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Efficient reconstitution of mitochondrial energy-transfer reactions from depleted membranes and F_1-ATPase as a function of the amount of bound oligomycin sensitivity-conferring protein (OSCP)

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(1) Pig heart mitochondrial membranes depleted of F_1 and OSCP by various treatments were analyzed for their content in α and β subunits of F_1 and in OSCP using monoclonal antibodies. Membrane treatments and conditions of rebinding of F_1 and OSCP were optimized to reconstitute efficient NADH- and ATP-dependent proton fluxes, ATP synthesis and oligomycin-sensitive ATPase activity. (2) F_1 and OSCP can be rebound independently to depleted membranes but to avoid unspecific binding of F_1 to depleted membranes (ASUA) which is not efficient for ATP synthesis, F_1 must be rebound before the addition of OSCP. (3) The rebinding of OSCP to depleted membranes reconstituted with F_1 inhibits the ATPase activity of rebound F_1, while it restores the ATP-driven proton flux measured by the quenching of ACMA fluorescence. The rebinding of OSCP also renders the ATPase activity of bound F_1 sensitive to uncouplers. (4) The rebinding of OSCP alone or F_1 alone, does not modify the NADH-dependent proton flux, while the rebinding of both F_1 and OSCP controls this flux, inducing an inhibition of the rate of NADH oxidation. Similarly, oligomycin, which seals the F_0 channel even in the absence of F_1 and OSCP, inhibits the rate of NADH oxidation. (5) OSCP is required to adjust the fitting of F_1 to F_0 for a correct channelling of protons efficient for ATP synthesis. (6) All reconstituted energy-transfer reactions reach their optimal value for the same amount of OSCP. This amount is consistent with a stoichiometry of two OSCP per F_1 in the F_0-F_1 complex.

Introduction

ATP synthesis in oxidative phosphorylation is catalyzed by the F_0-F_1 complex (see for recent

Abbreviations: ETP, inverted submitochondrial particles; F_1, nucleotide-depleted pig-heart mitochondrial ATPase; OSCP, oligomycin sensitivity-conferring protein; ASUA particles, ASU particles treated with NH_3 to remove OSCP; ASU particles, submitochondrial particles treated with ammonia, Sephadex and urea to remove F_1; ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); ACMA, 9-amino-6-chloro-2-methoxyacridine; IODOGEN, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; NEM, *N*-ethylmaleimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; P_i, inorganic phosphate.

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reviews Refs. 1–6). In mitochondrial membranes, this complex is made of two main parts F_0 and F_1. F_0 is responsible for the channeling of protons across the membrane. F_1 can be solubilized. It is made of five subunits arranged according to a stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ and contains nucleotide and phosphate binding sites. The coupling mechanism between proton flux through F_0 and ATP synthesis at the level of F_1 is not well understood. The oligomycin sensitivity-conferring protein (OSCP) has been described as a connecting link between F_0 and F_1 [7,8]. The structure of OSCP (M_r, 21 000) is now well known [9,10]. The stoichiometry of OSCP in the F_0-F_1 complex was controversial between 1 and 3 OSCP with respect to F_1 [11–14]. Recently, by precise immunotitration with monoclonal antibodies we determined a ratio of two OSCP per F_1 in intact mitochondria [15]. Although the presence of OSCP is mandatory to observe the oligomycin sensitivity of membrane-bound F_1-ATPase, the originally proposed role of OSCP as a connecting link between F_0 and F_1 has been discussed because F_1 can bind to F_0 in the absence of OSCP when cations are present in the reconstitution medium [12,16–18]. OSCP can form a complex with soluble F_1 [11–13,19] and interacts with the α and β subunits of F_1, whether F_1 is soluble or integrated in the membrane [13,20–21]. Recent studies permit the localization of OSCP in an external position as compared to F_1 and to the membrane [21].

As stressed by Hatefi [6], nobody knows as yet in what manner OSCP participates in proton translocation from F_0 to F_1 to drive ATP synthesis. Our aim was to understand, by using reconstituted systems, whether the two OSCP present in the mitochondrial membrane play a role in the proton flux related to ATP synthesis and to other energy transfer reactions.

Studies concerning the role of OSCP have been mainly performed by measuring the restoration of oligomycin sensitivity of F_1-ATPase activity. In this paper, the function of OSCP has been simultaneously investigated on ATP synthesis, ATP hydrolysis and its sensitivity to oligomycin, NADH oxidation and proton fluxes linked to these activities. These parallel reconstitutions required to work out optimized conditions of rebinding of F_1 and of OSCP to OSCP- and F_1-depleted membranes (ASU or ASUA particles). These membranes were well characterized for their endogenous activities and for their contents in α and β subunits of F_1 and in OSCP as determined by immunotitration with monoclonal antibodies [15].

Material and Methods

Materials

Lactate dehydrogenase, pyruvate kinase, NADH, ATP, ADP and oligomycin were obtained from Boehringer, FCCP and hexokinase (F300) from Sigma. [^{32}P]P_i, [^{125}I]NaI and [^{14}C]NEM (30 mCi/mmol) were purchased from the Commissariat à l'Energie Atomique, France. Ultra pure urea and IODOGEN were respectively obtained from Merck and Pierce. ACMA was a generous gift of Prof. Kraayenhof. All other reagents were of the highest purity available.

Biological preparations

Previously described procedures were used to prepare: pig heart mitochondria [22]; phosphorylating electron-transport particles (ETP) (method of Penin et al. [23] modified from that of Low and Vallin, [24]); nucleotide-depleted F_1-ATPase (F_1) [25]. The purification of OSCP described by Archinard et al. [26] was adapted to avoid the final ammonium sulfate precipitation, since ammonium ions interfere with the measurement of proton translocation in reconstitution experiments. After the ion-exchange chromatography, OSCP was dialyzed for 24 h with two changes of buffer (2.5 mM Hepes-NaOH, pH 7.5), lyophilized, dissolved in a minimal volume of distilled water (with 1/20 of OSCP volume before dialysis) and centrifuged for 10 min at 15 000 × g to eliminate any insoluble material. The solution containing about 3 mg OSCP per ml was kept in liquid nitrogen. The OSCP prepared under these conditions has properties identical to those of OSCP prepared under previous conditions [26].

'A particles' [27,28] prepared from pig heart mitoplasts [29] were treated with Sephadex and 2 M urea [30] to obtain the ASU particles depleted in F_1. Removal of OSCP from the ASU particles was performed essentially as described by Ernster et al. [31] by repeated treatments with 0.4 M NH_3. Sonicated ASUA were prepared according to Van

de Stadt et al. [11]. ASU and ASUA particles were homogenized in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5) at about 1 mg protein per ml and centrifuged at 0°C for 30 min at 250000 × g. This washing was repeated twice to remove urea and ammonia. The final pellet was homogenized in the same buffer at 10 mg protein per ml and aliquots of 100 μl were kept in liquid nitrogen.

