BBA 46299

$F_1 \cdot X$, A COMPLEX BETWEEN F_1 AND OSCP

R. J. VAN DE STADT, R. J. KRAAIPOEL* AND K. VAN DAM

*Laboratory of Biochemistry, B. C. P. Jansen Institute**, University of Amsterdam, Amsterdam (The Netherlands)*

(Received November 19th, 1971)

SUMMARY

1. F_1 and OSCP, soluble coupling factors isolated from beef-heart mitochondria, form a complex.

2. The binding between F_1 and OSCP is weakened by $(NH_4)_2SO_4$, but not enough to allow complete separation of F_1 and OSCP in a linear sucrose gradient. Complete separation is achieved when both $(NH_4)_2SO_4$ and deoxycholate are present.

3. $F_1 \cdot X$, a coupling factor also isolated from beef-heart mitochondria, can be separated in F_1 and OSCP activities in the presence of $(NH_4)_2SO_4$ and deoxycholate by centrifugation in a linear sucrose gradient or by gel filtration on a Sephadex G-100 column.

4. $F_1 \cdot X$ preparations of low specific activity contain a contaminating protein. This protein can be dissociated from the complex by $(NH_4)_2SO_4$. $F_1 \cdot X$ preparations with a high specific activity are virtually free of this contaminating protein.

5. $F_1 \cdot X$ preparations contain approximately 30 % free F_1.

6. Analytical gel electrophoresis of $F_1 \cdot X$, F_1 and OSCP in the presence of sodium dodecyl sulphate indicates that apart from F_1 and OSCP no other components are present in pure $F_1 \cdot X$.

INTRODUCTION

Mitochondrial ATPases have been isolated from beef heart[1], yeast[2] and rat liver[3], and the beef-heart and rat-liver enzymes have been purified to homogeneity (see refs 1, 3–6). The molecular weight is in each case about 360 000[3,7]. During gel electrophoresis in polyacrylamide–sodium dodecyl sulphate[8,9] the bovine-heart enzyme is split into 5 subunits[6,10] with different molecular weights. In addition

Abbreviations: F_1, mitochondrial ATPase; OSCP, oligomycin-sensitivity-conferring protein; A particles, submitochondrial particles prepared by sonication of heavy beef-heart mitochondria at an alkaline pH (ammonia); ASU particles, submitochondrial particles, prepared by treatment of A particles with Sephadex G-50 and urea; ASUA particles, submitochondrial particles prepared by exposure of ASU particles to sonic oscillation in the presence of ammonia; F_0 activity, property of mitochondrial membrane fragments to bind F_1 in such a way that the ATPase activity becomes sensitive to oligomycin.

* Present address: Sophia Children's Hospital, Neonatal Unit, Academic Hospital, School of Medicine, Gordelweg 160, Rotterdam, 3004, The Netherlands.

** Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

to these subunits, native F_1 probably contains a small peptide that can be identified with the mitochondrial ATPase inhibitor[5,10], described by Pullman and Monroy[11], with a molecular weight of 10 500.

Racker and co-workers have extensively studied the physiological role of the beef-heart enzyme, called by them F_1 (Factor 1) in oxidative phosphorylation. The soluble enzyme is cold labile and insensitive to energy-transfer inhibitors, such as oligomycin[1,12]. Reassociation of F_1 with mitochondrial membranes that are deficient in F_1, the so-called ASU (ammonia–Sephadex–urea) particles[13,14], restores the oligomycin sensitivity and cold stability of the ATPase and also enhances the capacity of the membrane system to catalyse energy-linked reactions (see refs 12–16).

When ASU particles are further resolved with ammonia, yielding ASUA particles[17], F_1 no longer binds to the membrane and the ATPase activity remains oligomycin insensitive. Oligomycin sensitivity (*i.e.* F_0 activity) can be restored by a second soluble coupling factor, called OSCP (Oligomycin-Sensitivity-Conferring Protein), isolated in nearly pure form by MacLennan and Tzagoloff[18]. A rather heterogeneous protein fraction with similar properties was isolated by Bulos and Racker[19]. Presumably OSCP is a highly purified preparation of the active component of the preparation of Bulos and Racker[19,20].

