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Stoichiometry of the oligomycin-sensitivity-conferring protein (OSCP) in the mitochondrial F_0F_1-ATPase determined by an immunoelectrotransfer blot technique

François Penin, Philippe Archinard, Mahnaz Moradi-Améli and Catherine Godinot *

Laboratoire de Biologie et Technologie des Membranes du Centre National de la Recherche Scientifique, Université Claude Bernard de Lyon, 43 Bd. du 11 Novembre 1918, 69622 Villeurbanne Cedex (France)

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The ratio between the amount of oligomycin-sensitivity-conferring protein (OSCP) and the amount of the α and β subunits of F_1-ATPase in the mitochondria has been determined by a method combining electrophoresis, electrotransfer and immunotitration with monoclonal antibodies. The peptides separated in SDS-polyacrylamide gel electrophoresis were blotted to nitrocellulose sheets by electrotransfer. The nitrocellulose sheets were incubated with ^{125}I-labelled purified monoclonal antibodies specific to various peptides. The ^{125}I-labelled immune complexes were located by immunodecoration using peroxidase-conjugated second antibodies and the blotted peptides were revealed with H_2O_2 and α-naphthol. The amount of immune complex present on the nitrocellulose was determined by counting the radioactivity present on the spots. The amount of peptide blotted is directly proportional to the amount of protein loaded on the electrophoresis. By comparing standard curves made with the isolated proteins to the values obtained in the presence of various amounts of the membrane-protein complex, one can calculate the content of this peptide in the membrane. It was found that the mitochondrial membrane contains 2 mol of OSCP per mol of F_1.

Introduction

The F_0F_1-ATPase complex is composed of a hydrophilic part F_1 bearing the nucleotide and phosphate sites and a membrane part F_0, involved in proton translocation. F_1 contains five different subunits organized according to a stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [1]. OSCP [2] is a small component (M_r = 20 967) [3] required for the interaction of mitochondrial F_1, with F_0 [4]. The presence of OSCP is necessary to render the ATPase activity sensitive to oligomycin in reconstituted F_0-F_1 complex [2,4].

The stoichiometry of OSCP in the F_0F_1-ATPase complex is still controversial. Hundal and Ernster [5] found that a ratio of 2–3 mol OSCP per mol F_1 was needed for maximal cold stabilization and for conferral of full oligomycin sensitivity in reconstitution experiments of particles depleted of F_1 and OSCP with purified F_1 and OSCP. They have also shown that OSCP protects all three α subunits of isolated F_1 against trypsin proteolysis [6]. These experiments suggested to Hundal and her co-workers that F_1 contains three binding sites for OSCP. Dupuis et al. [7], by assaying the amount of

* To whom correspondence should be addressed.

Abbreviations: OSCP, oligomycin-sensitivity-conferring protein; F_1-ATPase, pig heart mitochondrial ATPase prepared according to the procedure of Penin et al. [10].

OSCP necessary to improve maximally the ATP-P_i exchange activity, have measured that each mole of ATPase active site was able to bind 1.1 ± 0.5 mol of OSCP [7]. By equilibrium dialysis, they have also shown that isolated F_1 possesses a high-affinity OSCP binding site ($K_d = 0.08$ μM) and two low-affinity ones ($K_d = 6$–8 μM). They suggested that "the two latter sites are potential sites, not involved in the formation of the F_0-OSCP-F_1 complex" [8].

All the methods used above to estimate the amount of OSCP present in the mitochondrial membrane involving reconstitutions were indirect and could only suggest the possible stoichiometry of OSCP in the complex. We have set up a direct immunological method to titrate OSCP in mitochondria and found a stoichiometry of 2 mol OSCP per mol F_1. The method described consists in comparing the amount of OSCP to that of the α and β subunits present in the mitochondria.

