_d of 1 μM. It has to be borne in mind that all these titrations are either indirect or not performed under equilibrium conditions.

In this paper, we describe a method of chemical radiolabeling of OSCP that does not alter the biological efficiency of the protein. This method is based on the use of [^{14}C]-*N*-ethylmaleimide ([^{14}C]NEM), which alkylates the only cysteinyl residue present in the OSCP molecule (Ovchinnikov et al., 1984). The binding parameters of [^{14}C]OSCP with respect to isolated beef heart F_1-ATPase have been studied. The existence of three OSCP binding sites has been demonstrated. One of these sites has a much higher affinity (K_d = 80 nM) than the other two (K_d = 6–8 μM) and is probably responsible for the biological activity of OSCP.

Experimental Procedures

Materials. [^{14}C]-*N*-Ethylmaleimide (30 mCi/mmol) and dithiobis(nitro[^{14}C]benzoate) (19 mCi/mmol) were purchased from CEA (Saclay, France). All other chemical reagents were of the highest purity available. Pyruvate kinase was from Boehringer. Beef heart mitochondria were prepared as described by Smith (1967). The A particles and ASUA particles were prepared as described by Fessenden & Racker (1966) and Vadineanu et al. (1976). OSCP was isolated according to the procedure of Senior (1979) with a further step of purification by Sephadex G-50 chromatography (Dupuis et al., 1983b). Beef heart mitochondrial F_1 and *Escherichia coli* F_1 were purified as described by Knowles & Penefsky (1972) and Satre et al. (1982), respectively.

Biological Assays. ATPase activity was measured in 0.5 mL of an ATP-regenerating system to give the following final concentrations: 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 allowed to proceed for 10 min at 30 °C and was stopped by addition of 0.1 mL of 50% (w/v) trichloroacetic acid. P_i released by ATP hydrolysis was measured as described by Fiske & SubbaRow (1925), after the solution had been centrifuged for 15

[1] Abbreviations: OSCP, oligomycin sensitivity conferring protein; NEM, *N*-ethylmaleimide; DTNB, dithiobis(nitrobenzoate); TPCK–trypsin, trypsin treated by L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; ATPase, adenosinetriphosphatase; F_1, catalytic sector of H^+-dependent ATPase; F_0, membrane sector of H^+-dependent ATPase; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate.

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min at 600*g*. The activity of OSCP was determined either by its ability to confer oligomycin sensitivity to the F_1-ATPase or by its ability to stimulate ATP-[^{32}P]P_i exchange in mitochondrial particles devoid of OSCP, as previously described (Dupuis et al., 1983a).

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Gel electrophoresis on 12% polyacrylamide slab gel in 0.1% NaDodSO$_4$ was performed according to the method of Laemmli & Favre (1973). For autoradiography, Fuji NIF RX films were used.

Tryptic Digest of OSCP. OSCP was digested 2 h at 37 °C by TPCK–trypsin by using a ratio of trypsin to OSCP of 1/100 (w/w) in 0.1 M NH_4HCO_3, pH 7.8, followed by a further 22-h incubation at 37 °C with a trypsin to OSCP ratio of 1/20. After lyophilization, the digest was solubilized in a buffer composed of acetic acid, pyridine, acetone, and water (40/20/160/880), pH 4.5, and further subjected to electrophoresis in the same buffer followed by chromatography on cellulose F1440 TLC plates (Schleicher & Schuell) as previously described (Klein et al., 1980). Peptides were revealed by phenanthrenequinone and ninhydrin coloration. Plates corresponding to the tryptic digest of [^{14}C]NEM–OSCP were autoradiographed.

Limited Proteolysis of Beef Heart F_1 by Trypsin. Beef heart F_1 (2 mg/mL) was incubated for 5 min at 30 °C in a buffer consisting of 50 mM Tris–sulfate and 0.25 mM EDTA, pH 8.0, in the presence of TPCK–trypsin with a trypsin to F_1 ratio of 1/100 (w/w). The reaction was stopped by a 5-fold excess of soybean trypsin inhibitor.