In this laboratory, Vallejos *et al.*[21] isolated a soluble coupling factor, designated $F_1 \cdot X$, that closely resembles F_1 (ref. 1) with respect to its high ATPase activity, but has a much higher coupling activity. Groot and Meyer[22] showed that cold-treated $F_1 \cdot X$ induces F_0 activity in ASUA particles, and concluded that X is OSCP. Sani *et al.*[23], however, suggested that X may be a larger protein comprising OSCP and a factor described by them (Factor D).

The purpose of the present paper is to analyse $F_1 \cdot X$ more closely, using three techniques: analysis of the sedimentation velocity in a linear sucrose gradient, gel filtration on Sephadex G-100 and gel electrophoresis. We have compared the properties of F_1 and OSCP present in $F_1 \cdot X$ with those of isolated F_1 and OSCP and examined the possible presence of other protein components in $F_1 \cdot X$.

METHODS

Preparation of beef-heart mitochondria, submitochondrial particles and coupling factors

Heavy and light beef-heart mitochondria were prepared according to the method of Crane *et al.*[24] with slight modifications. A particles[25], ASU particles[13], coupling factor F_1 (ref. 5), coupling factor $F_1 \cdot X$ (ref. 21) and the ATPase inhibitor[5] were prepared according to the published procedures. OSCP was prepared essentially according to MacLennan and Tzagoloff[18]. The protein was purified further by elution from a carboxymethylcellulose column with a NaCl gradient. ASUA particles were prepared by exposure of about 10 ml ASU particles (20 mg/ml) to 0.5 vol. of 1.5 M ammonia for 30 min at 0°C[17]. After sonication for 2 min with a Branson sonifier, Model S125, equipped with a small probe set at Number 1, the particles were collected by centrifugation at 200 000 × *g* for 20 min and suspended in 0.25 M sucrose.

Measurement of the ATPase activity

An appropriate sample (not more than 0.3 unit (see below) of ATPase activity)

was preincubated at 30°C in a final volume of 0.8 ml containing 8 μmoles Tris–acetate buffer (pH 7.5), 1 μmole $MgCl_2$ and 25 μmoles sucrose. After 5 min the reaction was started by the addition of 0.2 ml of a solution containing 20 μmoles Tris–acetate buffer (pH 7.5), 5 μmoles ATP, 5 μmoles phosphoenolpyruvate, 3 μmoles $MgCl_2$ and 30 μg pyruvate kinase. After 5 or 10 min the reaction was stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid.

1 Unit of ATPase activity is defined as the amount of enzyme that hydrolyses 1 μmole ATP per min under the assay conditions. Specific activity is expressed as units per mg protein.

Assay of OSCP activity

Two methods were used:

(1) By the induction of F_0 activity in ASUA particles. 0.3 mg ASUA particles, 3 μg F_1, 5 μg oligomycin and an appropriate sample containing OSCP activity were preincubated at 30°C as described under *Measurement of the ATPase activity*. The ATPase activity in a control experiment without oligomycin was taken as a reference.

(2) By stimulation of the $^{32}P_i$–ATP exchange activity in A particles. 0.5 mg A particles, 40 μg F_1 and an appropriate sample with OSCP activity were preincubated at 30°C in a final volume of 0.75 ml containing 200 μmoles sucrose, 7.5 μmoles Tris–acetate buffer (pH 7.5) and 1 μmole $MgCl_2$. After 10 min the reaction was started by adding 0.25 ml of a solution containing 25 μmoles Tris–acetate buffer (pH 7.5), 4 μmoles $MgCl_2$, 4 μmoles ADP, 10 μmoles ATP, 2 μmoles Na_2S and 25 μmoles $^{32}P_i$ buffered to pH 7.5 ($2 \cdot 10^6$ counts/min). After 10 min incubation at 30°C the reaction was stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid. The ^{32}P incorporated into ATP was determined, after extraction of inorganic phosphate according to the method of Nielsen and Lehninger[26], by counting an aliquot of the aqueous layer in a Nuclear Chicago Gasflow Counter.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate

Electrophoresis was carried out vertically in gel columns immersed in buffer at room temperature as described by Groot *et al.*[27]. 30-μl samples were subjected to electrophoresis on 12% polyacrylamide gels containing 0.1% sodium dodecyl sulphate. The gels were fixed and stained with Coomassie blue. The stained gels were scanned at 600 nm with a scanning attachment for a Gilford spectrophotometer.

Centrifugation in a linear sucrose gradient

Linear sucrose gradients (5–15%) were prepared in a final volume of 5 ml, in standard Buffer I containing 10 mM Tris–acetate buffer (pH 7.5) and 1 mM EDTA. 0.20-ml samples, layered on top of the gradients, were centrifuged for 2.5 h at 50 000 rev./min in a Spinco 50 swing-out rotor, Type SW 50L, at room temperature. After puncturing the tubes at the bottom, ten fractions of 0.5 ml were collected. The following activities in the fractions were assayed: ATPase, the activity of a 20-μl sample was tested as described; OSCP, after cold inactivation of F_1 in the fractions for at least 3 h at 0°C, 0.20-ml samples were assayed for OSCP activity as described. The protein content of 0.20-ml samples was determined according to the method of Lowry *et al.*[28].

Gel filtration on Sephadex G-100

Samples of 0.5 ml were layered on top of a column (1.1 cm × 60 cm), previously equilibrated in standard Buffer II, containing 25 mM Tris–acetate buffer (pH 7.5) and 1 mM EDTA. Downward elution with the same buffer (15–20 ml/h) was continued until no more protein was eluted. The column was calibrated with cytochrome *c* and blue dextran 2000. Fractions of 1 ml were collected and assayed for the following activities: ATPase, the activity of a 5–10-μl sample was tested as described; OSCP, after cold inactivation of F_1 for at least 3 h at 0°C, 0.10-ml samples were assayed for OSCP activity as described. The absorbance at 280 nm was monitored as a measure of the protein content of the samples.

Analytical methods

Inorganic phosphate was determined by the method of Fiske and Subbarow as modified by Sumner[29]. Soluble protein was determined by the method of Lowry *et al.*[28] with bovine serum albumin as a standard, insoluble protein by the biuret method as described by Cleland and Slater[30].

MATERIALS

Carrier-free $H_3{}^{32}PO_4$ was purchased from Philips–Duphar. It was boiled in 0.1 M HCl for 3 h and neutralized with 1 M KOH before use.

ATP, ADP, pyruvate kinase and phosphoenolpyruvate were obtained from Boehringer und Söhne. Oligomycin was kindly provided by the Upjohn Chemical Co., dithiothreitol was purchased from Calbiochem and sodium dodecyl sulphate from Sigma. Deoxycholate was purchased from Koch-Light and recrystallized in ethanol.

Sephadex G-50 and G-100 were purchased from Pharmacia. All other chemicals were of analytical grade purity.

RESULTS

In preliminary experiments of G.S.P. Groot and M. Meyer (unpublished), using gel filtration on Sephadex G-100 with 0.05 M Tris–sulphate buffer (pH 7.5) as an eluent, it was not possible to separate $F_1 \cdot X$ into F_1 and OSCP. Both activities appeared in the same peak, together with the bulk of protein, close to the marker of the void volume. This suggested that F_1 and OSCP are strongly associated within $F_1 \cdot X$, a situation that bears some analogy with results described by Sani *et al.*[23], where Factor A and a new Factor D seem to be associated rather tightly, yielding Factor A·D.

To investigate the conditions under which $F_1 \cdot X$ can be separated, the behaviour of isolated F_1 and OSCP, both alone and in combination, were studied.