Materials and Methods

Materials

Fatty-acid-free serum albumin prepared from fraction V was obtained from Sigma. Acrylamide was purchased from Serva, bisacrylamide from Eastman Kodak, Coomassie blue R from Sigma. Electrophoresis were run in a Bio-Rad Protean double slab cell equipped with $160 \times 120 \times 1.5$ mm plates. Anti-mouse IgG antibodies conjugated with peroxidase were from Biosys, Compiègne, France. The 0.2 μm pore nitrocellulose sheets came from Schleicher and Schüll. Carrier-free ^{125}I (NaI) was obtained from the Commissariat à l'Energie Atomique (France). All other reagents were of the highest purity commercially available.

Methods

Previously described procedures were used to prepare pig heart mitochondria [9], F_1-ATPase after purification on Ultrogel ACA 34 [10], OSCP [11], β subunit of F_1 [12] and monoclonal antibodies against the α or β subunit of F_1 [13] and against OSCP [14]. The monoclonal antibodies were purified on protein A Sepharose as described by Ey et al. [15].

The iodination of OSCP and of the purified monoclonal antibodies was conducted as for F_1 in Ref. 13, according to the procedure of Fracker and Speck [16]. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [17], as described in detail in Ref. 18. After electrophoresis, the separated peptides were electrotransferred from the gels onto nitrocellulose sheets by the method of Towbin et al. [19]. After transfer, the gel was stained with Coomassie blue and destained as described in Ref. 20. The nitrocellulose sheets were incubated overnight at 4°C, in Tween-saline buffer (150 mM NaCl/10 mM sodium phosphate (pH 7.5)/0.05% Tween 20 (w/v)). The sheets were gently shaken for 4 h at room temperature in the presence of the purified monoclonal antibodies (5–10 μg of protein diluted in 1 ml of saline buffer containing 0.5% bovine serum albumin). The sheets were extensively washed 4 times for 10 min in Tween-saline buffer and then incubated for 1 h with anti-mouse IgG antibodies conjugated with peroxidase (diluted 1 : 500 in Tween-saline buffer). After washing in Tween-saline buffer (4 times for 10 min), the sheets were quickly rinsed with distilled water. The immune complexes were revealed as follows: the sheets were incubated with a solution of α-naphthol and H_2O_2 prepared extemporaneously (60 mg α-naphthol were dissolved in 20 ml methanol and adjusted to 100 ml with distilled water; then 200 μl of H_2O_2 (30%) were added (Monier, J.C., unpublished results). When the brownish purple spots corresponding to the immune complexes were clearly visible, the sheets were rinsed with distilled water and were dried with filter paper. For the quantitative estimation of the immune complexes, iodinated monoclonal antibodies were used and the radioactivity present in each spot was counted (see details in Fig. 3).

The protein contents of mitochondria and of F_1 were estimated by the technique of Lowry et al. [21], modified as in Ref. 22. The concentration of bovine serum albumin used as standard was checked spectrophotometrically either using an extinction coefficient E 1% of 6.6 at 280 nm [23] or taking into account the number of tryptophanes and tyrosines present in the serum albumin (M_r = 66 500; Trp = 2 and Tyr = 17 [24]) and measuring the optical density of serum albumin at 294.4 nm in 0.1 M NaOH. In the latter case, the concentration of serum albumin was calculated using an ϵ_m for tyrosine and tryptophane of 2390 at 294.4 nm

in 0.1 M NaOH [25]. Both techniques gave the same bovine serum albumin concentration. The concentrations of purified β subunit (12 tyrosines [26]) and of OSCP (5 tyrosines [3]) were also estimated by the latter technique. The amino acid analysis of OSCP and β subunit purified from porcine heart mitochondria are consistent with the amount of tyrosine found in bovine heart OSCP and β subunit (Julliard, J.H., unpublished results). The protein concentrations of the β subunit and of OSCP determined by the latter technique and by the technique of Lowry et al., using bovine serum albumin as a standard were compared. The Lowry technique overestimates the protein contents of the β subunit by 9% and of OSCP by 8.5%.

