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PROTEINS REQUIRED FOR THE BINDING OF MITOCHONDRIAL ATPase TO THE MITOCHONDRIAL INNER MEMBRANE

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SUMMARY

1. Isolated F_1 (mitochondrial ATPase) binds to urea-treated submitochondrial particles suspended in sucrose/Tris/EDTA with a dissociation constant of 0.1 μM.

2. About one-third of the F_1 and the oligomycin-sensitivity conferring protein (OSCP) are lost during preparation of submitochondrial particles prepared at high pH (A particles). None is lost from particles treated with trypsin (T particles).

3. After further treatment with alkali of urea-treated particles, binding of F_1 requires the addition of OSCP. Maximum binding is reached when both OSCP and Fc_2 are added. The concentration of F_1-binding sites in the presence of both OSCP and Fc_2 is about the same as that in TU particles.

4. After further extraction with silicotungstate of urea- and alkali-treated particles, OSCP no longer induces binding of F_1, unless Fc_2 is also present. Fc_2 induces binding in the absence of OSCP but with a lower binding constant and, in contrast to results under all the other conditions studied in this paper, the ATPase activity is oligomycin insensitive.

5. It is tentatively concluded that OSCP is the binding site for F_1 and Fc_2 is the binding site for OSCP.

INTRODUCTION

Racker and coworkers showed that extraction of a protein factor called F_1 from submitochondrial particles specifically impairs the ability of these particles to

Abbreviations: The different preparations of submitochondrial particles and coupling factors used are abbreviated in accordance with the original descriptions of these preparations. "A" submitochondrial particles are isolated in the presence of ammonia [1], AS, ASU and ASUA particles are A particles successively treated with Sephadex, Sephadex and urea, and Sephadex, urea and ammonia [2], respectively. "T" submitochondrial particles are extracted from mitochondria by sonication in the presence of pyrophosphate and then treated with trypsin [3]. TU, TUA and TUA-STA are T particles successively treated with urea, urea and ammonia, and urea, ammonia and silicotungstate, respectively. F_1 refers to coupling factor 1 of Pullman et al. [4], $F_1 \cdot X$ to an associated form of F_1 described by Vallejos et al. [5]. OSCP refers to oligomycin-sensitivity conferring protein [6]. Fc_2 is a protein fraction isolated by extraction of TUA particles with thiocyanate [7].

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catalyse oxidative phosphorylation without affecting their respiratory activity, and that the phosphorylative activity is restored by the addition of the purified factor (4, 8). The isolated phosphorylation coupling factor has ATPase activity. This activity, unlike that of submitochondrial particles, is insensitive to oligomycin and is cold labile but becomes oligomycin sensitive [9] and cold stable [10] when the F_1 is rebound to the particles.

In 1966, Kagawa and Racker [10] extracted from submitochondrial particles a particulate preparation, called F_o (o for oligomycin), to which F_1 binds with a parallel conferral of oligomycin sensitivity of the ATPase activity of the bound F_1. Binding of F_1 to TUA particles (submitochondrial particles treated successively with trypsin, urea and ammonia) and conferral of oligomycin sensitivity were found to require another protein fraction called F_4 [11]. In 1968, Bulos and Racker [12], using F_4 as starting preparation, purified the active factor responsible for the conferral of oligomycin sensitivity and called it F_c (c for conferral). They showed also that F_c increases the binding of F_1 to TUA particles.

In parallel work Tzagoloff and coworkers isolated an oligomycin-sensitive ATPase complex [13] and extracted from this complex a highly purified protein (called OSCP = oligomycin-sensitivity conferring protein), which increases the binding of F_1 to NaBr- and alkali-extracted ATPase complex and is necessary for the conferral of oligomycin sensitivity in the reconstituted complex [14, 6, 15]. Purified OSCP is 240 times as active as F_4. Although F_1 binds to alkali-extracted complex in the absence of OSCP the resulting ATPase activity is oligomycin insensitive and cold labile.

In 1968, Vallejos et al. [5] in our laboratory described the presence in an aqueous extract of an acetone powder of mitochondria of a coupling factor, called $F_1 \cdot X$, which contained an unknown protein loosely linked to F_1 and which was more active than F_1 in restoring oxidative phosphorylation to 'A' [1] particles. Subsequent studies [16, 17] showed that X is identical with OSCP.