Sedimentation of F_1, OSCP and F_1 plus OSCP in a linear sucrose gradient

The F_1 prepared according to the method of Horstman and Racker[5] had a specific activity of 73. This method is reported by Penefsky and Warner[4] and Lambeth *et al.*[7] to yield a homogeneous preparation with a molecular weight of 360000 (ref. 7). OSCP prepared according to the method of MacLennan and Tzagoloff[18] is reported to be nearly homogeneous, with a molecular weight of 18 000.

According to the upper profile of Fig. 1, F_1 behaves as a homogeneous protein. The specific activity of F_1 is somewhat higher (80–90) in the peak fraction, possibly due to activation of the ATPase during centrifugation, since it has been reported that this preparation contains endogenous ATPase inhibitor[5,7] that may dissociate in the absence of Mg^{2+}–ATP. No OSCP activity could be detected in F_1 alone.

The second profile of Fig. 1 shows that OSCP (15 μg) remains in the top of the gradient. For the third profile of Fig. 1, 400 μg of F_1 and 15 μg of OSCP were first preincubated for 15 min at room temperature and then subjected to centrifugation. It can be seen that the F_1 and OSCP activity appear in the same peak, indicating that they associate under the conditions employed. This is in contrast to the results reported by Tzagoloff[31], with F_1 and OSCP isolated from yeast mitochondria. The F_1 and OSCP activities remained separate in a linear glycerol gradient (5–15 %).

In the following experiment, conditions were established for the separation of F_1 and OSCP from the complex. Fig. 2A shows that in the presence of 0.25 M $(NH_4)_2SO_4$, the OSCP activity lags behind the F_1 peak, but complete separation is not obtained unless deoxycholate is also present. Even under these conditions some interaction remains, judged by the relative positions of the OSCP peak in Figs 1B and 2B.

Sedimentation of F_1·X in a linear sucrose gradient

The upper profile of Fig. 3 shows that, in the absence of $(NH_4)_2SO_4$ or deoxycholate, the ATPase and OSCP activities appear in a single peak. Some protein remains behind during sedimentation with the result that the specific ATPase activity in the peak fraction increased from the relatively low starting activity

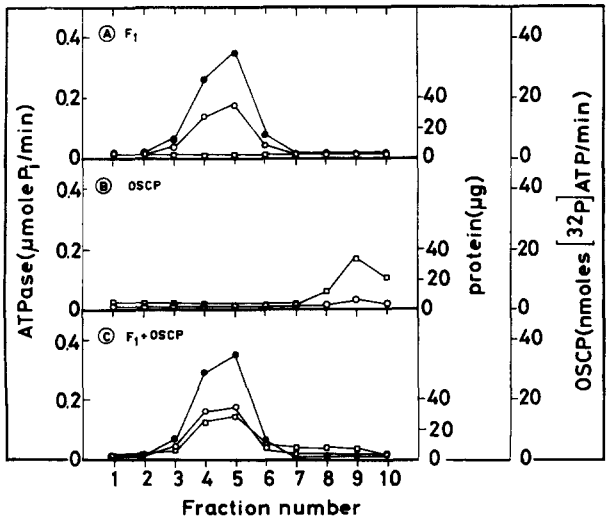

Fig. 1. Sedimentation pattern of F_1, OSCP and F_1 *plus* OSCP in a linear sucrose gradient. The gradients were prepared in standard Buffer I. (A) 400 μg F_1 were dissolved in 0.20 ml Buffer I and allowed to stand for 15 min at 25°C before centrifugation. (B) 15 μg OSCP were dissolved in 0.20 ml Buffer I. (C) 400 μg F_1 and 15 μg OSCP were dissolved in 0.20 ml Buffer I and allowed to stand for 15 min at 25°C before centrifugation. ●—●, ATPase; ○—○, protein; □—□, OSCP activity.