[^{14}C]NEM–OSCP Binding Assay. Two types of assay were used for measuring the binding of [^{14}C]NEM–OSCP to F_1. In the first one, F_1-bound [^{14}C]OSCP was separated from free [^{14}C]OSCP by centrifugation through a 1-mL column filled with 150 μL of CM-Sephadex C-25, as detailed in the legend of Figure 8, following the procedure of Penefsky (1977). F_1–OSCP passed directly through the column, whereas free OSCP was retained on the column due to its net positive charge. Because of its versatility, this method was used as a screening test. The second type of assay consisted of an equilibrium dialysis with a Dianorm apparatus equipped with microcells (2 × 250 μL) and M_r 160 000 cutoff Sartorius cellulose acetate membranes. One compartment of the microcells was filled with 180 μL of the F_1 preparation in 50 mM Tris–sulfate, 1 mM ATP, 0.5 mM EDTA, 200 mM KCl, and 0.02% Tween 20, pH 8.0; the other compartment contained [^{14}C]NEM–OSCP in the same medium. F_1 was used at the fixed concentration of 2 μM and [^{14}C]NEM–OSCP at varying concentrations. No loss of [^{14}C]NEM–OSCP or a decrease of ATPase activity could be detected after the 4-h period of dialysis required for complete equilibrium.

Radioactivity was measured by liquid scintillation (Patterson & Greene, 1965). Protein was estimated as described by Bradford (1976); the same results were obtained with another method based on the use of the Folin reagent (Zak & Cohen, 1961). In both cases, the data were consistent with the mass expected from amino acid analysis (Dupuis et al., 1983b).

Results

Chemical Modifications of OSCP by [^{14}C]NEM and [^{14}C]DTNB. OSCP (50 μg) was incubated for 30 min at 30 °C with increasing concentrations of [^{14}C]NEM or [^{14}C]DTNB in a 100 mM sodium phosphate buffer, pH 7.0, the final volume being adjusted to 50 μL. The pH was carefully checked during modification of OSCP by [^{14}C]DTNB. The solution was then applied to the top of a small column of 1 mL of Sephadex G-50 equilibrated with 50 mM Tris–sulfate, 15 mM K_2SO_4, and 5 mM $MgSO_4$, pH 8.0, and eluted by centrifugation (Penefsky, 1977). The protein recovery after centrifugation was between 70 and 80%.

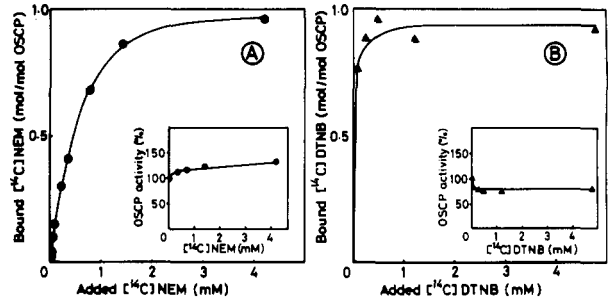

FIGURE 1: Titration of the chemical modification of OSCP by [^{14}C]NEM and [^{14}C]DTNB. Aliquots of 50 μg of OSCP (1 mg/mL) were incubated 30 min at 30 °C in a buffer consisting of 100 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, with increasing concentrations of [^{14}C]NEM (panel A) or [^{14}C]DTNB (panel B). The reaction was stopped by filtration–centrifugation through a 1-mL Sephadex G-50 column equilibrated with the same buffer. Protein and radioactivity were measured on aliquots to determine the extent of label incorporated in the protein. (Inset) OSCP was labeled by treatment with increasing amount of [^{14}C]NEM and [^{14}C]DTNB. The biological activity of the [^{14}C]OSCP recovered after this treatment was measured on aliquots of the G-50 eluates by means of its ability to confer oligomycin sensitivity to ATPase in a reconstituted system (cf. Experimental Procedures). This activity was normalized in percentage of the unmodified control.

The amount of [^{14}C]NEM or [^{14}C]DTNB incorporated was calculated on the basis of the radioactivity and the amount of protein recovered in the eluates, assuming a molecular weight of 20 967 for OSCP (Ovchinnikov et al., 1984). Incorporation of [^{14}C]NEM or [^{14}C]DTNB attained a plateau which corresponded to nearly 1 mol of labeled reagent incorporated per mol of OSCP (Figure 1). The high reactivity of [^{14}C]DTNB, a selective reagent of thiol groups, and the identical stoichiometries found for binding of [^{14}C]DTNB and [^{14}C]NEM to OSCP strongly suggested that both reagents reacted with the same amino acid residue, probably Cys-118, which is the only cysteine present in OSCP (Ovchinnikov et al., 1984). This was corroborated by the finding that preincubation of OSCP with 5 mM unlabeled NEM followed by removal of the unreacted NEM prevented by more than 90% radiolabeling of OSCP by 4 mM [^{14}C]NEM or 4 mM [^{14}C]DTNB.

