Interactions between the Oligomycin Sensitivity Conferring Protein (OSCP) and Beef Heart Mitochondrial F_1-ATPase. 2. Identification of the Interacting F_1 Subunits by Cross-Linking

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ABSTRACT: Interactions between oligomycin sensitivity conferring protein (OSCP) and subunits of beef heart mitochondrial F_1-ATPase have been explored by cross-linking at an OSCP/F_1 molar ratio close to 1 to ensure specific high-affinity binding of OSCP to F_1 [see Dupuis et al. [Dupuis, A., Issartel, J.-P., Lunardi, J., Satre, M., & Vignais, P. V. (1985) *Biochemistry* (preceding paper in this issue)]]. Cross-links between F_1 subunits and OSCP were established by means of two zero length cross-linkers, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline. The cross-linked products were separated by sodium dodecyl suflate–polyacrylamide gel electrophoresis. Coomassie blue staining revealed two cross-linked products of M_r 75 000 and 80 000 which could result from the binding of OSCP to the α and β subunits of F_1. Definite identification of the cross-linked products was achieved by chemical labeling with specific radiolabeled reagents and by blotting on nitrocellulose filters followed by immunocharacterization with anti-α, anti-β, and anti-OSCP antibodies. OSCP was found to cross-link with the α and β subunits of F_1.

In the preceding paper (Dupuis et al., 1985), it was shown through the use of chemically radiolabeled OSCP that mitochondrial F_1[1] possesses three OSCP binding sites, one of high affinity and the other two of low affinity. It is probably through its interaction with the high-affinity site of F_1 that OSCP confers oligomycin sensitivity to F_1 in an integrated F_1–F_0 complex. It remained to be determined which subunit(s) of F_1 interacts with OSCP. Cross-linking is a convenient approach to study oligomeric structures. Reagents routinely used like dimethyl suberimidate and dimethyl adipimidate introduce, however, arms of several angstrom length which may lead to inaccurate interpretations of the data. In the present work, we have determined which subunits of F_1 are in close contact with OSCP by means of the zero length cross-linkers EDAC and EEDQ. The components of the cross-linked products were identified by two approaches, namely, specific radiochemical labeling and immunocharacterization. The results obtained by these two approaches concurred to show that both the α and β subunits of beef heart F_1 interact with OSCP.

Experimental Procedures

Materials. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide (EDAC) and *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline (EEDQ) were obtained from Aldrich, and horse radish peroxidase conjugated antirabbit goat antibody was from Miles Laboratories. [^{14}C]dicyclohexylcarbodiimide (DCCD) and [^{14}C]*N*-ethylmaleimide (NEM) were purchased from CEA Saclay.

Biological Preparations. OSCP was prepared as described by Senior (1979) with a further step of purification by chromatography on a Sephadex G-50 column (Dupuis et al., 1983). Beef heart mitochondria F_1 was purified by the method of Knowles & Penefsky (1972a). Protein was determined by the method of Bradford (1976).

ATPase Assay. ATPase activity was measured spectrophotometrically at 340 nm by coupled NADH oxidation (Pullman et al., 1960). The assay was carried out at 30 °C. The medium consisted of 25 mM Tris–SO_4, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 25 μg/mL pyruvate kinase, 12.5 μg/mL lactate dehydrogenase, 15 mM KCl, 2.2 mM ATP, and 1.1 mM $MgCl_2$, pH 8.0.

Cross-Linking Assays. Beef heart mitochondrial F_1 (200 μg) was incubated for 15 min at 30 °C with 11–13 μg of OSCP in a buffer made of 50 mM Mops, 1 mM ATP, 0.5 mM EDTA, 15 mM K_2SO_4, and 0.02% Tween 20, final pH 6.5, in a volume of 0.1 mL. Concentrated methanolic solutions of EDAC or EEDQ were added to give final concentrations of 5 mM EDAC or 1 mM EEDQ and 1% methanol. After 30 min at 30 °C, cross-linking was stopped by 2% $NaDodSO_4$ and 5% mercaptoethanol to which were added 0.001% bromophenol blue and 10% glycerol. These concentrations were high enough to fully quench the cross-linking reaction. The samples were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis with a 12% polyacrylamide slab gel in 0.1% $NaDodSO_4$ (Laemmli & Favre, 1973). Fuji NIF RX films were used for detection of radioactivity labeled cross-linked products by autoradiography.