Protein contents were estimated by the method of Lowry et al. [32] as modified by Bensadoun and Weinstein [33]. SDS-polyacrylamide gel electrophoresis was made according to Laemmli [34], as detailed previously [35]. Electrotransfer [36] and quantitative immunotitration of the contents in α, β and OSCP in the various membrane preparations were performed as described [15] using iodinated purified monoclonal antibodies raised against α, β and OSCP [37,38].

Radiolabeling of OSCP and F_1

Radiolabeling of OSCP with [^{14}C]NEM was performed as described by Dupuis et al. [19], except that the labeled OSCP was finally dialyzed against 50 mM Hepes-NaOH (pH 7.5). The specific radioactivity of [^{14}C]OSCP was 1.24 μCi/mg. The radioactivity was measured by scintillation counting after mixing 100 μl of solution with 10 μl of Soluene 100 (Packard), incubating the vials at room temperature for 1 h and adding 5 ml of Ready-Solv HP/b (Beckman). [^{125}I]F_1 was prepared by iodination in the presence of IODOGEN [39] by a technique adapted to avoid a direct contact of F_1 with IODOGEN which induces a 30–50% loss of the ATPase activity of F_1: a series of glass test tubes coated with 20 μg IODOGEN were incubated with 100 μl 50 μM [^{125}I]NaI (1 mCi/μmol) for 2 min. Then, 90 μl of this solution were added to other tubes containing 730 μg of F_1 in 140 μl of buffer containing 70 mM Tris-H_2SO_4 (pH 8.0)/3.5 mM EDTA/35% glycerol. After 20 min of reaction, NaI was added at a concentration of 1.7 mM. The tubes containing F_1 were pooled and F_1 was filtered through a Sephadex G 50 fine column (1 × 60 cm) equilibrated in 100 mM Tris-H_2SO_4 (pH 8.0)/5 mM EDTA/50% glycerol at a flow rate of 5 ml per h to eliminate any unreacted [^{125}I]NaI. After concentration in an Amicon ultrafiltration cell equipped with a PM 10 membrane, [^{125}I]F_1 was kept at 4 mg protein per ml in liquid nitrogen, as 50 μl samples until used. Verification was made to ensure that the [^{125}I]F_1 preparation contained less than 1% free [^{125}I] by ascending paper chromatography developed in 10% trichloroacetic acid [40]. Under these conditions, 0.7 mol [^{125}I] were bound per mol of F_1 without significant loss of ATPase activity (less than 3%) and there was no detectable effect on the rate of ATP synthesis after reconstitution. The radioactivity of [^{125}I]F_1 was measured in a γ counter (Packard).

Conditions of reconstitution

The membranes (ASU or ASUA particles) were first incubated for 30 min at 30°C at a protein concentration of 1 mg/ml of reconstitution medium (0.25 M sucrose/1 mM EDTA/5 mM dithiothreitol/5% glycerol/50 mM Tris-acetate (pH 7.5)/5 mM $MgCl_2$/2.5 mM ATP) in the presence of F_1 or of OSCP, at the concentrations indicated in the legends of the table or figures. The mixture was then centrifuged either for 5 min in a Beckman airfuge at 172 kPa (140000 × g, micromethod) or for 20 min at 218000 × g (rotor 65, Beckman, large-scale method). The supernatant fluid was removed and each pellet was rinsed twice with the homogeneization medium (0.25 M sucrose/1 mM EDTA/50 mM Tris-acetate, pH 7.5) to remove unbound F_1 or OSCP. The pellets were then resuspended at 1 mg/ml of reconstitution medium in the presence of various concentrations of either F_1 or OSCP (membranes first reconstituted with F_1 and then incubated with OSCP or vice-versa). After 30 min at 30°C, the mixture was centrifuged for 5 min at 172 kPa in a Beckman airfuge. Each pellet was rinsed as above and finally homogenized with 200 μl of homogeneization medium with an epoxy resin pestle which had been specially adjusted to airfuge tubes. The homogenate was kept at 30°C and used within 1 h. No change could be detected in the rate of ATP hydrolysis, net ATP synthesis or proton translocation, during this time. It was checked that the second reconstitution did not remove significant amounts of F_1 or OSCP bound during the first one, as verified by using [^{14}C]OSCP or [^{125}I]F_1. In the experiments where F_1 and OSCP were reconstituted simultaneously only the first incubation was performed.

Assays of biological activity

ATPase activity of reconstituted particles was assayed at 30°C with an ATP-regenerating system coupled to NADH oxidation [41]. The assay medium (0.62 ml) contained 1.5 mM KCN in addition to the medium described by Glaser et al. [42] and 2–4 μg membrane protein. The percentage of inhibition induced by oligomycin was determined in the presence of 0.2 μg oligomycin in the ATPase assay. NADH-driven net ATP synthesis was measured in reconstituted particles at 30°C in a medium containing 0.25 M sucrose/50 mM Tris-acetate (pH 7.5)/1 mM EDTA/50 mM glucose/1 mM ADP/20 mM KH_2PO_4 ($2 \cdot 10^6$ cpm of $[^{32}P]P_i$ per μmol of P_i)/5 mM $MgCl_2$/1.5 mM NADH/40 units of hexokinase per ml (final concentrations). The assays were started by the addition of 30–50 μg of reconstituted particles (final assay volume, 0.7 ml) and stopped 4 min later by injection of 0.6 ml of the assay into a P_i extraction medium as previously described [43]. Net ATP synthesis was determined from the formation of glucose 6-$[^{32}P]$phosphate. The concentrations of ADP, NADH and hexokinase have been optimized to obtain maximal net ATP synthesis.

Proton translocation was followed by fluorescence quenching of ACMA as previously described [44] in 2 ml of medium containing 30 mM Tris/10 mM Mes/50 mM KCl/10 mM $MgSO_4$/1 mM dithiothreitol/10% glycerol, pH 7.5 (HCl)/1.25 μM ACMA/0.75 μM valinomycin. Each assay was performed with 50 μg of membrane protein/ml. NADH and ATP-dependent proton translocation were initiated by the addition of 0.1 mM NADH and 0.6 mM ATP, respectively.

The rate of NADH oxidation was measured spectrophotometrically at 340 nm under the conditions used for proton translocation, except that the final volume was 0.62 ml.