Results

Immunodecoration of the α and β subunits of F_1 and of OSCP

Fig. 1 shows an electrophoresis and an electrotransfer followed by an immunodecoration of the α and β subunits of F_1 and of OSCP with anti-α, anti-β and anti-OSCP monoclonal antibodies. This figure illustrates the basis of all further experiments. Known amounts of dissociated F1 and OSCP were loaded alone or together with dissociated mitochondrial proteins on SDS-polyacrylamide gels (Fig. 1A). The proteins were then transferred to nitrocellulose and immunodecorated by incubation first with anti-α, anti-β and anti-OSCP monoclonal antibodies and then with a peroxidase-conjugated anti-mouse IgG antibody followed by detection with the peroxidase substrates, H_2O_2 and α-naphthol (Fig. 1B). When purified F_1 or OSCP were present alone or in the presence of mitochondria, only one spot was detected by immunodecoration, corresponding to each peptide, as shown previously [13,14]. The intensity of the spots increases with the amount of protein. This increase is directly proportional to the amount of protein loaded on the gel and can be used to titrate the amount of OSCP in the membrane, as shown below.

Proportionality between the amount of proteins loaded on the gel and the amount of proteins blotted after electrotransfer

Fig. 2 shows that, within the studied range, the

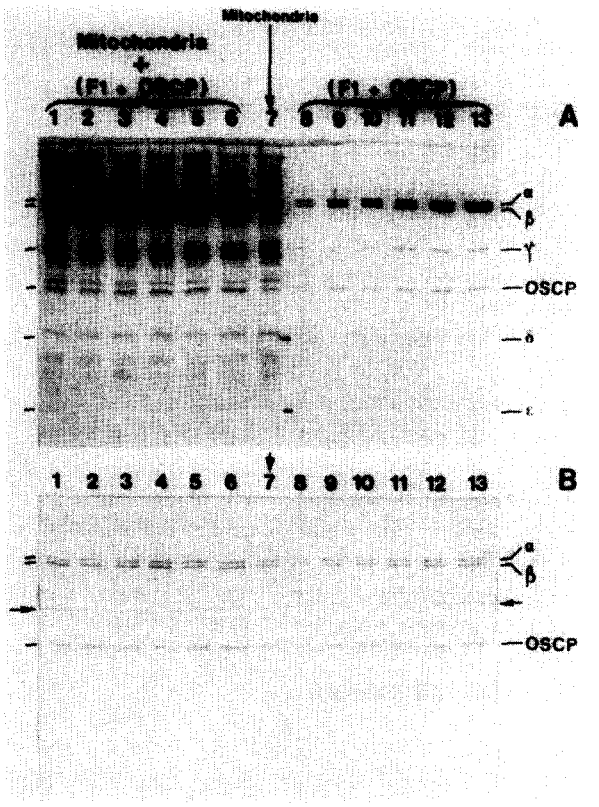

Fig. 1. Immunodecoration of subunits of F_1 (α, β) and of OSCP with monoclonal antibodies. (A) Electrophoresis – The samples were prepared by mixing 50 μl 0.125 M Tris-HCl (pH 8.8) containing either 120 μg of mitochondria or 29 μg of F_1 + 9 μg of OSCP with SDS (20 μl of a 10% solution), β-mercaptoethanol (5 μl) and 60 mg urea (final volume, 120 μl). Lanes 1–7: 10 μg mitochondria; lanes 1–6: increasing amounts of F_1 + OSCP: 2–7 μl of the above sample solution; lanes 8–13: increasing amounts of F_1 + OSCP: 2–10 μl of the above sample solution. After electrophoresis [18] and electrotransfer [19], the gel was stained and destained [20] as described. (B) Immunodecoration – After electrotransfer, the nitrocellulose sheets were incubated overnight with the saline buffer containing 0.05% Tween 20. The sheets were cut out into two parts, the upper part containing the α and β subunits and the lower part containing OSCP. (The location of the bands was determined by comparison with the bands stained on the acrylamide gel). The upper sheet was treated as described in Material and Methods with the monoclonal antibodies 7B3 and 14D5 (culture supernatants) reacting with the α and β subunits, respectively [34]. The lower sheet was treated with the anti-OSCP monoclonal antibody 2B1-B1 [14]. The immune complexes were revealed with peroxidase-conjugated second antibody (see Materials and Methods).