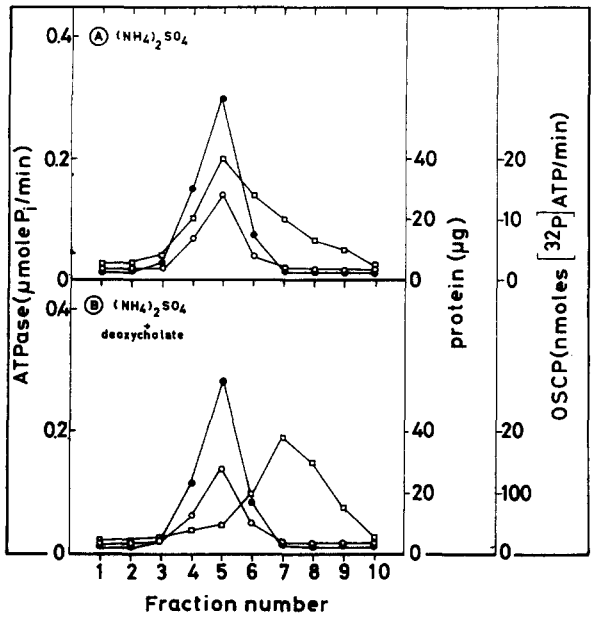

Fig. 2. The effect of $(NH_4)_2SO_4$ and deoxycholate on the sedimentation pattern of F_1 *plus* OSCP in a linear sucrose gradient. (A) The gradient was prepared in Buffer I, containing 0.25 M $(NH_4)_2SO_4$. 300 μg F_1 and 12 μg OSCP were dissolved in 0.20 ml Buffer I; after 15 min at 25°C, $(NH_4)_2SO_4$ was added to a final concentration of 0.25 M. The solution was centrifuged after 15 min at 25°C. (B) The gradient was prepared in Buffer I, containing 0.25 M $(NH_4)_2SO_4$ and 0.1% sodium deoxycholate. 300 μg F_1 and 12 μg OSCP were dissolved in 0.20 ml Buffer I and allowed to stand for 15 min at 25°C. After the addition of $(NH_4)_2SO_4$ and sodium deoxycholate to final concentrations of 0.25 M and 0.1 mg per mg F_1, respectively, centrifugation was carried out after a further 15 min. ●—●, ATPase; ○—○, protein; □—□, OSCP activity.

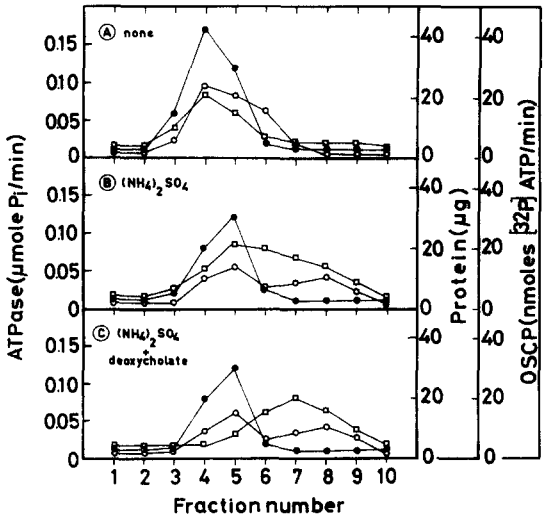

Fig. 3. The effect of $(NH_4)_2SO_4$ and deoxycholate on the sedimentation pattern of F_1·X in a linear sucrose gradient. (A) Same conditions as in Fig. 1A, 400 μg F_1·X (spec. act. 38). (B) Same conditions as in Fig. 2A, 300 μg F_1·X (spec. act., 38). (C) Same conditions as in Fig. 2B, 300 μg F_1·X (spec. act., 40). ●—●, ATPase; ○—○, protein; □—□, OSCP activity.

(35–40 units/mg protein) to 60 units/mg protein. $(NH_4)_2SO_4$, especially in the presence of deoxycholate (Fig. 3C), causes dissociation of the ATPase and OSCP activities. In the presence of $(NH_4)_2SO_4$ contaminating protein, already visible in Fig. 3A, is separated from both the ATPase and the OSCP. The specific ATPase activity in the peak fraction is increased to 83 units/mg protein. In a separate experiment it was shown that the attached protein can be split off also in the presence of 0.5 M urea.