Results

Peptide content and activities of various mitochondrial membrane preparations

Fig. 1 shows the electrophoretic pattern of mitochondrial membrane preparations after various treatments reputed to progressively decrease the content in F_1 and OSCP. The same protein amounts of the different membranes were loaded on the same slab. The relative amounts of each peptide can therefore be roughly compared. It is evident that the urea treatment (ASU) significantly depletes the membrane from the α subunit and even to a greater extent from the β subunit. Additional treatments with ammonia further decrease the OSCP content (ASUA particles). In contrast, the relative content in several peptides increases from ETP to ASUA particles, especially for peptides of M_r lower than 20000 and for peptides of M_r close to 30000.

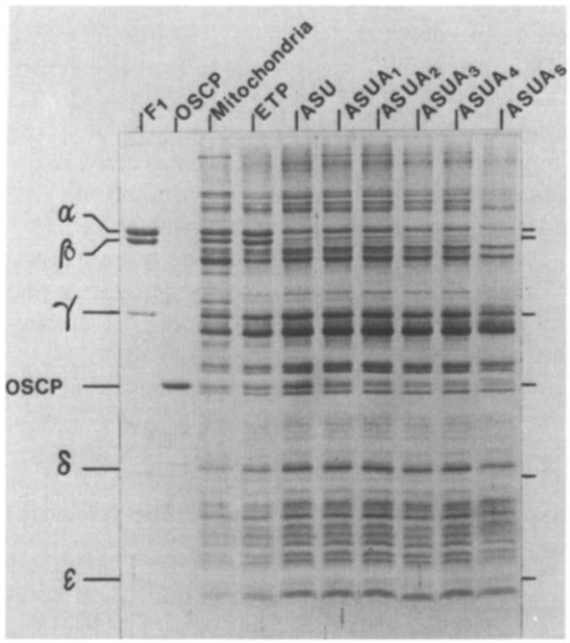


Fig. 1. SDS-polyacrylamide gel electrophoresis of various mitochondrial fractions more or less depleted in F_1 and OSCP. Mitochondria, ETP prepared from mitoplasts, ASU particles, ASUA particles or sonicated ASUA particles ($ASUA_s$) (24 μg protein); F_1 (5 μg protein), OSCP (1.3 μg protein) were submitted to SDS polyacrylamide gel electrophoresis (15% acrylamide) as detailed by Penin et al. [35]. The sub-indices following ASUA indicate the number of ammonia treatments made to extract OSCP. All fractions were loaded on the same slab.

A precise quantitation of the α and β subunits of F_1 and of OSCP has been performed by immunotitration with monoclonal antibodies (Table I).

The comparison between ETP, ASU particles and the various ASUA particles shows that β can

TABLE I

CHARACTERIZATION OF THE VARIOUS PREPARATIONS OF MITOCHONDRIAL MEMBRANES

	Native membranes				Membranes reconstituted with [d]			
					F_1		F_1 + OSCP	
	Contents in			NADH oxidase activity [c] (μmol per min per mg)	ATPase activity (μmol per min per mg)	Oligomycin sensitivity of ATPase activity (% inhibition)	Oligomycin sensitivity of ATPase activity (% inhibition)	Net ATP synthesis (μmol per min per mg)
	α [b] (nmol/mg membrane protein)	β [b]	OSCP [b]					
Mitochondria	0.97 ± 0.11 (12)	0.94 ± 0.1 (15)	0.58 ± 0.11 (26)	–	–	–	–	–
ETP	1.20 ± 0.04 (4)	1.14 ± 0.1 (4)	0.79 ± 0.06 (8)	0.32	–	–	–	–
ASU	0.43 (2)	0.21 (2)	0.85 ± 0.07 (4)	0.44	8.6	92	94	0.59
$ASUA_1$ [a]	0.37 (2)	0.12 ± 0.01 (3)	0.57 ± 0.11 (12)	0.56	5.9	70	92	0.26
$ASUA_2$ [a]	0.31 (2)	< 0.04	0.23 (2)	0.73	5.1	12	94	0.23
$ASUA_3$ [a]	0.32 ± 0.04 (4)	< 0.04	0.14 ± 0.03 (5)	0.69	6.6	0	95	0.135
$ASUA_4$ [a]	0.30 ± 0.02 (5)	< 0.04	0.09 ± 0.03 (5)	0.42	5.4	0	90	0.04
Sonicated ASUA	0.28 ± 0.03 (4)	< 0.04	0.27 ± 0.03 (6)	0.01	6.2	8	86	0.002

[a] The sub-indices following ASUA indicate the number of ammonia treatments made to extract OSCP (see Material and Methods).

[b] The contents in α, β and OSCP were measured by quantitative immunotitration [15]. The results are expressed in nmol per mg of membrane protein ± S.D., taking into account M_r of 55160; 51600 and 21000 for α, β [3] and OSCP [10], respectively. The number of determinations is given in parenthesis.

[c] The NADH oxidase activity is measured on the membranes without any reconstitution step.

[d] ASU or ASUA particles (200 μg protein) were reconstituted with F_1 or F_1 + OSCP under the conditions described in Material and Methods (micromethod). The amount of F_1 (330 μg/mg membrane protein) and OSCP (18–40 μg/mg of membrane protein) present in the reconstitution medium were optimized to induce a maximal rate of NADH-driven net ATP synthesis. The ATPase activity and the net ATP synthesis obtained under the optimal conditions reported here are expressed as μmol/min per mg reconstituted membrane protein.

be almost completely removed from the particles while α seems more effectively bound. After one ammonia treatment, ASUA particles are only partially depleted in OSCP. Four successive ammonia treatments appear necessary to remove most OSCP. However, one must not forget that peptides other than α, β and OSCP are eliminated during the Sephadex, urea and ammonia treatments and can be detected in the supernatant fluids (not shown here). The residual ATPase activity of ASU particles (approx. 1%, not shown) completely disappears after the first ammonia treatment. In contrast, NADH oxidase activity appears very little affected by these treatments except in the case of sonicated ASUA.

Reconstitution of membrane with F_1 and OSCP

Reconstitutions of these various membranes have been performed either with F_1 or with F_1 and OSCP. Under our conditions, F_1 easily reassociates with all particles, with an ATPase activity of 8.6 and 5.1–6.6 μmol ATP hydrolyzed per min per mg of membrane protein for ASU and ASUA, respectively. The oligomycin sensitivity of this reconstituted ATPase activity progressively decreases with the number of ammonia treatments. This oligomycin sensitivity can be almost fully restored in all cases when the reconstitution is performed in the presence of both F_1 and OSCP. Under the same conditions, an active net ATP synthesis is recovered, although it decreases with the number of ammonia treatments of the membrane made prior to reconstitution. In the absence of OSCP, the ATP synthesis is barely detectable with ASU and not at all with ASUA particles. With sonicated ASUA particles, even in the presence of OSCP, neither net ATP synthesis not ATP-P_i exchange (not shown) can be significantly restored. In contrast, for all other particles tested, ATP synthesis can always be reconstituted.