In 1971, Knowles et al. [7] extracted with thiocyanate or silicotungstate a second factor from TUA particles, which they called Fc_2 (F_c was renamed Fc_1). Both Fc_1 and Fc_2 are necessary for the reconstitution of an oligomycin-sensitive ATPase from silicotungstate (or thiocyanate)-extracted TUA particles. A previously described phosphorylation coupling factor, F_6 [18], was found to contain Fc_2 activity and this is presumably the reason for its coupling activity. Fc_2 was shown to be necessary for the binding of F_1 to silicotungstate-extracted TUA particles, but the binding was stronger in the presence of Fc_1.

From this survey it seems likely that both OSCP (= Fc_1) and Fc_2 (= F_6) are involved in the binding of F_1 to membranes stripped of these factors, in such a way that an oligomycin-sensitive ATPase is reconstituted, but the precise role of the two factors is not clear.

In view of the importance of the physical link between F_1, which catalyses the ATP-synthesizing step of oxidative phosphorylation, and the membrane which contains the electron-transfer pathway, it is desirable to have more information on these important factors. In this paper the effect of the two factors separately and together on the concentration of F_1-binding sites in stripped membranes and on the binding constant are described.

MATERIALS AND METHODS

Submitochondrial particles

Submitochondrial particles (ammonium or 'A' particles) were prepared from heavy beef-heart mitochondria [19] by the method of Fessenden and Racker [1]. So-called ammonium-Sephadex (AS) particles and ammonium-Sephadex-urea (ASU) particles were obtained by successive treatment of A particles with Sephadex G-75 and 3 M urea as described by Racker and Horstman [2], except that 3 M urea was used instead of 2 M urea. Ammonium-Sephadex-urea-ammonium (ASUA) particles were prepared by treating ASU particles with 0.4 M NH_3.

Trypsin or T submitochondrial particles were prepared by sonicating beef-heart mitochondria suspended in pyrophosphate, followed by incubation of the submitochondrial particles with trypsin as described by Kagawa and Racker [3]. Trypsin-urea (TU), trypsin-urea-ammonium (TUA) and trypsin-urea-ammonium-silicotungstate (TUA-STA) particles were prepared by successive treatment of T particles with urea, ammonia and silicotungstate as described by Knowles et al. [7].

Coupling factors

Coupling factor 1 (F_1), prepared by the procedure of Knowles and Penefsky [20], was kindly supplied by Mr. J. L. M. Muller. A molecular weight of 360 000 [21] was assumed.

Oligomycin-sensitivity conferring protein (OSCP) was prepared from $F_1 \cdot X$, which was isolated from beef-heart mitochondria by the method of Vallejos et al. [5], as modified by Van de Stadt et al. [17]. The $F_1 \cdot X$ was brought on to a carboxymethylcellulose column equilibrated with 20 mM Tris/H_2SO_4 buffer (pH 8). The F_1 ran through the column whereas the OSCP was retained and was eluted with a NaCl gradient. The OSCP fraction showed by gel electrophoresis in dodecyl sulphate one major band, with a relative molecular weight of 24 000, and several minor bands.

A preparation of Fc_2 was obtained from TUA particles by extraction with thiocyanate as described by Knowles et al. [7]. It was partially purified as described by these authors.

Measurement of activity of coupling factors

The ATPase activity of F_1 was measured by determining the phosphate liberated [22] from ATP in an ATP-generating system [4]. OSCP activity was measured by incubating 300 μg (protein) ASUA particles, 10 μg F_1 and the OSCP preparation, in the presence and absence of 5 nmol oligomycin, in 0.75 ml of 0.25 M sucrose, 50 mM Tris/TES buffer (pH 7.5). After 5 min at 30 °C, the reagents for the ATPase assay were added, bringing the volume to 1 ml, and the reaction was stopped with trichloroacetic acid after a further 10 min incubation. Identical incubations were carried out in the absence of the OSCP preparation. Only part of the sample was used for the phosphate determination.

Fc_2 activity was measured similarly using 400 μg TUA particles that had been extracted with thiocyanate, 10 μg F_1, an excess (10 μg) of OSCP and the Fc_2 preparation.

Binding experiments

The concentration of strong antimycin-binding sites was determined fluori-

metrically as previously described [23], in the presence of 1 mg/ml bovine serum albumin.

The concentration of aurovertin-binding sites was measured by determining the aurovertin fluorescence as will be described in detail in a later paper.