Gel filtration of $F_1 \cdot X$ on Sephadex G-100

The results obtained by centrifugation studies of $F_1 \cdot X$ are confirmed by analysis of the elution pattern from a Sephadex G-100 column. In the experiment shown in Fig. 4, OSCP activity was measured by two methods — stimulation of the $^{32}P_i$–ATP exchange in A particles and induction of F_0 activity in ASUA particles— with essentially the same results.

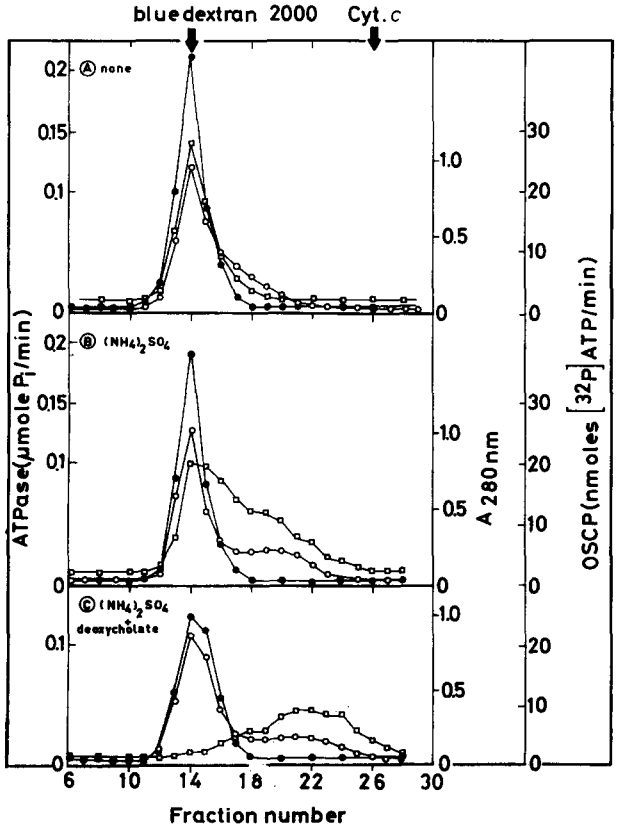

Fig. 4. The effect of $(NH_4)_2SO_4$ and deoxycholate on the elution pattern of $F_1 \cdot X$ from Sephadex G-100. (A) 2.1 mg $F_1 \cdot X$ (spec. act., 37), dissolved in 0.50 ml standard Buffer II, were layered on top of the column after 15 min at 25°C, and the elution was started with Buffer II. (B) Same conditions as under A, except that the preincubation mixture and the elution Buffer II were supplemented with 0.25 M $(NH_4)_2SO_4$. (C) Same conditions as under A, except that the preincubation mixture contained 0.25 M $(NH_4)_2SO_4$ and 0.1 mg sodium deoxycholate per mg $F_1 \cdot X$. The elution Buffer II was supplemented with 0.25 M $(NH_4)_2SO_4$ and 0.1 % sodium deoxycholate. ●—●, ATPase; ○—○, $A_{280\,nm}$; □—□, OSCP activity.

When $F_1 \cdot X$ was extracted with successive small amounts of water[21] from an acetone powder of heavy beef-heart mitochondria (150 mg powder in 1 ml water), the fourth and fifth extraction yielded a preparation with high specific activity (60 units/mg protein) that lacked the contaminating protein (Fig. 5).