Amount of F_1 and OSCP bound to ASUA particles correlated with net ATP synthesis

We see in Table I that the ATPase activity of F_1 rebound to the various ASUA particles does not seem to be directly correlated to the amount of endogenous OSCP. It must be precised that the reconstitutions were performed in the presence of $ATP + Mg^{2+}$. With $ASUA_1$ particles, it is shown (Fig. 2A) that, in the absence of added OSCP, the binding of labeled F_1 as a function of its concentration gives a typical saturation curve characteristic of specific binding. The plateau of about 100 μg of F_1 per mg of protein of particles indicates the maximal number of F_1 sites on $ASUA_1$ particles in the absence of added OSCP. Under these conditions, the ATPase activity per mol bound $[^{125}I]F_1$ is identical to the activity of $[^{125}I]F_1$ in solution (not shown). When OSCP was bound to $ASUA_1$ particles prior to F_1, saturation curves similar to that of Fig. 2A were obtained (not shown). However, the maximal number of F_1 sites was only slightly increased as a function of the amount of OSCP bound (Fig. 2B, ■). The maximal binding was of 120 μg of F_1 per mg of protein of $ASUA_1$ particles but, the maximal rate of ATP synthesis in the reconstituted system was reached for about 110 μg of F_1 rebound only. If F_1 and OSCP were incubated together with $ASUA_1$ particles, the maximal amount of F_1 bound (not shown) was not significantly different from that found when OSCP was prebound before addition of F_1.

The binding of OSCP (Fig. 2C) does not exhibit saturation curves in the absence of F_1 (●) or after maximal prebinding of F_1 (○) to $ASUA_1$ particles. When saturating concentrations of F_1 and variable amounts of OSCP were incubated together with $ASUA_1$ particles (▲) a biphasic curve was observed for the binding of OSCP as a function of its concentration in the incubation medium. This indicates two classes of sites, one being more specific and involved in ATP synthesis (0–10 μg OSCP bound per mg of membrane), the other being non specific (OSCP bound over 8–10 μg). Indeed, Fig. 2B shows that the maximal rate of ATP synthesis is reached when about 10 μg OSCP were bound to $ASUA_1$ before the reconstitution with F_1. Additional unspecific OSCP binding slightly increases F_1 binding in an unspecific manner, since it slightly decreases ATP synthesis and oligomycin sensitivity of the ATPase activity (not shown).

Similar binding curves were obtained with other types of ASUA particle although the exact amounts of bound F_1 and bound OSCP slightly varied: the more the particles were depleted from endogenous OSCP, the more OSCP could be rebound.

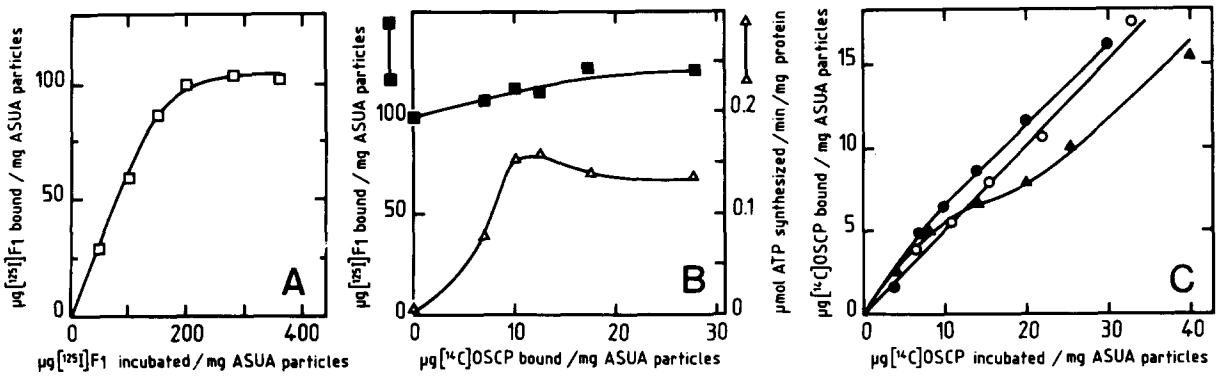


Fig. 2. Correlation between the binding of F_1 and OSCP to $ASUA_1$ particles and the reconstitution of net ATP synthesis. (A) Effect of F_1 concentration on its binding to $ASUA_1$ particles in the absence of OSCP. $ASUA_1$ particles (200 µg protein) were incubated at a final concentration of 1 mg/ml for 30 min, at 30°C with [^{125}I]F_1 (1.85 µCi/mg) at the concentration indicated on the figure, under the standard conditions of reconstitution. The particles were centrifuged, the pellets rinsed and homogenized (see Material and Methods). The radioactivity and the protein contents were measured on several aliquot samples of the homogenate. The protein amount due to ASUA particles in the homogenates was calculated after subtraction of the protein contents of F_1 (deduced from its radioactivity). Then the amount of F_1 bound per mg of $ASUA_1$ particles was calculated. (B) Effects of the previous binding of OSCP to $ASUA_1$ particles on the binding of F_1 to these particles and on the rate of ATP synthesis. $ASUA_1$ particles (270 µg protein) were first incubated 30 min at 30°C in the presence of 0–15 µg of [^{14}C]OSCP (1.24 µCi/mg). After centrifugation the pellets were rinsed and homogenized as in Fig. 2A. The amount of [^{14}C]OSCP bound was measured. Fractions of the homogenates containing 200 µg of ASUA particles were incubated again 30 min at 30°C with 66 µg of [^{125}I]F_1 in the reconstitution medium, centrifuged and the pellets rinsed and homogenized as above. Aliquot samples of the latter homogenates were used in parallel to estimate the amount of F_1 bound per mg of $ASUA_1$ particles (■) and to measure the rate of net ATP synthesis (△) catalyzed by these reconstituted particles (see Material and Methods). (C) Effect of OSCP concentration on its binding to $ASUA_1$ particles in the absence or the presence of F_1 added together with OSCP or bound to membranes prior to OSCP. $ASUA_1$ particles (200 µg protein) were incubated as in Fig. 2B with [^{14}C]OSCP at the indicated concentrations in the absence (●) or the presence of 66 µg of F_1 (▲). In another series of experiments (○), the $ASUA_1$ particles (1.6 mg protein) were first incubated with 530 µg of F_1, centrifuged and homogenized under the same conditions as that of Fig. 2A. Aliquot samples of the homogenate containing 200 µg of $ASUA_1$ particles were then incubated in the reconstitution medium with increasing amounts of [^{14}C]OSCP and centrifuged. The pellets were rinsed and homogenized as in Fig. 2A. The protein contents and the amount of [^{14}C]OSCP bound per mg of $ASUA_1$ particles were estimated as in Fig. 2A for all three sets of conditions (●, ▲, ○).