The binding of F_1 to particles was measured by incubating 1–2 mg particles with various amounts of F_1 in a medium containing 250 mM sucrose, 10 mM Tris/TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid, pH 7.5) and 2 mM EDTA in a total volume of 1 ml. After 20 min at 22 °C the samples were centrifuged for 20 min at 105 000 × *g* and the ATPase activity and protein content of the supernatants were determined. In some experiments, the amount of F_1 in the supernatant was determined by measuring the binding of aurovertin to F_1 fluorimetrically, but, in general, measurement of the ATPase activity was found to be the more convenient method. A control experiment, in which the particles were omitted, was run in the same way. The ratio of ATPase activity in the experimental and control supernatants yielded the ratio of free ATPase to total ATPase, from which the amount of bound could be calculated.

The oligomycin sensitivity of the ATPase activity of F_1 bound to the particles was tested after suspending the sediment (1–2 mg protein) in 1 ml of the sucrose/Tris/EDTA medium at 0 °C, followed by centrifugation and resuspending the sediment in 1 ml of medium.

Protein determination

Soluble protein was determined by the method of Lowry et al. [24], using bovine serum albumin as standard. Particulate protein was determined by the biuret method after precipitation with trichloroacetic acid and extraction with acidic ethanol [25].

Materials

ATP, phospho*enol*pyruvate and pyruvate kinase were obtained from Boehringer; Sephadex G-75, G-50 and G-25, diethylaminoethyl Sephadex A-50 and carboxymethylcellulose were obtained from Pharmacia, and antimycin from Boehringer. Aurovertin was prepared in our laboratory by Dr. R. M. Bertina [26].

RESULTS

Concentration of aurovertin-, oligomycin- and antimycin-binding sites

In Table I, the concentration of aurovertin-binding sites in AS particles is compared with the concentration of antimycin- and inhibitory oligomycin-binding sites. The concentration of antimycin-binding sites (equal to the concentration of cytochrome c_1 [27]) was measured by fluorescence quenching [23] and inhibition of electron transfer. The concentration of inhibitory oligomycin-binding sites was determined as the minimum amount of oligomycin required maximally (60–70 %) to inhibit NADH oxidation, after a 3 min induction period (cf. Bertina et al. [28]), in the absence of ADP and phosphate. The table also includes the concentration of F_1-binding sites in particles derived from the AS, and T particles, determined as described below.

TABLE I

CONCENTRATION OF F_1-, AUROVERTIN-, ANTIMYCIN- AND OLIGOMYCIN-BINDING SITES IN SUBMITOCHONDRIAL PARTICLES

Particle	Concentration (μmol/g) of binding sites for			
	F_1	Aurovertin	Antimycin	Oligomycin*
AS		0.35	0.55	0.21
ASU	0.32		0.58	
ASU+OSCP	0.46			
ASUA	0.05			
ASUA+OSCP	0.36			
ASUA+OSCP+Fc_2	0.53			
T			0.57	
TU	0.56		0.59	
TUA	0.09			
TUA+OSCP+Fc_2	0.53			

* Concentration required for maximal inhibition of respiration.

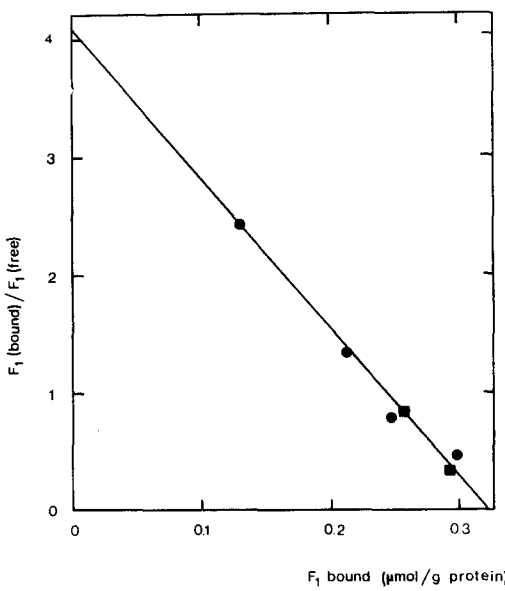

Fig. 1. Scatchard plot of binding of F_1 to ASU particles. Two experiments are shown (● and ■, respectively). The concentration of ASU particles was 1.35 mg protein/ml.