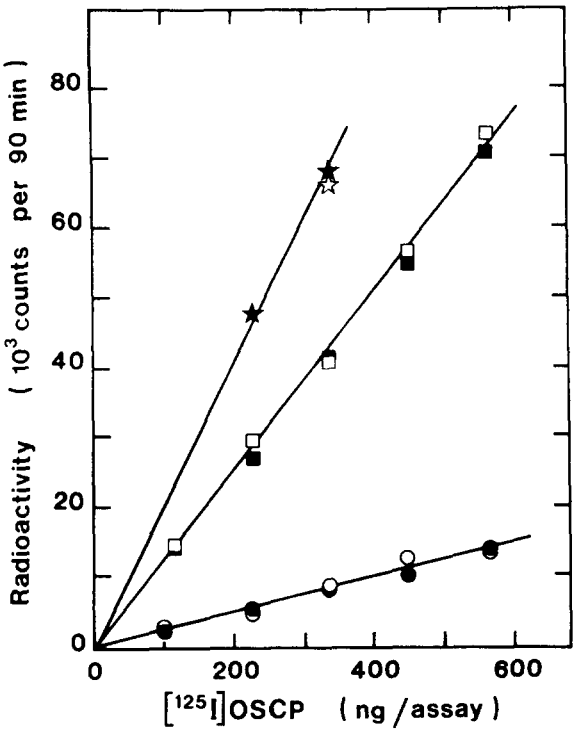

Fig. 2. Proportionality between the amount of protein loaded on the electrophoresis and the amount of protein recovered on the nitrocellulose sheet after electrotransfer. Electrophoresis was performed as in Fig. 1 except that ^{125}I-OSCP (1 μCi/mg) was used. After running the electrophoresis, two lanes containing 0.22 and 0.34 μg of ^{125}I-OSCP respectively and one lane containing 0.34 μg of ^{125}I-OSCP and 10 μg of mitochondria were directly cut out, stained and destained [20]. The remaining part of the slab was submitted to an electric field (190 mA for 100 min) in order to transfer the proteins from the acrylamide gel to a nitrocellulose sheet, as described by Towbin et al. [19]. After transfer, the acrylamide gel was stained and destained with Coomassie blue [20] and the OSCP was located on the nitrocellulose sheet using the anti-OSCP antibody, as in Fig. 1. The bands corresponding to OSCP in the acrylamide gel and in the nitrocellulose sheet were cut out and counted in a γ-counter (Packard) for 90 min. The counts were plotted as a function of the amount of ^{125}I-OSCP loaded in each well. Nitrocellulose sheet (○,●); acrylamide gel after electrotransfer (□, ■); acrylamide gel without electrotransfer (☆, ★); ^{125}I-OSCP alone (closed symbols); ^{125}I-OSCP in the presence of mitochondria (open symbols).

amount of ^{125}I-OSCP blotted onto nitrocellulose by electrotransfer from acrylamide gels is directly proportional to the amount of protein loaded on the gel before running the electrophoresis. The presence of mitochondria together with the ^{125}I-OSCP does not modify the amount of ^{125}I-OSCP blotted onto the nitrocellulose sheet (lower curve). A comparison between the upper curve (amount of ^{125}I-OSCP in the gel which has not been submitted to electrotransfer) and the middle curve (amount of ^{125}I-OSCP remaining in the gel after electrotransfer) indicates that about 62% ^{125}I-OSCP has not been electroeluted, under the conditions used. Although only about 12% of the ^{125}I-OSCP loaded on the gel is counted on the nitrocellulose (lower curve), this percentage remains constant whatever the amount of OSCP loaded, in the presence or absence of mitochondria. Therefore, it is possible to titrate OSCP or any other peptide in mitochondria whenever this peptide available in a purified form, can be used to make a standard curve.

Immunotitration of β in F_1

A few wells of a SDS gel were loaded with increasing amounts of purified β subunit (standard curve). Other wells were loaded with variable amounts of purified F_1. The concentration of proteins loaded in electrophoresis must be known with good precision for the standard curve and for the protein mixture under study. To avoid an underestimation of proteins by adherence of these proteins to the walls of the tubes in which they are stored [8], aliquots of standard solutions of each protein were simultaneously withdrawn for protein concentration determination in the presence of deoxycholate [22] and for electrophoresis.