Effects of the binding of OSCP to $ASUA_3$ particles on the sensitivity of ATP hydrolysis to oligomycin and uncouplers. Correlation with ATP synthesis

To specify the functions of OSCP in the system, $ASUA_3$ particles were used for several reasons: they are very poor in OSCP; after rebinding of F_1 the ATPase activity is not sensitive to oligomycin in the absence of added OSCP, but becomes fully sensitive after reconstitution with OSCP; after reconstitution with F_1 and OSCP, they exhibit fairly good rates of ATP synthesis coupled with a high rate of NADH oxidation. Besides, to avoid unspecific F_1 binding in the presence of excess OSCP, the particles were first reconstituted with F_1 and then with increasing amounts of OSCP. Under these conditions, 100–107 µg F_1 were bound per mg $ASUA_3$ particles (i.e., 0.27–0.29 nmol/mg $ASUA_3$-F_1, taking into account an M_r of 371 000 for F_1 [3]). The maximal rate of ATP synthesis reached a plateau for 8 µg of rebound OSCP, which corresponds to 10 µg OSCP incubated per mg of reconstituted $ASUA_3$-F_1 particles. This rate was not affected by further addition of OSCP (Fig. 3A). The inset of Fig. 3A shows that the correlation between the rates of net ATP synthesis and of ATP-P_i exchange was very good. Fig. 3B shows that the ATPase activity of F_1 rebound to $ASUA_3$ particles in the absence of OSCP was insensitive to uncouplers. Reconstitution of $ASUA_3$-F_1 with increasing amounts of OSCP progressively decreased the ATPase activity of bound F_1 up to a value corresponding to about 80% of the original activity. This plateau was observed for 8 µg OSCP

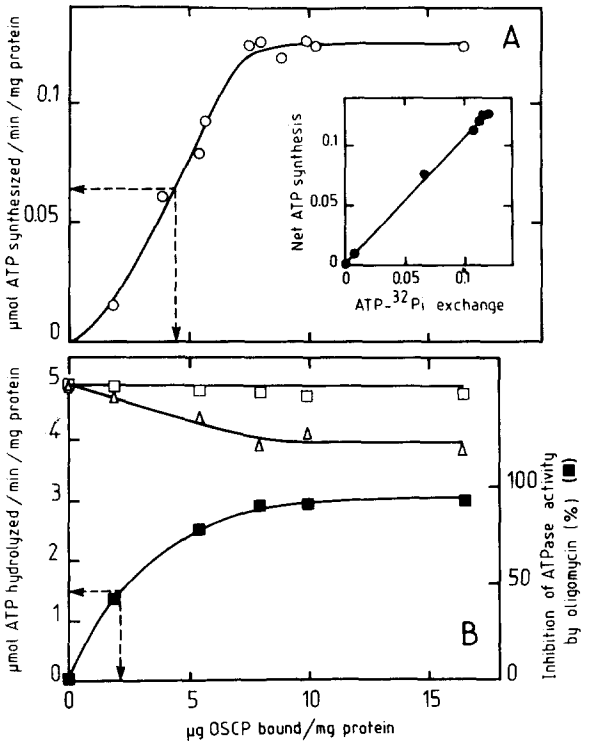


Fig. 3. Rates of ATP synthesis and ATP hydrolysis as a function of the amount of OSCP reconstituted with $ASUA_3$ particles. $ASUA_3$ particles (1 mg protein/ml) were first reconstituted with F_1 (0.33 mg/ml) as described in Material and Methods (large-scale method). The pellets ($ASUA_3$-F_1 were then reconstituted with increasing amounts of [^{14}C]OSCP. The amount of bound OSCP was determined as described in Material and Methods. The rate of NADH-driven net ATP synthesis, of ATP-$^{32}P_i$ exchange (A) and of ATP hydrolysis ± inhibitors (B) was measured on the $ASUA_3$-F_1 reconstituted with OSCP. ATPase activity tested in the absence of inhibitor (controls, △), in the presence of 1.6 µM FCCP (□) or of 0.2 µg oligomycin/assay (■). Since FCCP and oligomycin were added as an ethanolic solution, the same amount of ethanol was added in the controls. Inset: correlation between the rate of NADH-driven net ATP synthesis and ATP-$^{32}P_i$ exchange activity. All rates are expressed as µmol ATP synthesized or hydrolyzed/min per mg protein of reconstituted particles. The arrows at the end of the dashed lines indicate the amount of bound OSCP required to observe 50% of the maximal ATP synthesis (A) or inhibition of ATPase activity by oligomycin (B).

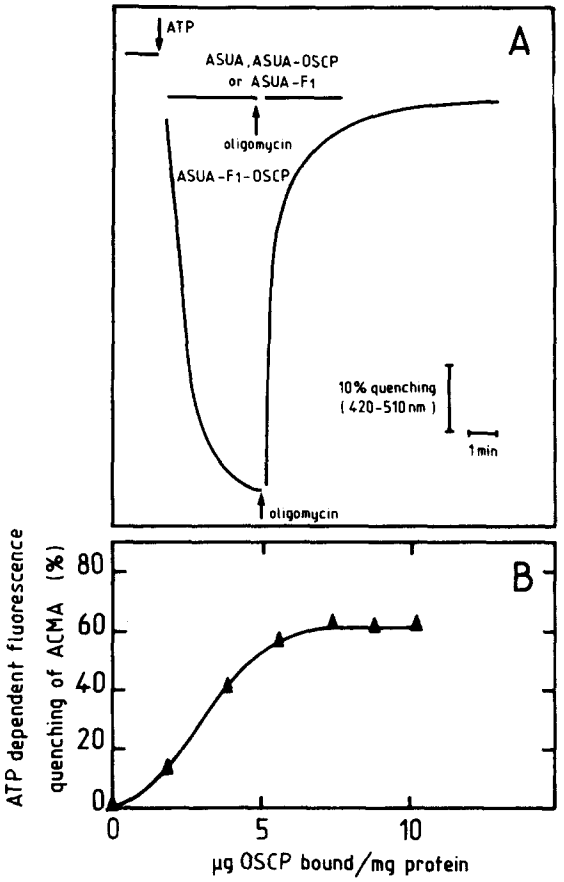


Fig. 4. Effects of the reconstitution of $ASUA_3$ particles with F_1 and OSCP on the ATP-dependent proton translocation as measured by the fluorescence quenching of ACMA. ASUA-OSCP and ASUA-F_1: $ASUA_3$ reconstituted with OSCP (2–50 µg/mg $ASUA_3$ protein) and with F_1 (50–400 µg F_1/mg protein), respectively. ASUA-F_1-OSCP: $ASUA_3$ particles reconstituted first with 0.33 mg F_1/mg $ASUA_3$ protein and then with 10 µg OSCP/mg ASUA-F_1 protein (A) or with increasing amounts of [^{14}C]OSCP (B). The amount of bound OSCP was determined as described in Material and Methods. When indicated, 0.5 µg oligomycin were added in the assays. In (B) the maximal quenching observed at steady-state (see Ref. 44) is plotted as a function of OSCP bound after the reconstitution. ATP-dependent fluorescent quenching of ACMA: see Material and Methods.

rebound per mg of $ASUA_3$-F_1. Addition of FCCP to $ASUA_3$-F_1-OSCP complex restored the original ATPase activity of $ASUA_3$-F_1, independently of the OSCP rebound. Therefore, OSCP rendered the ATPase activity of bound F_1 sensitive to uncouplers.