The effect of labeling OSCP by [^{14}C]NEM or [^{14}C]DTNB on the biological activity of the protein was examined with a reconstituted F_0–F_1 system through an assay based on the sensitivity of ATPase to oligomycin. The amount of OSCP used was adjusted to give about 50% inhibition in the conditions of the assay. The data presented in the insets of Figure 1-A,B showed that labeling of the cysteine residue present in OSCP by saturating concentrations of [^{14}C]NEM or [^{14}C]DTNB had no significant effect on the biological activity of OSCP with respect to the hydrolytic activity of the F_1–F_0 complex. As reaction of cysteine residues with DTNB is reversible, NEM modification was preferred. In the following, OSCP modified by [^{14}C]NEM will be designated as [^{14}C]OSCP for convenience.

The competence of [^{14}C]OSCP compared to unlabeled OSCP in improving the rate of ATP-[^{32}P]P_i exchange in a reconstituted system made of A particles and F_1 is illustrated in Figure 2. The titration curves relative to OSCP and [^{14}C]OSCP were virtually superimposable. In summary, by two different types of assay, namely, the conferral of oligomycin sensitivity to ATP hydrolysis in an ATPase complex and the improvement of the synthetic activity of a reconstituted

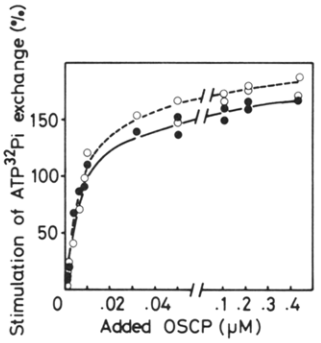

FIGURE 2: Comparison of native and [^{14}C]OSCP by titration of their stimulatory effect on ATP–[^{32}P]P_i exchange. Different amounts of unmodified OSCP (○) or [^{14}C]OSCP (●) were incubated 10 min at 25 °C with 250 μg of A particles and 50 μg of F_1 in a buffer consisting of 30 mM Tris–sulfate (pH 7.5), 10 mM $MgCl_2$, and 0.12 mg of bovine serum albumin, at a final volume of 0.45 mL. The reaction was initiated by addition of 50 μL of 100 mM ATP and 100 mM [^{32}P]P_i. After 2 min, the reaction was quenched by 50 μL of 30% perchloric acid. [[^{32}P]P_i] incorporated into ATP was measured as previously described (Dupuis et al., 1983a).

F_0–F_1 system, [^{14}C]OSCP proved to be as efficient as native OSCP. Further, we found that [^{14}C]OSCP is able to protect F_1 against cold inactivation as does native OSCP (Hundal & Ernster, 1979).

For large scale preparation of radiolabeled OSCP, about 4 mg of OSCP was dialyzed against 0.1 M sodium phosphate, pH 7.0, for 5 h and then incubated for 1 h at about 2.5 mg/mL with 8 mM [^{14}C]NEM at 0 °C. Alkylation was stopped with 10 mM dithiothreitol. [^{14}C]NEM–OSCP was then precipitated by 50% ammonium sulfate at 0 °C, and the precipitate was recovered by centrifugation. The pellet was solubilized in 0.05 M Tris–sulfate, pH 7.5. Insoluble material was removed by centrifugation. The [^{14}C]OSCP preparation was dialyzed exhaustively for 20 h against 0.05 M Tris–sulfate, pH 7.5. [^{14}C]OSCP was stored in this buffer in liquid nitrogen. The mean average labeling of OSCP in 10 experiments was 1.01 mol of [^{14}C]NEM/mol of OSCP ● 0.15. The specific radioactivity of [^{14}C]OSCP was 70.10^6 dpm/μmol.

Chemical Characterization of [^{14}C]NEM–OSCP. The covalent nature of the bond between OSCP and [^{14}C]NEM and the radiochemical purity of [^{14}C]OSCP were ascertained by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography. As previously reported (Dupuis et al., 1983b), only one protein band was observed after staining by Coomassie blue, corresponding to a M_r 21 000–22 000 polypeptide. Likewise, after autoradiography, only one radioactive spot was revealed, coincident with the band of OSCP stained by Coomassie blue.