After electrotransfer, the nitrocelulose sheet was incubated with the ^{125}I-labelled anti-β. The immune complexes were revealed using a peroxidase labelled second antibody and the spots corresponding to each complex was cut out and counted. Fig. 3A shows an example of standard curve. The increase in ^{125}I-labeled antibody found in each spot increases as a function of the amount of purified β-subunit loaded in each electrophoresis well. This curve does not intersect the X-axis at the origin. This shift of the curve can be related to the affinity of the antibodies. Indeed, the antibodies having the highest affinity exhibited the smallest shift (experiments not shown). Besides, the shift decreased when the time of incubation of the antibodies with the nitrocellulose sheets was increased, and it increased when the number and duration of washings was increased. For high

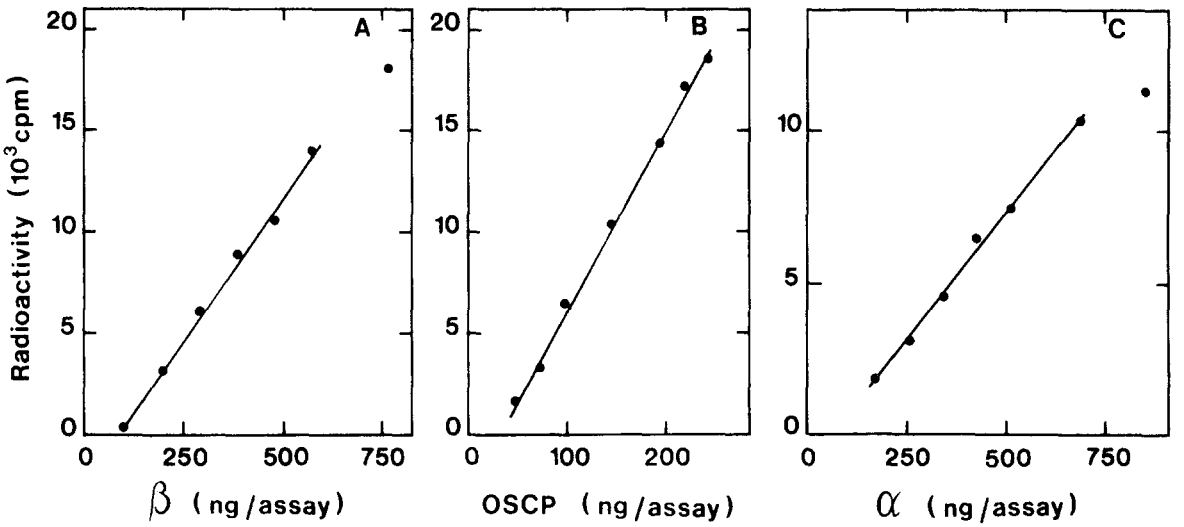

Fig. 3. Immunotitration of α subunit, of β subunit and of OSCP in mitochondria, using purified β subunit, purified OSCP or α present in purified F_1 as standards. An electrophoresis was conducted as in Fig. 1, running a standard curve either with purified β subunit + purified OSCP or with purified F_1 + purified OSCP and several samples containing variable amounts of either F_1 or mitochondria on the same slab. The samples for electrophoresis were prepared as in Fig. 1. The samples loaded on the wells for making the standard curves contained the indicated amount of purified β subunit (A), of purified OSCP (B), or of α present in purified F_1 (taking into account that F_1 contains 43.4% of α subunit) (C). On the same slab, other wells were loaded either with purified F_1 (0.5–1.5 μg protein) or mitochondria (5–13 μg protein). After electrophoresis and electrotransfer as in Fig. 1, the nitrocellulose sheet was incubated overnight with the saline buffer containing 0.05% Tween 20. The sheets were cut out into two parts as in Fig. 1. The upper part containing the α subunit and the β subunit was incubated with either the anti-α (7B3) or the anti-β (14D5) monoclonal antibodies. The lower part containing OSCP was incubated with anti-OSCP (2B1B1) monoclonal antibody. All antibodies had been purified and labeled with ^{125}I (see Methods). The immune complexes were located with peroxidase conjugated second antibody. Each spot (2 mm × 10 mm) was cut out, solubilized in 400 μl of dimethylsulfoxide and counted in a γ-counter (Packard). Blanks were made by cutting out and counting similar pieces of nitrocellulose taken randomly on the same sheet. After subtraction of the average blank, the radioactivity of each spot (bound antibodies) was plotted as a function of the amount of β subunit (Fig. 3A), OSCP (Fig. 3B) or α present in purified F_1 (Fig. 3C) loaded in each well.