Binding of F_1 to urea-treated particles

ASU and TU particles contained only a small amount of residual ATPase activity, amounting to 6 % and 1 %, respectively, of the activity of the parent particles. Assuming that the ATPase activity of the residual F_1 is the same as that in the parent particles, the concentration of F_1 in ASU and TU particles is 0.02 and 0.005 μmol/g protein, respectively.

Fig. 1 shows a Scatchard plot of two experiments in which the binding of F_1

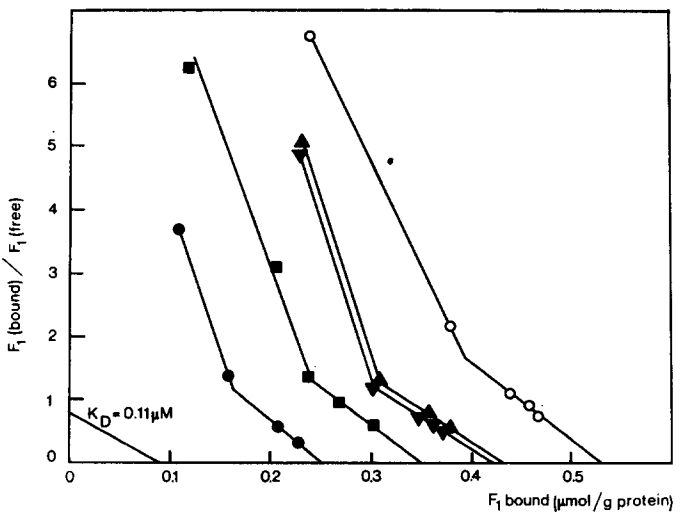

Fig. 2. Scatchard plot of binding of F_1 to TUA particles in the presence and absence of OSCP and Fc_2. The concentration of TUA particles was 1 mg/ml. The curve in the bottom left is for no OSCP, others show effects of 6 (●), 9 (■), 12 (▼) and 15 (▲) μg OSCP present, respectively, and of 18 μg OSCP+40 μg Fc_2 (○).

to ASU particles was measured. From this plot it may be calculated that ASU particles contain 0.32 μmol free F_1-binding sites per mg protein, or 0.34 μmol/g total allowing for the residual F_1. This is about the same as the concentration of aurovertin-binding sites in the parent AS particles (0.35 μmol/g, see Table I). The dissociation constant of the rebound F_1, calculated from the slope of the straight line in the Scatchard plot, is 0.11 μM.

The ATPase activity of the reconstituted particles, washed once by recentrifugation at 0 °C, was inhibited by 90 % by oligomycin, from which it may be concluded that there was little dissociation of the rebound F_1 from the dilute particle suspension during the 5 min assay period, at least in the presence of oligomycin. Suspending the reconstituted particles at room temperature resulted in some of the F_1 dissociating again, as would be expected from a binding constant of 0.11 μM.

A similar experiment with TU particles showed that these contained 0.56 μmol/g F_1-binding sites, compared with 0.59 μmol/g antimycin-binding sites (see Table I). The dissociation constant of the rebound F_1 was the same as for ASU particles.

Binding of F_1 to urea- and alkali-treated particles

Treatment of ASU and TU particles with ammonia resulted in a reduction of the concentration of F_1-binding sites to 0.05 and 0.09 μmol/g, respectively (Table I). The K_D for binding to residual sites was the same as to the urea-treated particles. These residual sites probably represent unaltered F_1-binding sites that escaped extraction with ammonia. The concentration of F_1-binding sites was increased by adding OSCP. In the presence of OSCP, a biphasic Scatchard plot was obtained, suggesting two dissociation constants (Fig. 2). The concentration of the tighter binding sites increased with increasing amount of OSCP added, whereas the concen-

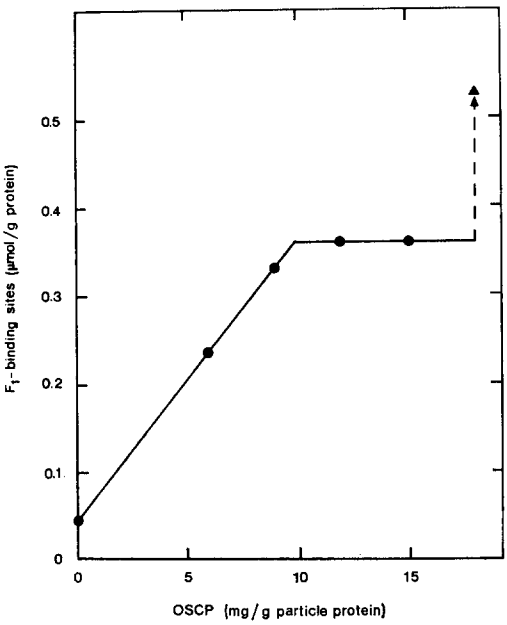

Fig. 3. Concentration of F_1-binding sites in ASUA particles as a function of the amount of OSCP added. Each point represents the intercept with the abscissa in Scatchard plots carried out in the same way as in Fig. 2. The point represented by ▲ was obtained in the presence of 18 μg OSCP and 40 μg Fc_2. The concentration of protein was 1 mg/ml.