The autoradiography of the tryptic map of [^{14}C]OSCP illustrated in Figure 3 showed one major radioactive peptide containing 80% of the radioactivity of the peptide digest and corresponding to a spot revealed by ninhydrin. This peptide did not react with phenanthrenequinone, and was therefore considered as arginine negative. The fingerprint of the native OSCP did not present ninhydrin-positive material at this level, which is likely explained by a different mobility of the [^{14}C]NEM-bearing peptide compared to the same unlabeled peptide. Minor radioactive spots not revealed by ninhydrin were detected on the autoradiograph after prolonged exposure; they probably corresponded to residual overlapping peptides resulting from incomplete trypsinolysis of OSCP. The quasi-homogeneous radiolabeling of OSCP by [^{14}C]NEM and the fact that 1 mol of [^{14}C]NEM binds at saturation to 1 mol of OSCP corroborate the conclusion that [^{14}C]NEM binds exclusively to the only cysteinyl residue present in OSCP.

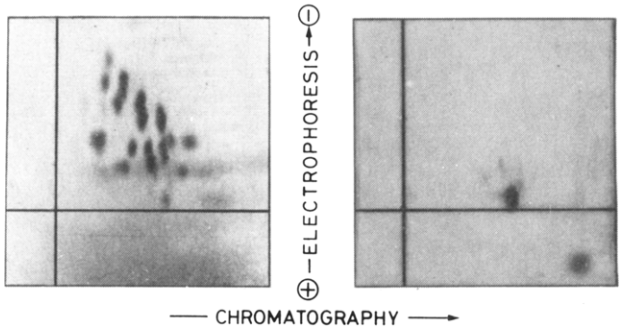

FIGURE 3: Tryptic map of [^{14}C]OSCP. A total of 50 μg of [^{14}C]OSCP was digested for 2 h at 37 °C by 0.5 μg of TPCK–trypsin in 0.1 M NH_4HCO_3, pH 7.8, followed by a further step of digestion for 24 h at 37 °C with 2.5 μg of TPCK–trypsin. The digest was lyophilized, solubilized in acetic acid/pyridine/acetone/water (40/20/160/88), and then subjected to electrophoresis followed by chromatography on cellulose plates (cf. Experimental Procedures). The peptides were revealed by phenanthrenequinone (not shown) or ninhydrin (panel A). The plate was then autoradiographed (panel B).

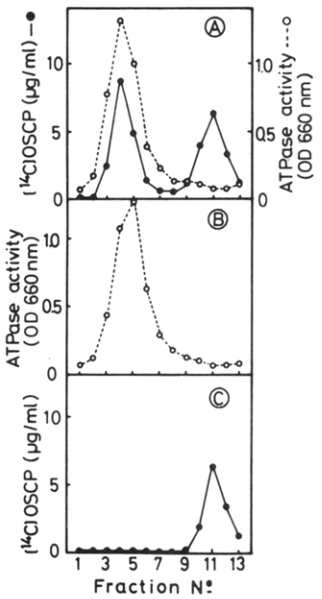

FIGURE 4: Isolation of the [^{14}C]OSCP–F_1 complex by centrifugation in a glycerol gradient. (Panel A) A total of 40 μg of [^{14}C]OSCP was incubated with 150 μg of F_1 in 100 μL of buffer consisting of 50 mM Tris–sulfate, 2 mM ATP, and 1 mM EDTA, pH 7.5, for 10 min at 25 °C. This solution was layered onto 5 mL of a 5–15% linear gradient of glycerol. The tubes were centrifuged for 180 min at 50000 rpm in a Beckman SW65 Ti rotor. After centrifugation, 13 fractions were collected and assayed for radioactivity (●), ATPase activity (○), and protein concentration (not shown). (Panel B) Control F_1 without OSCP. (Panel C) Control [^{14}C]NEM–OSCP.

Demonstration of an F_1–[^{14}C]OSCP Complex by Centrifugation on a Glycerol Gradient. In the experiment illustrated in Figure 4, beef heart F_1 was allowed to react with [^{14}C]OSCP. The F_1–[^{14}C]OSCP complex was separated from free F_1 and [^{14}C]OSCP components by centrifugation on a 5–15% glycerol gradient. During centrifugation, equilibrium between bound and free [^{14}C]OSCP was continually displaced, which made calculation of a K_d value inaccurate. Nevertheless, the well-separated peak of F_1–[^{14}C]OSCP afforded direct evidence for binding of [^{14}C]OSCP to F_1. Interaction between OSCP and mitochondrial F_1 was previously reported by Van de Stadt et al. (1972) using a linear sucrose gradient and unlabeled beef heart OSCP. However, with the yeast system, no interaction could be demonstrated (Tzagoloff, 1970).