Radiolabeling of the F_1 Subunits. α and β subunits were labeled by [^{14}C]NEM and [^{14}C]DCCD, respectively (Senior, 1975; Pougeois et al., 1979). Control experiments were carried out to ensure that [^{14}C]DCCD did not label either free OSCP or OSCP complexed to F_1. Therefore, [^{14}C]DCCD labeling was performed either on F_1 prior to cross-linking or on the F_1–OSCP cross-linked products. No difference in [^{14}C]DCCD labeling or in the cross-linking patterns was found. As

[1] Abbreviations: EDAC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxydihydroquinoline; DCCD, dicyclohexylcarbodiimide; OSCP, oligomycin sensitivity conferring protein; ATPase, adenosinetriphosphatase; F_1 catalytic sector of H^+-dependent ATPase; F_0, membrane sector of H^+-dependent ATPase; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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[^{14}C]NEM labeling of the α subunit occurs under denaturating conditions (Senior, 1975), it was performed only on the F_1–OSCP cross-linked products. In this case, OSCP was first saturated with unlabeled NEM by incubation for 1 h at 0 °C with 5 mM NEM to prevent [^{14}C]NEM incorporation into OSCP during incubation of [^{14}C]NEM with F_1–OSCP cross-linked products. Under these conditions, no further labeling of OSCP by [^{14}C]NEM was observed.

Immunocharacterization of Cross-Linked Products. Pure α and β subunits for production of anti-α and anti-β antibodies were obtained by means of electrophoretic isolation. F_1 (5 mg) was first submitted to 7.5% urea–polyacrylamide slab gel electrophoresis (Knowles & Penefsky, 1972a). Gels were slightly stained by Coomassie blue for detection of the subunits (Fairbanks et al., 1971). The β band was recovered and electroeluted. The gel areas corresponding to the α–γ band was cut out and submitted to a 12% $NaDodSO_4$–polyacrylamide slab gel electrophoresis (Laemmli & Favre, 1973) to separate α from γ. Specific antisera to pure α and β subunits and OSCP were raised in rabbits. The sera were tested by the method of Ouchterlony (1967).

To identify the components of the cross-linked products formed between OSCP and F_1 subunits, electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose sheets was performed under 24 V for 150 min 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. For this purpose, the blots were soaked for 16 h at 4 °C in 10 mM sodium phosphate, 150 mM NaCl final pH 7.4 and 0.1% Tween 20 (w/v) (saline–Tween medium) supplemented with 3% bovine serum albumin (w/v) to saturate additional protein binding sites in nitrocellulose. Filters were then incubated for 2 h at 20 °C with the specific antisera diluted in saline–Tween medium and 3% bovine serum albumin. After three washes in the saline–Tween medium, the nitrocellulose sheets were incubated for 2 h at room temperature with a horse radish peroxidase conjugated antibody where the antibody was an antirabbit IgG raised in goat. The peroxidase activity attached to the immunoreactive proteins on the nitrocellulose sheets was revealed by staining with diaminobenzidine in the presence of nickel and cobalt salts (De Blas & Cherwinski, 1983).

Results

EDAC and EEDQ Cross-Linking Patterns of OSCP–F_1 Complex. In all experiments dealing with cross-linking, beef heart F_1 and OSCP were allowed to react at equimolar concentrations, a condition under which the only one high-affinity OSCP binding site of F_1 is titrated [see Dupuis et al. (1985)]. After cross-linking with EDAC or EEDQ as described under Experimental Procedures, the cross-linked products were separated from F_1 subunits and OSCP by $NadodSO_4$–polyacrylamide gel electrophoresis, and the protein bands were stained by Coomassie blue (Figure 1).

When beef heart F_1 alone was treated by EDAC or EEDQ, two major cross-linked products of M_r 105 000–110 000 were found to accumulate. Cross-linked products of the same size obtained after treatment of beef heart F_1 by the long-chain cross-linkers dimethyl suberimidate, dimethyl adipimidate, and methyl mercaptobutyrimidate were identified as $\alpha\alpha$ and $\alpha\beta$ dimers on the basis of the molecular weights of the α subunit $\simeq$55 000 and the β subunit $\simeq$50 000 (Knowles & Penefsky, 1972b) and specific radiolabeling of α and β by [^{14}C]NEM and [^{14}C]nitrobenzofurazan (Satre et al., 1976). One may anticipate that the same cross-linked products accumulated