It is noteworthy that the same amount of 8 µg OSCP rebound per mg of $ASUA_3$-F_1 corresponds to the lowest ATPase activity, the maximal rate of ATP synthesis and the maximal sensitivity to oligomycin. Also, this same amount of OSCP reconstitutes an optimal proton flux through F_0 (see below). In contrast, the rebinding of 2 µg OSCP is sufficient to produce half maximal inhibition of

ATPase activity by oligomycin, while 4.5 μg are required to restore half maximal rate of ATP synthesis.

Reconstitution of proton fluxes with $ASUA_3$ particles, F_1 and OSCP

Fig. 4A shows that with $ASUA_3$ reconstituted in the presence of optimal amounts of F_1 and OSCP, an important proton flux could be observed as measured by the ATP-driven fluorescence quenching of ACMA in the presence of valinomycin + K^+ (i.e., membrane potential collapsed as much as possible). After reconstitution of ASUA with either F_1 or OSCP alone, no proton flux could be observed. The maximal extent of quenching was also observed for 7–8 μg OSCP bound per mg of $ASUA_3$-F_1 (Fig. 4B). When the proton flux was initiated in $ASUA_3$ particles by NADH oxidation (Fig. 5), a small quenching of ACMA fluorescence was observed; it was not modified after reconstitution with either F_1 or OSCP (Fig. 5A). On the contrary, after reconstitution with optimal amounts of F_1 and OSCP, an important quenching was observed close to the maximum quenching observed in the presence of oligomycin (Fig. 5A). As before, the maximal effect of OSCP was obtained for 7–8 μg OSCP bound per mg $ASUA_3$-F_1 (Fig. 5B).

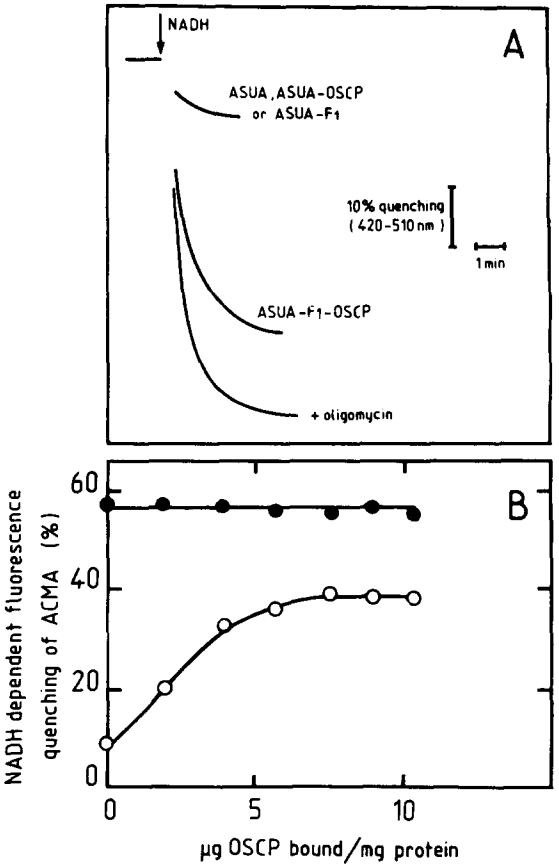


Fig. 5. Effects of the reconstitution of $ASUA_3$ particles with F_1 and OSCP on the NADH-dependent proton translocation as measured by the fluorescence quenching of ACMA. (A) and (B): Conditions as in Fig. 4, except that NADH-dependent fluorescent quenching of ACMA was measured as described in Material and Methods. The trace shown in the presence of oligomycin is identical for ASUA, ASUA-F_1, ASUA-OSCP and ASUA-F_1-OSCP. (B) The assays were made in the absence (○) or in the presence (●) of 0.5 μg oligomycin per assay.

Effects of OSCP on the rate of NADH oxidation by $ASUA_3$ particles

The rate of NADH oxidation was of about 0.42 μmol per mg of $ASUA_3$ particles. It was lower than the rate measured in Table I because ethanol present as a control decreased this activity. This rate was not modified by reconstitution with OSCP alone or F_1 alone and by addition of FCCP (Fig. 6). In contrast, oligomycin decreased this activity

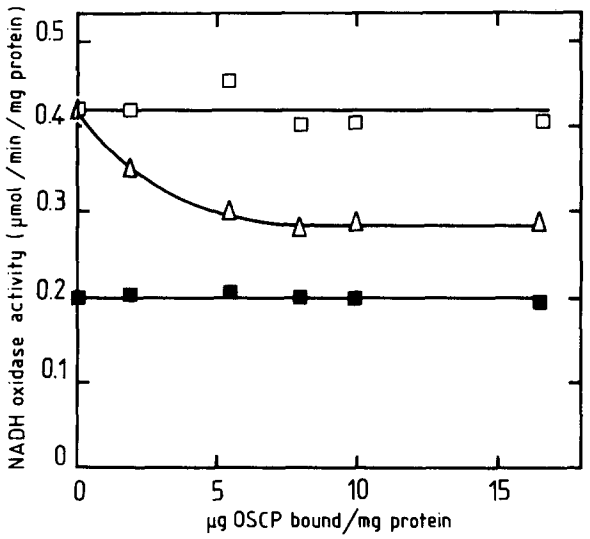


Fig. 6. Effects of the reconstitution of $ASUA_3$-F_1 particles with OSCP on the rate of NADH oxidation, Conditions as described in Fig. 3. The rate of NADH oxidation was measured in the absence of inhibitors (control, △), in the presence of 1.6 μM FCCP (□) or in the presence of 0.2 μg oligomycin/assay (■), as described in Materials and Methods. The rate is expressed as μmol NADH oxidized/min/mg protein of reconstituted particles.

about 50% in the presence or the absence of F_1 and/or OSCP. The addition of OSCP to $ASUA_3$ reconstituted with F_1 decreased the rate of NADH oxidation with a maximal effect at 8 μg OSCP bound per mg $ASUA_3$-F_1. Addition of FCCP to $ASUA_3$ reconstituted with F_1 and OSCP always restored the maximal rate of NADH oxidation, and abolished all effects of oligomycin. The presence of KCN completely inhibited the NADH oxidase activity and the concomitant proton flux (not shown).