amounts of proteins, a flattening of the standard curve can be observed (not shown). This is probably explained by steric hindrance. Therefore, for better accuracy, only the linear part of the curves was used in the titration of the β subunit of F_1.

F_1 contains 440 ± 40 μg of β subunit per mg of F_1 (not shown on Fig. 3A). Taking respectively 51 300 [26] and 380 000 [1] as M_r of the β subunit and of F_1, one could calculate a stoichiometry of 3.26 ± 0.3 β subunit per mole of F_1. This value approximately coincides with the known stoichiometry of purified F_1: $\alpha_3\beta_3\gamma\delta\epsilon$ [1,13] and demonstrates the validity of the technique within a precision of about 10%.

Stoichiometry of OSCP as compared to F_1 in mitochondria

Similar experiments have been conducted to estimate the amount of OSCP, α and β subunits present in mitochondria. Standard curves for OSCP were made with purified OSCP as in the case of β subunit. Standard curves for the α subunit were made with purified F_1. The contents of α in F_1 was calculated by assuming a stoichiometry of 3α per mol of F_1 [1,13] and M_r of 55 100 [27] and 380 000 [1] for α and F_1, respectively.

After electrophoresis and electrotransfer the α, the β and the OSCP were approximately located on the nitrocellulose sheet by comparison with the stained acrylamide gel in order to cut the nitrocellulose in two parts, the upper part containing the α and β subunits and the lower part containing OSCP. The upper part was incubated with the ^{125}I-labeled anti-α or anti-β antibody and the lower part with the anti-OSCP antibody. The immune complexes were revealed using a peroxidase con-

TABLE I

RELATIVE AMOUNTS OF F_1 AND OSCP IN MITOCHONDRIA

	μg [a]	subunit nmoles [b]	F_1 nmoles [c]	mol OSCP / mol F_1
α	53.5 ± 6.3(12)	0.97	0.32	1.81
β	48 ± 5.2(15)	0.94	0.31	1.87
OSCP	12.1 ± 2.3(26)	0.58		

[a] The results are expressed as μg of protein per mg of mitochondrial protein ± S.D. The number of determinations is given in parenthesis.

[b] The nmoles of α, β and OSCP were calculated assuming an M_r of 55100, 51300 and 21000, respectively for α [27], β [26] and OSCP [3].

[c] The nmoles of F_1 were calculated from that found for α or β assuming a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ [1,13].

jugated second antibody and the spots corresponding to each complex were cut out and counted. Fig. 3 shows the increase in radioactivity found in each spot as a function of the amount of purified β subunit (Fig. 3A), purified OSCP (Fig. 3B) or α present in purified F_1 (Fig. 3C) loaded in each electrophoresis well. The shape of the standard curves observed for OSCP and for α are similar to that described for β (see above). Standard curves for OSCP, α and β subunits, were also made in the presence of a constant amount of mitochondria. The same increase in radioactivity due to the addition of OSCP, α or β subunit was observed whether the mitochondria were present or not (results not shown).

In each experiment, the amount of OSCP was titrated and compared either to the α subunit or to the β subunit. Table I summarizes the results obtained in various experiments. 1 mg of mitochondrial protein contains 0.97 nmol of α subunit and 0.94 nmol of β-subunit which corresponds to 0.31–0.32 nmol of F_1 or 120 μg of F_1 per mg mitochondrial protein. 1 mg mitochondrial protein contains 0.58 nmol of OSCP. The ratio between the amount of OSCP to that of F_1 equals 1.81–1.87. The stoichiometry of OSCP as compared to F_1 is of the same order of magnitude when using the anti-α or anti-β antibodies. Therefore one can conclude that mitochondria contain about 2 mol OSCP per mol F_1.