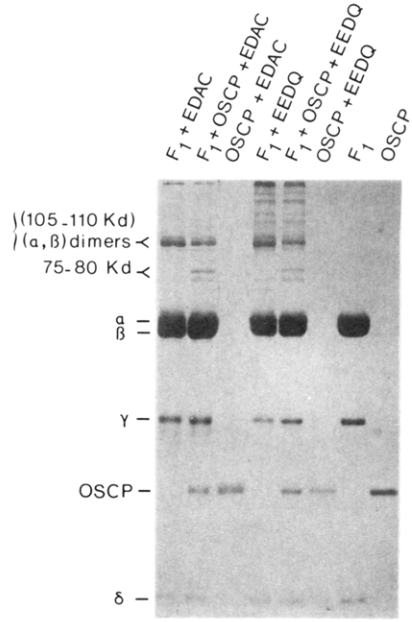

FIGURE 1: Cross-linking of OSCP and F_1 subunits by EDAC and EEDQ and separation of the cross-linked products by $NaDodSO_4$–polyacrylamide slab gel electrophoresis. OSCP (13 μg) and F_1 (200 μg) were preincubated for 10 min at 30 °C in 0.1 mL of a buffer composed of 50 mM Mops, 1 mM ATP, 0.5 mM EDTA, 15 mM K_2SO_4, and 0.02% Tween 20; then EEDQ or EDAC were added at the final concentration of 1 and 5 mM, respectively. Control incubations of EDAC or EEDQ with OSCP alone and F_1 alone were performed under the same conditions as above. After 30 min at 30 °C, the reaction was quenched by addition of 5% mercaptoethanol, 2% $NaDodSO_4$, and 10% glycerol. The original subunits and the cross-linked products were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. Lane 1, F_1 + EDAC; lane 2, F_1 + OSCP + EDAC; lane 3, OSCP + EDAC; lane 4, F_1 + EEDQ; lane 5, F_1 + OSCP + EEDQ; lane 6, OSCP + EEDQ; lane 7, F_1; lane 8, OSCP.

with the zero chain length cross-linkers EDAC or EEDQ used in the present work. Coomassie blue staining did not reveal any significant cross-links of OSCP alone by EDAC or EEDQ. However, as shown in the next section, through the use of radiolabeled OSCP, minute amounts of OSCP dimer could be revealed after cross-linking. By treatment of a mixture of F_1 and OSCP by EDAC or EEDQ, two additional cross-linked products of apparent M_r 78 000 and 80 000 accumulated beside the major cross-linked products of M_r 105 000–110 000 obtained with F_1 alone. On the basis of a molecular weight of $\simeq$21 000 for OSCP, the M_r 75 000 and 80 000 products were tentatively identified as β-OSCP and α-OSCP. This was in accordance with the fact that accumulation of these products required the presence of OSCP and was accompanied by a decrease of the M_r 105 000–110 000 products arising from cross-links between α and β. The cross-linking patterns with EDAC and EEDQ were essentially similar, with the difference that, with EEDQ, accumulation of products of M_r >100 000 was more marked than with EDAC.

In the following section, it is demonstrated by specific chemical labeling and immunocharacterization that the F_1 subunits present in the M_r 75 000 and 80 000 products are indeed β and α, respectively.

Characterization of the Components of the M_r 75 000 and 80 000 Cross-Linked Products by Radiochemical Labeling. As shown in the preceding paper (Dupuis et al., 1985), OSCP can be readily labeled with [^{14}C]NEM. On the other hand, as described under Experimental Procedures, the α and β subunits of F_1 can be distinguished by their selective reactivity toward [^{14}C]NEM and [^{14}C]DCCD, since α is labeled by [^{14}C]NEM under our experimental conditions and β by