Mitochondrial [^{14}C]OSCP did not react with *E. coli* F_1 (not shown). Although this result was surprising since mitochon-

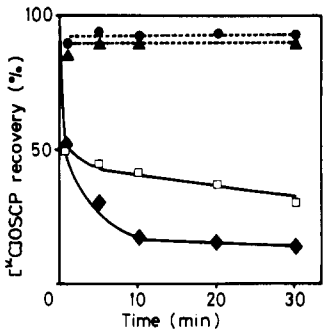

FIGURE 5: Kinetics of unspecific adsorption of [^{14}C]OSCP to polystyrene tubes. [^{14}C]OSCP was diluted at a final concentration of 0.005 mg/mL (○, △, ♦) or 0.05 mg/mL (□) in 2 mL of 50 mM Tris–sulfate, pH 7.5, without Tween 20 (♦, □) or with 0.01% (●) or 0.005% (▲) Tween 20. The incubation was carried out at 30 °C in polystyrene tubes. At the indicated time, 0.2-mL aliquots were withdrawn after vigorous vortex shaking and assayed for radioactivity.

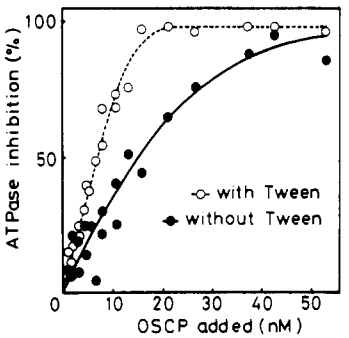

FIGURE 6: Effect of Tween 20 on the ability of OSCP to confer oligomycin sensitivity to F_1-ATPase in a reconstituted system. OSCP was incubated for 10 min at 30 °C with 1 μg of F_1, 0.13 mg of ASUA particles, and 5 μg of oligomycin in 0.45 mL of a medium consisting of 50 mM Tris–sulfate, 10 mM KCl, and 5 mM $MgSO_4$, pH 8.0, with (○) or without (●) 0.01% Tween 20. The ATPase activity was measured as described under Experimental Procedures. The P_i released was calculated by calibration with P_i standards in the absence or presence of Tween 20.

drial OSCP is supposed to be the counterpart of the δ subunit of the *E. coli* F_1 (Walker et al., 1982; Ovchinnikov et al., 1984), it clearly points to the specific nature of the binding of mitochondrial [^{14}C]OSCP to mitochondrial F_1. On the other hand, cross-reactivity between beef heart OSCP and rat liver mitochondrial F_1 was observed as previously reported by Liang & Fisher (1983).

During the course of the binding experiments with [^{14}C]OSCP, it was repeatedly observed that the amount of radioactivity recovered from the gradient was substantially smaller than that applied to the gradient prior to centrifugation. This led to the unexpected finding, not yet mentioned in literature, that OSCP has a strong propensity to adsorb to the walls of glass tubes and plastic tubes.

Prevention by Tween 20 of Unspecific Adsorption of OSCP. After a 30-min incubation of solutions containing 5 μg of [^{14}C]OSCP/mL in a glass tube or a polystyrene tube, the radioactivity recovered in the solutions was 5 and 15%, respectively. Most of [^{14}C]OSCP had adsorbed to the walls of the tubes. The adsorption was less severe but still important—about 60%—in the case of Minisorp tubes. As shown in Figure 5, unspecific adsorption was a rapid process, half of the total adsorption occurring in less than 1 min.

Among a number of chemicals tested, Tween 20 at a very low concentration was found to prevent fully unspecific OSCP adsorption. Bovine serum albumin and cytochrome *c* at 0.05% (w/v) had only a minor effect. The data in Figure 6 show that

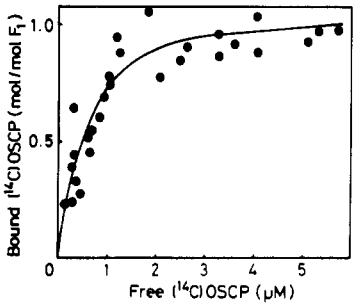

FIGURE 7: Determination of ion-exchange chromatography of the binding parameters of [^{14}C]OSCP with respect to F_1. [^{14}C]OSCP was incubated for 10 min at 30 °C with 50 μg of F_1 in 150 μL of a buffer consisting of 50 mM Tris–sulfate, 1 mM ATP, 0.5 mM EDTA, 50 mM KCl, and 0.02% Tween 20, pH 8.0. The medium was then centrifuged through a 1-mL tuberculin syringe filled with 150 μL of CM-Sephadex C-25 equilibrated with the same buffer. Protein and radioactivity were determined in the eluates. Controls for elution of free [^{14}C]OSCP gave values lower than 5% of the bound OSCP.