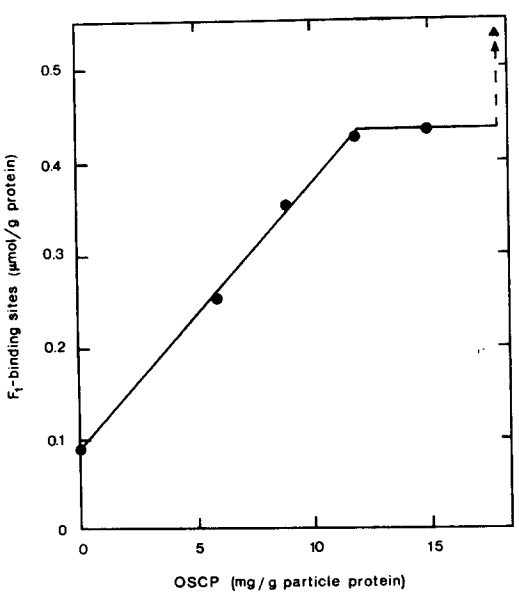

Fig. 4. Concentration of F_1-binding sites in TUA particles as a function of the amount of OSCP added. Each point represents the intercept with the abscissa in the Scatchard plots shown in Fig. 2. The point represented by ▲ was obtained in the presence of 18 μg OSCP and 40 μg Fc_2. The concentration of protein was 1 mg/ml.

tration of the weaker binding sites did not vary. The former presumably represent F_1-binding sites reconstituted by binding of OSCP to the membrane, the latter represent the unextracted binding sites. The dissociation constant of the tight binding site was found to be at least 0.02 μM with TUA particles + OSCP (Fig. 2) and 0.04 μM with ASUA particles + OSCP (not shown).

In Figs. 3 and 4 the total concentration of F_1-binding sites is plotted for ASUA and TUA particles, respectively, as a function of OSCP concentration. In the former case, 0.36 μmol/g binding sites are reconstituted, about the same as in the ASU particles. However, 0.46 μmol/g F_1-binding sites can be obtained by adding excess OSCP to ASU particles (not shown). The maximum concentration of F_1-binding sites reconstituted with OSCP in TUA particles (0.43 μmol/g) is somewhat lower than found in TU particles. It appears, then, that the treatment with ammonia after urea removes another factor that limits binding of F_1 to the particles, even in the presence of excess OSCP. The last points in Figs. 3 and 4 show that the addition of Fc_2, in the presence of excess OSCP, raises the concentration of F_1-binding sites in the urea- and ammonia-treated particles to 0.53 μmol/g F_1 in both cases.

The titrations with OSCP shown in Figs. 3 and 4 give information on the purity of the OSCP preparation, if it is assumed that it reacts mole for mole with F_1, and that the molecular weight of OSCP is 18 000* [6]. The slopes of the lines in Figs. 3 and 4 correspond to 1 nmol F_1 per 32 and 35 μg, respectively, of the OSCP prepa-

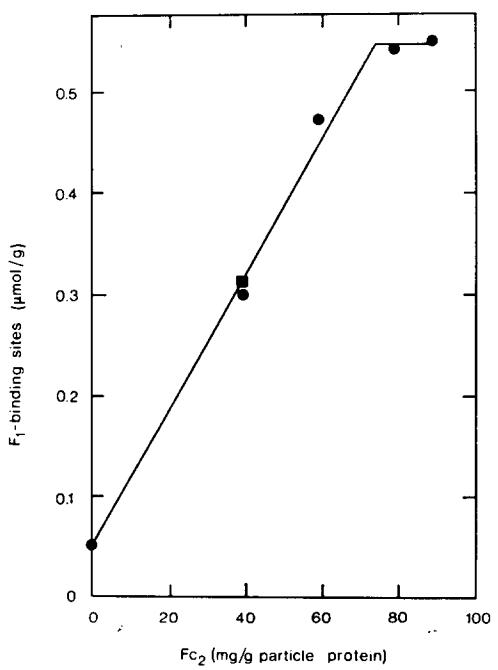

Fig. 5. Concentration of F_1-binding sites in TUA-STA particles as a function of the amount of Fc_2 added. ■, no OSCP added; ●, 18 μg OSCP/mg particle protein added.