Discussion

In this study, an immunoelectrotransfer blot technique has been used to quantify the amount of α subunit, of β subunit and of OSCP in mitochondria after electrophoretic resolution of the mitochondrial proteins. As shown in other reports (see the review of Towbin and Gordon [28]), the assay is quantitative if the amount of protein transferred is proportional to the amount of protein loaded on the gel, if the amount of antibody bound to the nitrocellulose is proportional to the amount of protein present and if the presence of other components does not modify the assay results. The standard curves made in the presence or absence of mitochondria demonstrate that the last two conditions are fulfilled. The proportionality between the amount of peptide found on nitrocellulose after electrotransfer and that loaded on the electrophoresis gel is demonstrated in Fig. 2 even though only 12% of the loaded ^{125}I-OSCP is recovered on nitrocellulose. A comparison between the amount of ^{125}I-OSCP present in the acrylamide gel before or after electrotransfer shows that 40% of ^{125}I-OSCP were eluted during electrotransfer. The fact that only about 1/3 of this eluted ^{125}I-OSCP was present on the nitrocellulose can be explained by a migration of OSCP in the electrotransfer or washing medium.

Several authors have reported that the amount of protein transferred depends on the size and charge of the protein [28,29]. When using standard curves made with purified peptides, the exact amount of protein transferred does not have to be taken into account in the final calculation. The technique can be applied to various peptides as long as a reference curve can be made with each peptide. In this way, problems due to differences in transfer between the peptides of various size and charge can be overcome. Under such conditions, it is possible to determine with good precision the relative amount of various peptides in a complex protein mixture. The validity of this technique has been assessed by controlling that purified F_1 contains 3 β subunits, as expected [1,13].

The use of monoclonal antibodies is much better suited to this type of experiment than the use of polyclonal antibodies, particularly when mem-

brane proteins are concerned. It is always difficult to ascertain if a band visible in SDS-polyacrylamide gel corresponds to a single peptide and therefore the monospecificity of polyclonal antibodies is always more questionable than that of monoclonal ones.

This technique permits to work with intact mitochondria, thus avoiding harsh treatments such as sonication susceptible to deplete partially the mitochondria from some peptides.

The data presented in this study have permitted the direct titration of the amount of F_1 present in the mitochondria. The value of 0.31–0.32 nmol of F_1 per mg of mitochondrial protein estimated here is in good agreement with that found indirectly in rat heart mitochondria (0.27) by Bertina et al. [30] who have titrated the number of tight binding sites of aurovertin on F_1.

The most important result found here is that mitochondria contain about 2 OSCP per mole of F_1. This estimation is based upon the M_r and protein concentration of each peptide. The M_r of β (51.3 kDa) and that of OSCP (20.9 kDa) are well established from their amino-acid sequence [26,3]. Moreover, their respective contents in tyrosine being known, the protein concentration of the solutions used to make the standard curves can be determined with good precision. Although the protein concentration of F_1 is known with less accuracy than that of the β subunit, the results obtained with the α subunit present in F_1 is not very different from the value of about 2 mol OSCP per mol F_1.

Walker et al. [27] have shown that there exists a striking homology between the amino-acid-sequence of the δ subunit of *Escherichia coli* F_1 and OSCP. Besides, Ovchinnikov et al. [31] have observed a homology between the sequence of OSCP and the central part of the b subunit of *E. coli* [32], although this homology is poor. In addition, Foster and Fillingame have shown that the *E. coli* membrane contains 1 mol of δ subunit and 2 mol of b subunit per mole of F_1 [33]. Our demonstration of a stoichiometry of 2 mol OSCP per mol F_1 would rather suggest that the b subunit of *E. coli* might have a role similar to OSCP in the structure and function of the mitochondrial F_0-F_1 complex. However, since some homology also exists between OSCP and the adenine nucleotide translocator [31] or the γ subunit of bovine F_1 [27], the significance of these homologies must be taken with caution.

Acknowledgments

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