total prevention of unspecific adsorption of OSCP could be achieved with as little as 0.005% Tween 20. Routinely, Tween 20 was added at final concentrations ranging between 0.01% and 0.02%. To ascertain the absence of side effects of Tween 20 on the biological activities of OSCP or F_1, we compared the conferral by unlabeled OSCP of oligomycin sensitivity to F_1-ATPase in a reconstituted system in the absence and presence of 0.01% Tween 20 (Figure 6). The assay carried out in the presence of Tween 20 showed a marked enhancement (more than 2-fold) of the OSCP activity with respect to the assay in which Tween 20 was omitted. Adsorption of OSCP to the tube walls decreased its effective concentration, and Tween 20 efficiently opposed this unspecific adsorption.

Determination of Binding Parameters of [^{14}C]*OSCP with Respect to Beef Heart Mitochondrial* F_1. A rapid and convenient method to study the binding parameters of [^{14}C]OSCP with respect to F_1 consists in the separation of the [^{14}C]OSCP–F_1 complex by an ion-exchange chromatography, accelerated by centrifugation (cf. Experimental Procedures). This method was used in the experiment illustrated in Figure 7. A saturation plateau was observed, which corresponded to the binding of 1 mol of [^{14}C]OSCP/mol of F_1. The K_d value was 0.6 μM. Binding kinetics could not be resolved because maximal binding was attained in less than 2 min; that was the minimal period of time required for this assay. Similar results were obtained with a nucleotide-depleted preparation of F_1, suggesting that tightly bound nucleotides do not interfere with the binding of OSCP to F_1.

Binding of [^{14}C]OSCP to mitochondrial F_1 is fully reversible, as illustrated by the following experiment. After incubation of F_1 with [^{14}C]OSCP in a molar ratio of 1/10 for 10 min at 30 °C, the F_1–[^{14}C]OSCP complex was separated by chromatography on CM-Sephadex C-25 and incubated with a 10-fold excess of unlabeled OSCP. Under these conditions, less than 5% radioactivity remained bound to F_1 after rechromatography on CM-Sephadex C-25.

Binding of [^{14}C]OSCP to F_1 was also studied by equilibrium dialysis. Under equilibrium conditions, three OSCP binding sites were titrated on F_1 (Figure 8). The nonlinear Scatchard plot corresponding to the binding of [^{14}C]OSCP to F_1 clearly showed the presence of a high-affinity binding site (K_d = 0.08 μM), the other two sites having a much lower affinity (K_d = 6–8 μM). It was checked that the ATPase activity was not modified after 4 h of dialysis, a period of time required for full equilibrium. In a control assay, equilibrium dialysis did not reveal any significant interaction between [^{14}C]OSCP and

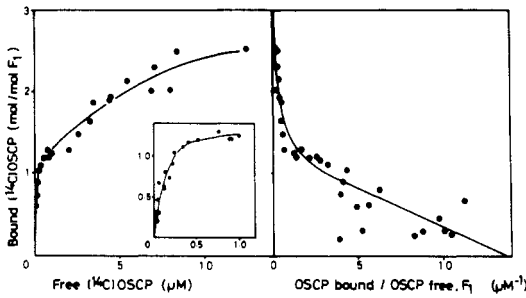

FIGURE 8: Determination by equilibrium dialysis of the binding parameters of [^{14}C]OSCP with respect to F_1-ATPase. Experiments were carried out as described under Experimental Procedures in 50 mM Tris–sulfate, 1 mM ATP, 0.5 mM EDTA, 200 mM KCl, and 0.02% Tween 20, pH 8.0. F_1 was used at the final concentration of 2 μM. (Panel A) Direct binding curve: the inset shows an expansion of the high-affinity portion of the [^{14}C]OSCP binding curve. (Panel B, Scatchard plot) The curve drawn through the points is a theoretical curve calculated for 1 site of K_d = 80 nM, 1 site of K_d = 6 μM, and 1 site of K_d = 8 μM.