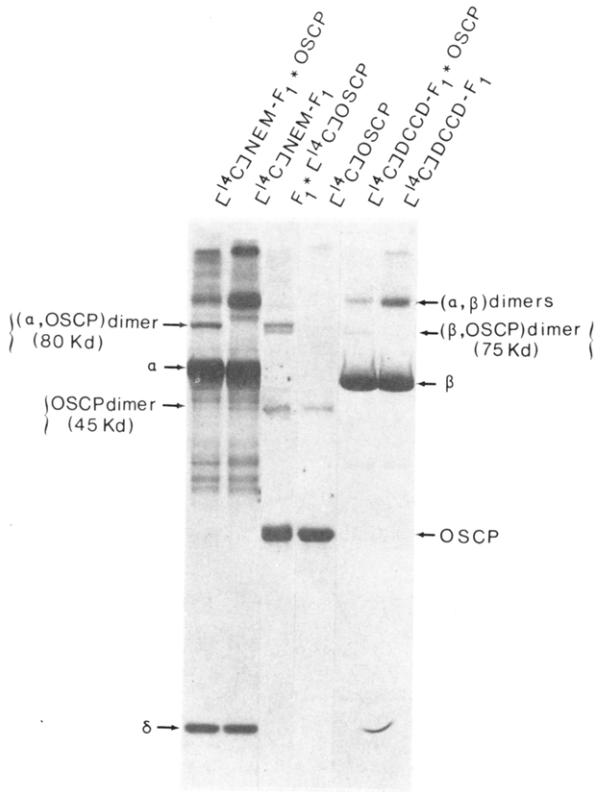

FIGURE 2: Topographical study of interaction between OSCP and F_1 subunits by means of cross-linking with EDAC and radiochemical labeling. The radiochemical labeling was carried out as described in the test by using the same amounts of F_1 and OSCP and the same buffer as in the experiment of Figure 1. The cross-linked products were separated by $NaDodSO_4$–polyacrylamide slab gel electrophoresis. The gel was autoradiographed. [^{14}C]NEM–F_1* OSCP (lane 1) corresponds to a preincubation of OSCP with unlabeled NEM followed by cross-linking with F_1 and subsequent treatment with [^{14}C]NEM. [^{14}C]DCCD–F_1*OSCP (lane 5) corresponds to a preincubation of OSCP with unlabeled NEM followed by cross-linking with F_1 and subsequent treatment with [^{14}C]DCCD. Finally, F_1*[^{14}C]OSCP (lane 3) corresponds to cross-linking of unlabeled F_1 with OSCP previously labeled with [^{14}C]NEM.

[^{14}C]DCCD. Two cross-linking experiments with EDAC were carried out. In the first one, [^{14}C]OSCP was cross-linked with unlabeled F_1; in the second one, OSCP pretreated by unlabeled NEM was cross-linked with F_1; this was followed by treatment of the cross-linked products with [^{14}C]NEM or [^{14}C]DCCD. Labeling with [^{14}C]NEM was expected to occur on the putative α subunit of the M_r 80 000 cross-linked product and labeling with [^{14}C]DCCD on the putative β subunit of the M_r 75 000 product. The autoradiograph corresponding to cross-linking by EDAC is presented in Figure 2. The α and β subunits at the level of the M_r 105 000–110 000 bands were detected by [^{14}C]NEM and [^{14}C]DCCD, respectively. In the same way, the M_r 80 000 and 75 000 products were found to contain the [^{14}C]NEM–α subunit and the [^{14}C]DCCD–β subunit, respectively. In addition, both the M_r 75 000 and the M_r 80 000 products contained [^{14}C]NEM–OSCP. Autoradiography allowed the detection of minute amounts of a radiolabeled cross-link product of M_r 45 000, even after cross-linking of [^{14}C]OSCP alone; for this reason, the M_r 45 000 radiolabeled product could be reasonably identified with an OSCP dimer. All these data strongly suggest that the M_r 80 000 product contains OSCP and the α subunit and that the M_r 75 000 product contains OSCP and the β subunit. From the protein-bound radioactivity in four different cross-linking experiments, it could be calculated that between 10 and 12% of the added OSCP was engaged in single cross-linking with

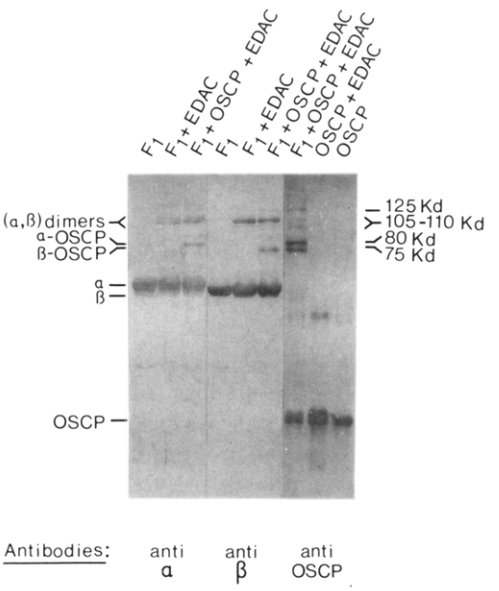

FIGURE 3: Transfer of cross-linked products of OSCP and F_1 subunits from polyacrylamide slab gel onto nitrocellulose sheet followed by immunodetection of OSCP, α, and β subunits. A 200-μg sample of F_1 and 11-μg sample of OSCP were cross-linked with EDAC as described in the legend of Figure 1. Aliquots corresponding to 10 μg of F_1 and 0.55 μg of OSCP were submitted to electrophoresis on a 12% polyacrylamide slab gel. Blotting and immunodetection are described under Experimental Procedures. Lanes 1–3, reaction with anti-α antibodies. Lane 1, control F_1 (10 μg); lane 2, cross-linked F_1 (10 μg); lane 3, cross-linked F_1 (10 μg) and OSCP (0.55 μg). Lanes 4–6, reaction with anti-β antibodies. Lane 4, control F_1 (10 μg); lane 5, cross-linked F_1 (10 μg); lane 6, cross-linked F_1 (10 μg) and OSCP (0.55 μg). Lanes 7–9, reaction with anti-OSCP antibodies. Lane 7, cross-linked F_1 (10 μg) and OSCP (0.55 μg); lane 8, cross-linked OSCP (0.55 μg); lane 9, control OSCP. The immunoreactive polypeptides were revealed by a peroxidase-conjugated antibody followed by reaction with diaminobenzidine, as described under Experimental Procedures.

either the α or β subunit. This yield of cross-linking compares favorably with that obtained with EDAC in a closely related system, namely, beef heart mitochondrial F_1 cross-linked with the natural ATPase inhibitor (Klein et al., 1980).