* In our hands, the apparent molecular weight, measured by polyacrylamide gel electrophoresis, of isolated OSCP and of OSCP in the ATPase complex is 24 000.

ration, suggesting that the latter is about 50–55 % pure. The course of the titration shows that it is free from other factors that might affect the binding, in particular of Fc_2.

Binding of F_1 to silicotungstate-treated particles

In order further to study the role of Fc_2 in the binding of F_1 to particles, the TUA particles were extracted by silicotungstate, the procedure introduced by Knowles et al. [7] to isolate Fc_2. According to these authors, silicotungstate-extracted particles are more stable than thiocyanate-extracted particles, which are also deficient in Fc_2. In our hands silicotungstate-extracted particles can be kept in liquid nitrogen for several weeks without loss of reconstitutive activity. TUA-STA particles bind little F_1, even in the presence of excess OSCP. Fig. 5 shows that Fc_2 induces binding of F_1 to these particles, a concentration of 0.53 μmol/g protein being obtained with excess Fc_2. The dissociation constant was 0.1 μM, the same as with ASU and TU particles. In agreement with Knowles et al. [7], F_1 was found to bind to the particles in the presence of Fc_2 even in the absence of OSCP (see Fig. 5), but the dissociation constant was somewhat larger (0.2 μM) and, more important ,the ATPase activity of F_1 bound in the absence of OSCP is oligomycin-insensitive.

The data with the various particle preparations derived from TU particles are summarized in Table II.

TABLE II

BINDING PARAMETERS OF F_1 TO TU PARTICLES AND DERIVED PARTICLES AND OLIGOMYCIN SENSITIVITY

System	F_1-binding sites (μmol/g)	K_D (μM)	Oligomycin sensitivity of ATPase activity (%)
TU	0.56*	0.1	92
TUA	0.09	0.11	–
TUA+OSCP	0.43	0.025	91
TUA+OSCP+Fc_2	0.53	0.025	90
TUA STA	0.014	0.1	–
TUA STA+OSCP	0.05	0.1	–
TUA STA+Fc_2	**	0.2	12
TUA STA+Fc_2+OSCP	0.54	0.1	88

* Including residual F_1 in TU particles.

** Measured only with sub-optimal amounts of Fc_2 where OSCP had no effect on the concentration of F_1-binding sites (see Fig. 5).

DISCUSSION

It must be emphasized that all the binding studies described in this paper were carried out in a low-salt medium free from added Mg^{2+} and containing 2 mM EDTA. Bulos and Racker [12] and Tzagoloff [29] have shown that, in the presence of salt, F_1 binds to submitochondrial particles in the absence of OSCP and that the resulting

ATPase activity is oligomycin-insensitive. When the EDTA was replaced by the same concentration of $MgCl_2$ in our suspending medium, F_1 was found to bind very strongly (K_D 3–4 nM) to TUA and ASUA particles in the absence of OSCP (Berden, J. A. and de Metz, M., unpublished observations). This finding explains why F_1 does not dissociate from the particles in the assay medium for measuring the ATPase activity which contained 5 mM $MgCl_2$.

Submitochondrial particles successively treated with alkali, Sephadex, urea and alkali (ASUA particles) are lacking the ATPase inhibitor (removed by Sephadex), F_1 (removed by urea) and OSCP (removed particularly by the second alkali treatment). The reconstituted oligomycin-sensitive ATPase obtained by incubating ASUA particles with OSCP and F_1 contains more F_1 than the AS particles, but the F_1 is less firmly bound than before treatment with urea.

Both ASUA and TUA particles show an absolute requirement for OSCP for F_1 binding. TUA particles extracted with the chaotropic reagent, silicotungstate, known from the work of Knowles et al. [7] to extract Fc_2, show an absolute requirement for Fc_2, but can bind F_1 in the absence of OSCP. The reconstituted ATPase is however, oligomycin insensitive and the reconstitution of an oligomycin-sensitive ATPase with TUA-STA particles requires both Fc_2 and OSCP, as well as F_1.