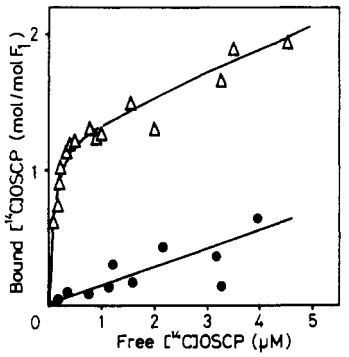

FIGURE 9: Effect of mild tryptic proteolysis of F_1 on its binding affinity for OSCP. The experiment was carried out as described in Figure 8 with control F_1 (Δ) or trypsinized F_1 (●). Treatment of F_1 by trypsin was performed as described under Experimental Procedures. Cleavage of the α subunit led to disappearance of α and accumulation of a smaller peptide which migrated with an apparent molecular weight close to that of the β subunit on gel electrophoresis. Under our conditions, cleavage of α was virtually 100%. ATPase activity of the trypsinized F_1 was stable over the dialysis period.

E coli F_1, corroborating results obtained by centrifugation on glycerol gradient.

Mild digestion of F_1 by trypsin fully inhibited the high-affinity binding of [^{14}C]OSCP to the isolated mitochondrial F_1-ATPase (Figure 9). It is not known whether the failure of OSCP to bind with high affinity to F_1 is due to removal by trypsin of a critical portion of α (Leimgruber & Senior, 1976) or β subunits (Hollemans et al., 1983) containing binding sites for OSCP or to a conformational change of the trypsinized α and β subunits.

Discussion

Radiochemical labeling of a protein without loss of biological activity is the method of choice to study interactions of this protein when integrated into a multicomponent system. In the case of the mitochondrial ATPase complex, this method was successfully applied to study the interaction of the natural ATPase inhibitor with F_1 (Klein et al., 1980). Since OSCP is devoided of directly measurable biological activity, its functional characterization requires the presence of both F_1 and F_0, leading to complex interactions difficult to control. It was, therefore, of interest to obtain OSCP under a radiolabeled form to investigate its binding properties. As a matter of fact, indirect evidence based on biological titrations showed interaction between OSCP and F_1, but no concordant results with regard to the OSCP binding stoichiometry could be obtained. This is illustrated by the fact that stoichiometric ratios ranging from 1 (Van de Stadt et al., 1972; Liang & Fisher, 1983; Dupuis et al., 1983) to 2–3 (Hundall & Ernster, 1979) had been reported.

Chemical radiolabeling of OSCP was achieved by use of [^{14}C]NEM. The fact that the only cysteine residue present in OSCP, Cys-118, is alkylated by [^{14}C]NEM is based on the following lines of evidence: (1) [^{14}C]NEM and [^{14}C]DTNB are incorporated in OSCP with a maximal 1/1 stoichiometry. (2) [^{14}C]NEM and [^{14}C]DTNB compete for binding to one amino acid residue. (3) NEM and DTNB are highly selective thiol reagents. (4) Only one cysteine residue, Cys-118, is present in OSCP (Ovchinnikov et al., 1984). (5) The peptidic map of a tryptic digest of [^{14}C]OSCP shows only one major radiolabeled spot. Biological characterization of the [^{14}C]OSCP showed that the radiolabeled OSCP exhibits the same properties as the native protein. Thus, alkylation of the only cysteine residue present on OSCP by [^{14}C]NEM does not alter functional efficiency of OSCP. In contrast, Liang & Fisher (1983) reported that disulfide bond formation between 2 mol of OSCP prevented binding of the dimeric OSCP to F_1. It is likely that failure of dimeric OSCP to bind to F_1 is due to sterical hindrance of the OSCP binding site on F_1 rather than to a deleterious consequence of the modification of the cysteine residue.

The use of [^{14}C]OSCP has revealed the dramatic propensity of OSCP to adsorb to test tube walls. Adsorption occurred with glass and polystyrene tubes and even Minisorp tubes which are reported not to interact with proteinaceous material. [^{14}C]OSCP was used to quantify the unspecific OSCP adsorption and to screen different reagents which might remedy to it. Among a number of reagents tested, Tween 20 gave the most satisfactory results; at the final concentration of 0.01% or 0.02%, Tween 20 totally prevented the unspecific adsorption of OSCP without interfering in the binding assays or changing the biological activity of OSCP. With 0.01% Tween 20, the efficiency of OSCP in conferring of oligomycin sensitivity to F_1-ATPase in a reconstituted system was increased more than 2-fold, compared to an assay performed in the absence of Tween 20. This is readily explained by the higher actual concentration of OSCP in the medium in the presence of Tween 20 which prevents unspecific adsorption. The strong unspecific adsorption of OSCP in the absence of Tween 20 casts serious doubt on the validity of previously reported titrations of OSCP which did not take unspecific adsorption of OSCP into account.