The β–OSCP band of M_r 75 000 revealed either by Coomassie blue staining or by autoradiography using [^{14}C]NEM–OSCP was fainter than the α–OSCP band of M_r 80 000. It must be borne in mind, however, that in oligomeric proteins cross-linking efficiency depends on the proper alignment of reactive amino acid residues. In the present experiment, not only the proximity of carboxyl and amino groups in OSCP and adjacent F_1 subunits but also the reactivity of these groups are important factors that control the formation of covalent cross-links.

Another zero length cross-linker, EEDQ, was used to cross-link OSCP and the subunits of mitochondrial F_1. The cross-linked products were similar to those found with EDAC (not shown).

Immunoenzymatic Characterization of the Components of the M_r 75 000 and 80 000 Cross-Linked Products. To ascertain the α and β nature of the F_1 subunits linked to OSCP in the M_r 75 000 and 80 000 bands, the cross-linked products were further analyzed by means of electrotransfer onto nitrocellulose filters followed by immunoenzymatic characterization with specific anti-α, anti-β, and anti-OSCP antibodies as described under Experimental Procedures. The same results were obtained with the two cross-linkers EDAC and EEDQ. For the sake of simplicity, we will report here only the EDAC cross-linking data. As shown in Figure 3, peroxidase activity revealed the bands of M_r 105 000 and 110 000 after treatment

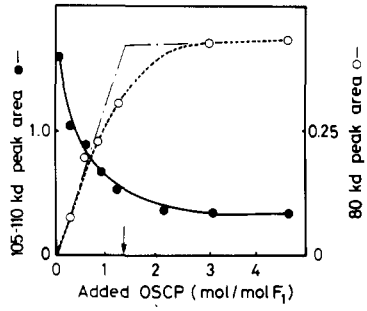

FIGURE 4: Effect of the OSCP concentration in the presence of a fixed concentration of F_1 on the accumulation of the EDAC cross-linked products of M_r 105 000–110 000 and 80 000. OSCP was incubated and cross-linked at different concentrations with 200 μg of F_1 in 0.1 mL of buffer, as described in the legend of Figure 1, so that the molar ratio of added OSCP to added F_1 increased up to 5. Ten micrograms of bovine serum albumin (BSA) in 10 μL was added to each sample as internal standard, and 10 μL of each sample was submitted to electrophoresis on a 12% polyacrylamide slab gel. After staining, the gels were scanned. The areas of the peaks corresponding to the cross-linked products of M_r 105 000–110 000 and 80 000 were determined. The ratios of the peak areas of the cross-linked products to the peak area of BSA were calculated and plotted vs. the molar ratio of added OSCP to F_1.

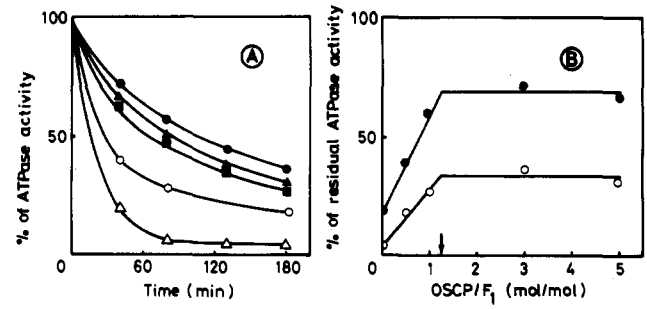

FIGURE 5: Protecting effect of OSCP against cold inactivation of F_1. (A) Effect of the period of incubation and OSCP concentration: F_1 (50 μg) was incubated with different concentrations of OSCP in 0.05 mL of 50 mM Mops, 0.5 mM EDTA, 15 mM K_2SO_4, and 0.02% Tween 20, final pH 6.5 at 0 °C; the molar ratios of OSCP to F_1 were zero (△), 0.5 (○), 1 (■), 3 (▲), and 5 (●). At various intervals of time, aliquots of this solution were withdrawn and tested for ATPase activity. ATPase activities were normalized, taking as 100% activity that of a control carried out at 20 °C. (B) Titration of the protecting effect of OSCP: This plot was obtained from the data in panel A for incubation periods of 40 (●) and 180 min (○).