Discussion

Membrane characterization

In many studies, various submitochondrial particles have been used in reconstitution experiments. They were essentially characterized by losses of activity. In this work, we present a clear cut view of the content in α and β subunits of F_1 and in OSCP after immunotitration with monoclonal antibodies in various pig heart mitochondrial membrane preparations known either to lack ATPase activity or to need addition of OSCP to restore oligomycin sensitivity of ATPase activity.

The results show that the ATPase activity is readily lost from the membrane by a single urea treatment while the removal of most β subunits needs two additional ammonia treatments. In contrast, significant amounts of α subunits always remain linked to the membrane, whatever the treatment made, and several ammonia treatments are necessary to progressively remove OSCP.

Conditions of reconstitution

The precise knowledge of the membrane contents in α, β and OSCP was compulsory to establish correlations between the rebinding of F_1 and OSCP to depleted membranes and the reconstitution of all activities involved in oxidative phosphorylations. Indeed, to our knowledge, this study is the first report describing the reconstitution of all energy-transfer reactions from well characterized ASUA particles, OSCP and F_1. To observe efficient reconstitutions, several factors must be carefully controlled.

(1) The particles must be sufficiently depleted in F_1 and OSCP. Otherwise, it is difficult to assign the recovery of activity to endogeneous or added OSCP. This problem occurs with ASU particles and also partly with ASUA particles after only one ammonia treatment.

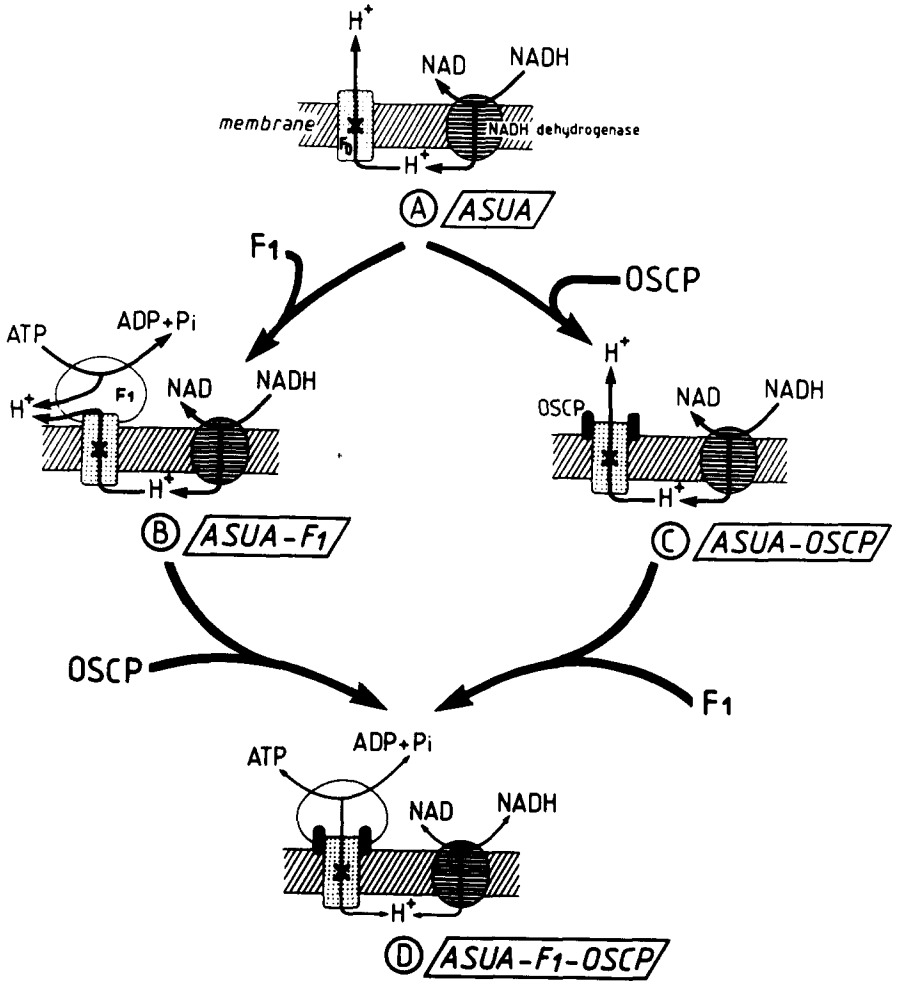
(2) The particles must still contain active respiratory chain components. This requirement excludes the use of sonicated ASUA particles and of ASU particles after four ammonia treatments ($ASUA_4$) when the membrane is too damaged. Therefore, three successive ammonia treatments of ASU particles provide membranes ($ASUA_3$) exhibiting an optimal ability to study the role of OSCP and F_1 in energy transfer reactions.

(3) The reconstitution of functional particles requires the rebinding of F_1 and OSCP in sufficient amounts to seal at the best the F_0 channels leaky to protons. F_1 specifically binds to the membrane even in the absence of OSCP provided cations are present in the reconstitution medium [16–18]. On the contrary, an unspecific binding of OSCP may occur (Fig. 2C), eventually at the level of the F_0-α complexes that are still present in depleted membranes. Therefore, OSCP must not be added in excess because the unspecific OSCP could bind additional F_1 (Fig. 2B) not properly related to F_0. This extra F_1 can hydrolyze ATP in an oligomycin-insensitive manner and competes with hexokinase present in ATP synthesis assay medium to consume newly synthesized ATP (due to F_1 specifically reconstituted to the membrane). Therefore, the rate of ATP synthesis measured by glucose 6-phosphate formation apparently decreases (Fig. 2B).

The best way to avoid this extra binding of F_1 is to reconstitute first F_1 with the membrane at saturating concentration of F_1 (330 μg F_1 per mg of $ASUA_3$ particles) in the presence of ATP + Mg^{2+}. Free F_1 must then be eliminated by spinning down the membranes prior to the addition of OSCP. Under these conditions, and extra binding of OSCP will not decrease the reconstitution of ATP synthesis. This procedure is the most suitable for studying the function of OSCP.