In binding studies, free and bound [^{14}C]OSCP were measured by ionic chromatography combined with centrifugation or by equilibrium dialysis. Ionic chromatography using short columns filled with CM-Sephadex C-25 submitted to centrifugation to increase the rate of elution allowed quantitative recovery of the [^{14}C]OSCP–F_1 complex but did not enable us to undertake a kinetic study of the interaction between [^{14}C]OSCP and F_1; in fact, binding reached completion within less than 2 min. One OSCP binding site per F_1 with a K_d value of 0.6 μM was revealed. These binding parameters are close to those reported by Liang & Fisher (1983). These authors using a nondenaturating gel electrophoresis to separate bound and free OSCP reported the formation of an OSCP–F_1 complex with an OSCP to F_1 stoichiometry of 1 and a K_d value of about 1 μM. It is evident, however, that the ionic chromatography technique, although rapid and easy to perform, and the nondenaturating gel electrophoresis are nonequilibrium techniques; they cannot therefore provide accurate binding data

because of dissociation of bound OSCP, and it is probable that the K_d values calculated by these techniques are overestimated. In contrast, by equilibrium dialysis, three specific OSCP binding sites per F_1 were demonstrated, namely, one high-affinity site (K_d = 0.08 μM) and two low-affinity sites (K_d = 6–8 μM). The K_d of 0.6 μM obtained by ionic chromatography may correspond to the high-affinity site (K_d = 0.08 μM) demonstrated by equilibrium dialysis. On the other hand, the dissociation constant of 6–8 μM found by equilibrium dialysis for two of the three OSCP binding sites of F_1 reflects a rather low affinity for protein–protein interaction; it is suggested that the corresponding OSCP binding sites are potential sites, not actively involved in the formation of the F_0–OSCP–F_1 complex. The presence of one high-affinity binding site for OSCP out of the three sites present on F_1 recalls the $^1/_3$ site reactivity exhibited by F_1 toward a number of ligands [for review, Vignais et al. (1984)].

When assayed with rat liver mitochondrial F_1, beef heart OSCP exhibited a strong cross-reactivity, indicating a functional homology between the two mammalian mitochondrial systems in agreement with a previous report by Liang & Fisher (1983). On the other hand, no interaction of [^{14}C]OSCP with *E coli* δ depleted F_1 could be demonstrated in spite of considerable homologies of primary sequence between *E. coli* F_1 δ subunit and beef heart OSCP (Walker et al., 1983; Ovchinnikov et al., 1984). Failure of [^{14}C]OSCP to bind to *E. coli* F_1 was substantiated by the observation that beef heart OSCP was unable to induce oligomycin sensitivity to the *E. coli* F_1 in the presence of OSCP and F_1-depleted submitochondrial membranes (not shown).

Correlation between binding of [^{14}C]OSCP to F_1 and conferral of oligomycin sensitivity to F_1 in a reconstituted system is clearly demonstrated in the case of trypsinized F_1. As reported by Hundal et al. (1983), OSCP no longer brings about oligomycin sensitivity to trypsinized F_1 rebound to F_1-depleted submitochondrial particles. As shown in the present paper, loss of OSCP activity is well correlated with loss of high-affinity binding of OSCP to the trypsinized F_1. This result together with the fact that nanomolar concentrations of OSCP are sufficient to confer oligomycin sensitivity to F_1 clearly suggests that OSCP exerts a control on F_1 through its binding to the high-affinity OSCP site on F_1.

In a previous report an apparent K_d value of 1.7 nM for binding of OSCP to the F_0–F_1 complex was deduced from oligomycin sensitivity titration assays in a reconstituted F_0–F_1 system (Dupuis et al., 1983a). It is known that there are direct interactions between F_1 and F_0 in the absence of OSCP (Tzagoloff & MacLennan, 1968; Vadineanu et al., 1976). The difference between the very low K_d value for binding of OSCP to F_0–F_1 and the K_d value fo the high-affinity OSCP binding site on F_1 reported in this paper (0.08 μM) illustrated the role played by F_0–F_1 direct interactions in tightening the bond between OSCP and the F_0–F_1 complex.

Registry No. ATPase, 9000-83-3.

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