with the anti-α and anti-β antibodies, corroborating the assignment of $\alpha\alpha$ and $\alpha\beta$ to these two bands (see above). The band at M_r 80 000 reacted with the anti-α antibody and that at 75 000 with the anti-β antibody. The anti-OSCP antibody reacted with the OSCP band as expected and also with the bands of M_r 75 000 and 80 000. These data taken together afford definite evidence for identification of the M_r 80 000 product with an α–OSCP dimer and of the M_r 75 000 product with a β–OSCP dimer. Besides these cross-linked products consisting of two subunits only, a higher molecular weight product of M_r ~125 000, reactive to the anti-OSCP, anti-α, and anti-β antibodies, was revealed. On the basis of its size and its immunological reactivity, the M_r 125 000 product was tentatively identified as the cross-linked trimer, α–β–OSCP. The low amount of the M_r 125 000 product compared to the M_r 75 000 and 80 000 products is expected from the decreased probability for a cross-linking reagent to cross-link more than two subunits in an oligomeric complex.

Dependence of OSCP–F_1 Cross-Linking on OSCP Concentration. The cross-linker used in the present experiment was EDAC. The amounts of cross-linked products of M_r 105 000–110 000, i.e., $\alpha\alpha$ and $\alpha\beta$, and of the M_r 80 000 product, i.e., α–OSCP, were assessed on the basis of the areas of the corresponding peaks in the densitometric pattern (Figure 4). A satisfactory correlation was found to exist between the decrease of the 105 000–110 000-dalton peak area and the increase of the 80 000-dalton peak area. For both processes, a plateau was attained at 1.3 mol of added OSCP/mol of F_1. The saturable nature of the OSCP–F_1 cross-linking above a molar ratio of OSCP to F_1 of 1 and the fact that OSCP binds to both α and β suggested that the high-affinity OSCP binding site in F_1 is shared by one α and one β subunits out of the three α and β subunits that are present in F_1.

Effect of Increasing Concentrations of OSCP on the Protection of F_1 against Cold Inactivation. Another means to investigate the biological significance of each OSCP binding site on F_1 was to use the protecting effect of OSCP against cold inactivation of F_1. Indeed, OSCP prevents inactivation of F_1 left to stand at 0 °C (Hundal & Ernster, 1979). Protection against cold inactivation of F_1 increased with the concentration of OSCP used (Figure 5A). The dependence of the protection on the OSCP/F_1 molar ratio (Figure 5B) showed an end point of titration at 1.2 mol of added OSCP/mol of F_1, similar to that found in the case of accumulation of the M_r 80 000 cross-linking product (Figure 4). From binding experiments based on equilibrium dialysis [see Dupuis et al. (1985)], a ratio of 1.2 mol of added OSCP/mol of F_1 corresponds to 1 mol of bound OSCP/mol of F_1. Hundal & Ernster (1979) found three OSCP binding sites per F_1 in a similar protection experiment. The difference with our results is probably due to the presence of Tween 20 in our medium which prevents unspecific adsorption of OSCP [see Dupuis et al. (1985)].

Effect of Mild Tryptic Digestion of F_1 on Cross-Linking between OSCP and F_1 Subunits As Assayed with EDAC. The α subunit in F_1 is particularly prone to mild digestion by trypsin resulting in a product α' of molecular weight close to that of β (Leimgruber & Senior, 1976). Partial digestion of F_1 by trypsin, presumably at the α subunit, was shown to hamper the ability of OSCP to convey to F_1 sensitivity to oligomycin bound to the F_0 sector of the ATPase complex (Hundal et al., 1983); this was taken as indicative that OSCP could have contact with α, when interacting with the F_1 sector of the ATPase complex. However, as recently reported (Hollemans et al., 1983), trypsin digestion is not strictly specific for α; β is also partially digested by trypsin with release of a pentapeptide to give a product referred as β'. In the preceding paper (Dupuis et al., 1985), we showed that such tryptic treatment suppressed the high-affinity OSCP binding site on F_1. It was therefore of interest to study the effect of mild trypsin treatment on cross-linking by EDAC. As shown in Figure 6, cross-linking between α' and β' was as efficient as cross-linking between α and β; the cross-linked products obtained from α' and β' migrated a little faster than those obtained from α and β because of their lower molecular weights. On the other hand, cross-linking between OSCP and either α' or β' was abolished, as shown by the absence of products of molecular weights in the range of 75 000–80 000. Furthermore, there was a slight protection of OSCP by F_1 and F_1 by OSCP against tryptic proteolysis (not shown), which suggested some masking of trypsin-sensitive regions both on F_1 and OSCP in the OSCP–F_1 complex. However, it is equally possible that OSCP and F_1 may act as competing substrates for trypsin digestion leading to an unspecific kinetic protection.