Relationships between rebinding of OSCP and reconstitution of energy-transfer reactions

This study has permitted to correlate the binding of OSCP to $ASUA_3$-F_1 particles with the rates of NADH oxidation, of ATP synthesis or ATP-P_i exchange, of ATP hydrolysis and with the



Scheme I. Reconstitution of inverted ASUA particles with F_1 and OSCP. Role of OSCP in ATP- or NADH- driven proton fluxes. (——) high reaction rate (A,B,C); (———) slow reaction rate (D); (✖) indicates inhibition by oligomycin. The aim of this scheme is not to discriminate between localized or delocalized protons.

oligomycin-sensitivity of ATPase activity. All these activities are controlled by the proton flux through F_0 which is specifically directed to F_1 by OSCP, as shown in Scheme I. ASUA particles depleted from F_1 and OSCP (A) or reconstituted with F_1 alone (B) or with OSCP alone (C) are leaky to protons (Fig. 5A) and exhibit a maximal rate of NADH oxidation (Fig. 6). Therefore, neither F_1 nor OSCP alone prevents the leakage of protons through F_0. By sealing all F_0 channel including the F_0-α complexes behaving as 'leaking holes', oligomycin (✖) increases the NADH-dependent quenching of ACMA fluorescence (Fig. 5A), which indicates an accumulation of protons inside the particles. This accumulation of protons inhibits the rate of NADH oxidation by shifting the equilibrium of the reaction.

The protons produced by ATP hydrolysis in ASUA-F_1 particles (B) do not cross the membrane through F_0, since neither ATP synthesis nor oligomycin sensitivity of ATPase activity (Fig. 3) or ATP-dependent proton flux (Fig. 4), or inhibition of NADH oxidase activity (Fig. 6) can be detected. On the contrary after reconstitution of ASUA-F_1 with OSCP (D), an active ATP synthesis can be measured (Fig. 3A), the ATPase activity is inhibited by oligomycin, the ATP-dependent quenching of ACMA fluorescence is restored (Fig.

4), while the NADH oxidase (Fig. 6) and the ATPase activity (Fig. 3B) are decreased. The latter inhibitions are due to the accumulation of protons inside the particles, as explained above for oligomycin. This inhibition of ATPase activity of bound F_1 by addition of OSCP was observed as early as 1968 [7] but had never been clearly explained. The progressive removal of F_1 and OSCP increased the rate of NADH oxidation (Table I) as uncouplers do, while the inhibition of NADH oxidation upon rebinding of F_1 + OSCP appears similar to a State 3–State 4 transition. FCCP which creates proton leaks in the membrane restores both the rates of ATP hydrolysis (Fig. 3B) and of NADH oxidation (Fig. 6) observed in the absence of OSCP. FCCP also reverses the inhibitory effects of oligomycin. All the experiments presented here demonstrate that OSCP plays the role of a 'clamp' ensuring the good fitting of F_1 on F_0. The fact that OSCP added alone does not affect the proton leak through the membrane and the external localization of OSCP relative to F1 [21] (which is in agreement with the reconstitution of F_1 first, then OSCP to ASUA) might mean that OSCP is not directly involved in the H^+ channel of F_0. However, it cannot be absolutely decided as yet whether protons finally transit through OSCP to reach F_1 catalytic sites or if OSCP changes the conformation of F_1 to specifically orientate the protons between F_0 and F_1.

Stoichiometry of F_1 and OSCP in reconstituted coupled particles

Another point raised by these experiments is the minimal ratio OSCP/F_1, necessary to fully reconstitute energy-transfer reactions. The minimal amount of OSCP required to reconstitute the best rate of ATP synthesis (NADH-driven or measured by ATP-P_i exchange) is very well correlated with that necessary to obtain either the lowest rates of ATP hydrolysis and NADH oxidation or the maximal sensitivity to oligomycin of the ATP hydrolysis or the largest accumulation of protons (ATP- or NADH-dependent) as measured by fluorescence quenching of ACMA. This minimal amount of OSCP is of about 8 μg of rebound OSCP, i.e., 0.38 nmol/mg $ASUA_3$-F_1-OSCP. The amount of F_1 bound per mg of $ASUA_3$-F_1-OSCP is 100–107 μg, i.e., 0.27–0.29 nmol/mg $ASUA_3$-F_1-OSCP. The endogenous OSCP present in $ASUA_3$ particles before reconstitution measured by immunotitration with monoclonal antibodies (Table I), 0.14 nmol/mg $ASUA_3$, corresponds, after reconstitution, to 0.126 nmol/mg $ASUA_3$-F_1-OSCP. Therefore the total amount of OSCP in the reconstituted membrane, 0.38 + 0.126 = 0.506 nmol/mg $ASUA_3$-F_1-OSCP gives a ratio OSCP/F_1 varying between 1.7 and 1.9. This value is consistent with the ratio OSCP/F_1 equal to 1.81–1.87 measured in intact mitochondria [15]. Consequently, it appears that about 2 OSCP/F_1 are involved in the reconstitution of energy-transfer reactions. It could be argued that the ratio 1.7–1.9 could be considered as an upper limit, since some unspecific binding of OSCP occurs, and since endogenous or added OSCP might not be equivalent. The question is to know whether one or two OSCP are implied in ASUA-F_1-OSCP complexes efficient for all energy transfer reactions. Fig. 3 shows that the amount of bound OSCP necessary to reconstitute 50% of the rate of ATP synthesis is more than two-fold higher than that observed for 50% of inhibition of the ATPase activity by oligomycin. A first explanation could be that an optimal reconstitution of ATP synthesis requires a good sealing by OSCP of all F_1-F_0 complexes previously leaky to protons, while the oligomycin sensitivity of ATPase activity is directly dependent on the amount of F_0-F_1 complexes which have bound OSCP. Another possibility is that the binding of the first OSCP to F_0-F_1 induces a partial inhibition of the ATPase activity by oligomycin and the binding of a second OSCP brings this inhibition to 100%. Since in the reconstituted system the inhibition by oligomycin barely exceeds 90–95% as compared to 99–100% in mitochondria or ETP one can wonder whether the second OSCP required is more difficult to adequately bind. The fact that isolated F_1 contains both high and low affinity binding sites for OSCP [19] supports this interpretation. Besides it would not be surprising after all the treatments applied to the membranes, that a few F_0, F_1, OSCP do not fully reconstitute, due for example to damages to F_0.

In conclusion, it can be proposed as a working hypothesis that the binding of the first mole of OSCP per mole of F_0-F_1 can induce a large but

incomplete oligomycin sensitivity of the ATPase activity, while the binding of a second mole of OSCP would be necessary to observe a complete sealing of the F_0 channel, and hence the maximal rate of ATP synthesis. This interpretation could explain why several authors have estimated values favouring a stoichiometry of one OSCP per F_1 by measuring oligomycin sensitivity of the ATPase activity in reconstitution experiments [11,14], while two OSCP per F_1 are present in mitochondria [15].

Acknowledgments

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