It may be concluded from this and previous work that an intact oligomycin-sensitive ATPase requires F_o, which is not extracted from the membrane by any of the treatments given here, in addition to the F_1, OSCP and Fc_2 which are successively extracted by urea, alkali and silicotungstate. F_o does not seem to bind F_1, but both OSCP and Fc_2 can. A complex of F_1 and OSCP was isolated by Vallejos et al. [5] under the name of $F_1 \cdot X$. The binding site for F_1 on Fc_2 is presumably buried in ASUA and TUA particles, since these particles, although they contain Fc_2, do not bind F_1. Since the ATPase activity of particles reconstituted from TUA-STA particles, OSCP, Fc_2 and F_1 is oligomycin sensitive, the F_1 is presumably bound to the OSCP rather than to the Fc_2 in the reconstituted particles.

It seems reasonable to conclude, then, that OSCP is the binding site for F_1 in the intact particle, and that Fc_2 is necessary for the binding of the $F_1 \cdot$ OSCP complex to F_o.

Electron micrographs of sub-mitochondrial particles after negative staining show a globular protein projecting outside the membrane and linked to it with a stalk [30]. Racker and co-workers have shown that the globular protein is lacking in membranes stripped of F_1 and is restored in particles reconstituted with F_1 [31, 3]. Since, moreover, the diameter of the globular protein is consistent with a molecule of about 300 000 daltons, the size of F_1, they have suggested that the globular protein is F_1. McLennan and Asai [15] have shown that when OSCP as well as F_1 is stripped from the membranes, reconstitution of the characteristic electron microscopic pattern requires both F_1 and OSCP, and have suggested that OSCP is identical with the stalk. As Racker [32] has pointed out it is, however, possible that OSCP is not itself the material of the stalk but is necessary for the specific binding of F_1 to the membrane which yields the structure seen in negatively stained electron micrographs. He suggests that the stalk might be a stretched out subunit of F_1. In any case, Fc_2 is unlikely to be the stalk material, since it is much less easily extracted from the membrane than either F_1 or OSCP.

Bertina et al. [28] have shown that in both rat-heart and rat-liver mitochon-

dria, the concentration of F_1, measured by aurovertin binding, is equal to the concentration of cytochrome c_1, the latter being measured by antimycin binding. In agreement with this conclusion, the concentration of F_1-binding sites in TU particles is equal to that of antimycin-binding sites (Table I). AS particles, however, contain considerably less F_1, and ASU particles contain less F_1-binding sites than antimycin-binding sites. It seems, then, that during their preparation A particles lose not only F_1, but also some F_1-binding sites. The latter cannot be fully restored by OSCP, but can by Fc_2 and it is striking that the concentration of F_1-binding sites in ASUA particles in the presence of OSCP and Fc_2 is the same as that of the antimycin-binding sites. TUA particles have also lost some F_1-binding sites that are not restored by OSCP and these are also restored to the original level by adding Fc_2. The 1 : 1 stoicheiometry between F_1 and cytochrome c_1 found with intact mitochondria also seems to hold, then, for submitochondrial particles, provided that factors lost during preparation are restored. It should be mentioned, however, that the number of F_1-binding sites calculated from the Scatchard plots, is dependent upon the molecular weight of F_1, and that there is some uncertainty about the accuracy of the 360 000 assumed.

Bertina et al. [28] found also one specific inhibitory oligomycin-binding site for each molecule of F_1 in both liver and heart mitochondria. Lee and Ernster [33] have shown that, in addition to the inhibition of phosphorylation by oligomycin seen with both intact mitochondria and submitochondrial particles, low concentrations of oligomycin induce coupling in sub-mitochondrial particles, probably by blocking a "leak" of some description [32, 1, 34]. It is interesting that the concentration of oligomycin necessary to bring about this coupling in AS particles, as measured by induction of respiratory control (Table I), is equal to the difference between the concentration of antimycin-binding sites and F_1 in the AS particles. This suggests that oligomycin blocks the "leak" by combining with those F_o molecules that have lost their F_1 and that oligomycin combines preferentially with these molecules. This is in good agreement with the suggestion that F_o is the source of the leak, possibly acting as a proton channel [35]. Ernster et al. [36] have shown that the oligomycin titre for coupling in AS particles is increased by extraction of F_1 from the particles by urea.

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