Discussion

OSCP Interacts with the α and β Subunits of F_1. In topographical studies, cross-linkers of about 10-Å span like

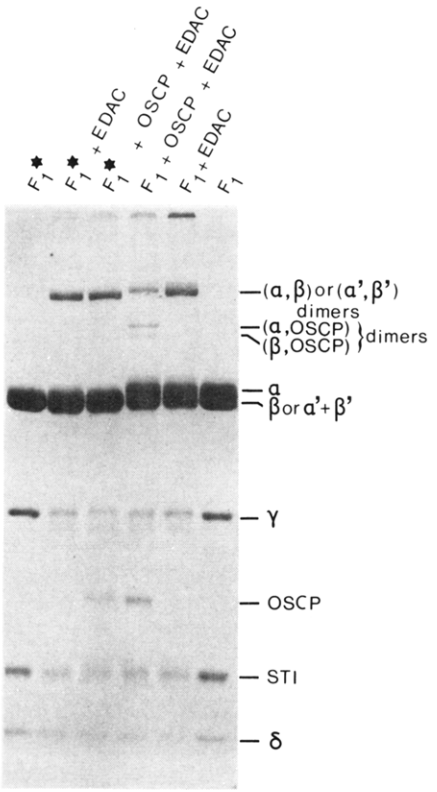

FIGURE 6: Effect of mild tryptic treatment of F_1 on cross-linking between OSCP and the α and β subunits of F_1. F_1 (800 μg of protein) was incubated with TPCK–trypsin by using a trypsin/F_1 ratio of 1/100 for 5 min at 30 °C in 0.2 mL of 50 mM Tris–sulfate and 0.25 M EDTA, pH 8.0. The reaction was stopped by addition of a 5-fold excess of soybean trypsin inhibitor over trypsin. The trypsin-treated F_1 was freed of small peptide fragments by centrifugation filtration through a 1-mL Sephadex G-50 column equilibrated with 50 mM Mops, 1 mM ATP, 0.5 mM EDTA, 15 mM K_2SO_4, and 0.02% Tween 20, pH 6.5. Trypsin inhibitor was again added at the same concentration as before, to ensure no reactivation of trypsin due to removal by gel filtration of the trypsin inhibitor firstly added. A 200-μg sample of the trypsinized F_1 was incubated with 14 μg of OSCP, and cross-linking with EDAC was conducted as detailed in the legend of Figure 1. In the control, soybean trypsin inhibitor was added prior to trypsin. Lane 1, trypsinized F_1 (F_1*); lane 2, cross-linked trypsinized F_1; lane 3, cross-linked OSCP and trypsinized F_1; lane 4, cross-linked OSCP and control F_1; lane 5, cross-linked control F_1, lane 6, control F_1. The gel was stained with Coomassie blue.

dimethyl suberimidate and dimethyl adipimidate are currently used. Such cross-linkers are less appropriate than zero-length cross-linkers to identify proximal subunits in an oligomeric protein like F_1 whose maximal dimensions are about 100 Å and where the major subunits are quite close to each other because of their hexagonal arrangement. In this work, contacts between OSCP and the α and β subunits of F_1 were revealed by the use of the zero-length cross-linkers EDAC and EEDQ. Furthermore, because of the molar ratio of added OSCP to F_1 close to 1, the cross–links observed reflected binding of OSCP to a high-affinity OSCP binding site on F_1. Immunochemical characterization and specific radiochemical labeling of the components of the cross-linked products indicated specific interactions between OSCP and both the α and β subunits of mitochondrial F_1. In this context, it is worth mentioning that in mitochondrial F_1 the α and β subunits are probably juxtaposed (Satre et al., 1976). Therefore, OSCP may bind at the $\alpha\beta$ interface to both subunits. This would explain why the accumulation of α–OSCP and β–OSCP cross-linked products is concomitant with the decrease of the $\alpha\beta$ cross-linked products. Bragg & Hou (1982) have treated a rat liver ATPase complex with a cleavable cross-linking reagent; by two-dimensional polyacrylamide gel electrophoresis, they identified two products that both contain a M_r 26 500 protein apparently similar to beef heart OSCP; one of the products contained the α subunit and the other the β subunit. This is consistent with our results.

Since 85% of the molecular mass of F_1 is contained in the α and β subunits and since OSCP is a sticky molecule, one may wonder whether the cross-linked products in which OSCP is engaged reflect specific topographical interactions. The following results speak in favor of specific interactions. (1) The zero-length cross-linkers used, EDAC and EEDQ, are expected to cross-link adjacent polypeptides. (2) Unspecific binding of OSCP to F_1 was prevented by addition of Tween 20 [see Dupuis et al. (1985)]. (3) Cross-linking of OSCP with α and β was saturable, the plateau of saturation being attained when the high-affinity binding site on F_1 was filled with OSCP (Figure 4). (4) Limited proteolysis of F_1 at the level of the α subunit prevented the accumulation of the cross-linked products between OSCP and the α or β subunits; on the other hand, the $\alpha\beta$ cross-linked products that accumulated in the absence of OSCP and disappeared in its presence were found again to accumulate after limited proteolysis of α even in the presence of OSCP (Figure 6). (5) Finally, cross-linking was carried out at a concentration of OSCP just sufficient to saturate the high-affinity binding site of F_1 measured under reversible conditions. Less than 10% of OSCP was left free under these conditions (Dupuis et al., 1985), which makes an unspecific binding by random collision very unlikely.

Trypsin treatment of F_1 may affect binding of OSCP to α and β in two ways. The clipped α and β subunits may no longer bind OSCP if the removed portions of these subunits contain specific binding sites for OSCP. Another possibility is that the clipped α and β subunits have a different conformation compared to the native ones, the new conformations being unable to recognize OSCP. As the catalytic site of F_1 is most probably located in the β subunit, it is tempting to imagine that direct interaction of OSCP with β is central for the function of OSCP. On the other hand, there are indications that, although α is not directly involved in catalysis, it controls the efficiency of catalysis at the level of β [for review, see Vignais & Satre (1984)]. Thus, OSCP by interacting with α could indirectly convey to β the signal of the binding of oligomycin to F_0 in an F_1–F_0 complex. We must recall here that OSCP is not the only link between F_1 and F_0 and that direct contacts between F_1 and F_0 probably exist, as discussed in the preceding paper (Dupuis et al., 1985).

Does OSCP Conform to the Partial Site Reactivity Typical of F_1? The binding data obtained by equilibrium dialysis (Dupuis et al., 1985) showed that F_1 possesses one high-affinity OSCP binding site (K_d = 0.08 μM) and two low-affinity OSCP binding sites (K_d = 6–8 μM). The high-affinity binding site appeared to be the site essentially involved in the functioning of OSCP for the following reasons: (1) The dissociation constant of 6–8 μM corresponds to a low affinity for a protein–protein interaction and is not compatible with the function of OSCP. (2) In titration of the OSCP effect on F_1 sensitivity to oligomycin or on [^{32}P] ATP–P_i exchange activity, the apparent dissociation constant was in the nanomolar range, which indicated high-affinity binding. (3) When F_1 was submitted to mild tryptic treatment, the restitution by OSCP of oligomycin sensitivity to F_1 was lost, and concomitantly the high-affinity OSCP binding site disappeared. (4) Titration of the effect of OSCP on the F_1–OSCP cross-linking pattern and titration of protection by OSCP against F_1 cold inactivation showed an end point close to 1 mol of OSCP/mol of

F_1. Since OSCP binds to both α and β and that there are three α and three β subunits alternately arranged in F_1 (Senior & Wise, 1983), it is likely that the high-affinity binding site for OSCP is shared by two juxtaposed α and β subunits in F_1 and that binding of one OSCP to this high-affinity site confers full efficiency to OSCP activity. This typical $^1/_3$ of the site reactivity may be explained by cooperative interaction between α and β subunits in F_1, the binding of one OSCP to $\alpha\beta$ couple leading to a decrease in affinity of the other two $\alpha\beta$ couples for OSCP. On the other hand, a difference in site affinity in F_1 might exist prior to addition of OSCP; this preexisting difference in site affinity may come from an asymmetric arrangement of subunits in F_1, as recently suggested by X-ray diffraction data (Amzel et al., 1982) and scanning electron micrographs (Vignais et al., 1984).

It is noteworthy that the $^1/_3$ of the site reactivity of F_1 with respect to OSCP is consistent with the alternative site mechanism postulated for F_1 (Boyer et al., 1977). Although the possibility remained that, due to the size of the OSCP molecule, the three α and β subunits in F_1 are covered by one OSCP molecule bound to this high-affinity site, one cannot but be impressed by the many other examples of $^1/_3$ of the site reactivity of F_1; they concern reversible ligands like aurovertin and ATPase inhibitor and irreversible ligands like photoactivable derivatives of nucleotides and P_i and chemical modifiers which all react with the β subunit in such a way that binding of 1 mol of ligand to one β subunit fully inhibits the enzymic activity of F_1 [for review, see Vignais et al. (1984)].

Registry No. ATPase, 9000-83-3.

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