Estimation of the molar ratio of F_1 and OSCP in the $F_1 \cdot X$ preparations

To estimate the content of OSCP in various fractions of $F_1 \cdot X$, obtained after successive extractions of an acetone powder, 0.4 mg ASUA particles were

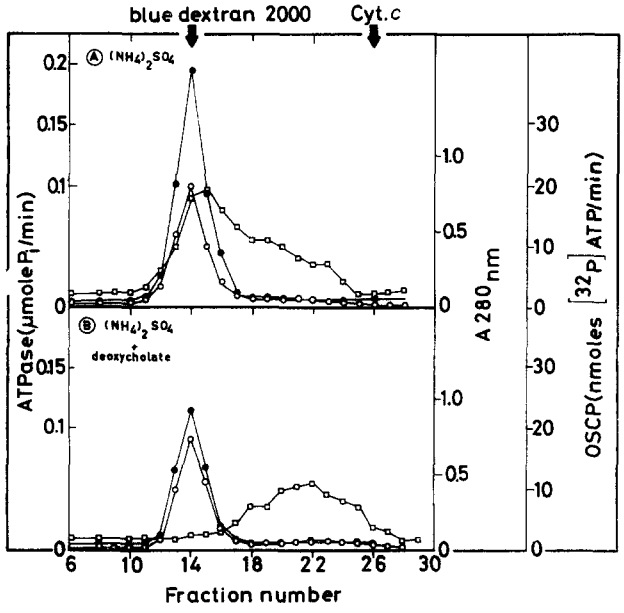

Fig. 5. The effect of $(NH_4)_2SO_4$ and deoxycholate on the elution pattern of $F_1 \cdot X$ with high specific activity from Sephadex G-100. Same conditions as described under Figs 4B and 4C, respectively. (A) 1.7 mg $F_1 \cdot X$ (Spec. act., 60). (B) 1.7 mg $F_1 \cdot X$ (spec. act., 60). ●—●, ATPase; ○—○, $A_{280\ nm}$; □—□, OSCP activity.

TABLE I

ESTIMATION OF THE FREE F_1 IN $F_1 \cdot X$

0.4 mg ASUA particles were preincubated with the various fractions of $F_1 \cdot X$, each containing about 0.2 ATPase unit, in the presence and absence of 5 μg oligomycin. After 5 min the ATPase activity was assayed. The inhibition by oligomycin was calculated from the formula, [(% inhibition −5)/(94−5)]·100, which takes account of the facts that the ATPase activity of 0.27 unit of F_1 in the presence of 0.4 mg ASUA particles was inhibited by oligomycin by 5%, and the ATPase activity of the $F_1 \cdot X$ tested (0.2 unit) was inhibited to the extent of 94% by oligomycin in the presence of 0.4 mg ASUA particles and 0.6 μg OSCP.

Fraction	*ATPase activity (units/mg protein)*	*Inhibition by oligomycin* (%)	*F_1 in $F_1 \cdot X$* (%)
1	11	45	53
2	26	58	35
3	49	63	26
4	59	59	32
5	59	64	24

preincubated with the $F_1 \cdot X$ fraction, containing about 0.2 ATPase unit (Table I). The percentage inhibition of the ATPase by oligomycin was calculated, allowing for the endogenous F_0 activity of the particles (5%) and the maximal inhibition that can be obtained in the presence of an excess of OSCP (94%).

In a separate experiment 0.4 mg of the same ASUA particles were preincubated with 3.6 μg F_1 (0.27 ATPase unit) and increasing amounts of OSCP (Fig. 6). The titration curve of the inhibition of the ATPase activity with OSCP is not linear, so that one cannot assume that the percentage oligomycin-resistant ATPase activity

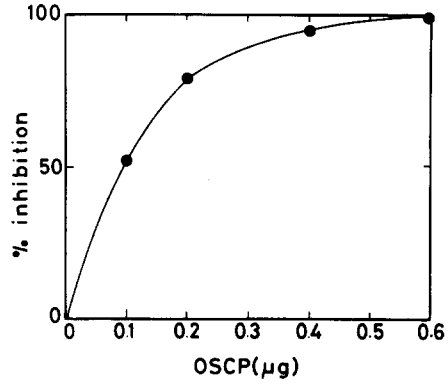

Fig. 6. Induction of F_0 activity in ASUA particles by OSCP in the presence of F_1. 0.4 mg ASUA particles were preincubated with 3.6 μg F_1 and increasing amounts of OSCP in the presence and absence of 5 μg oligomycin. After 5 min at 30°C the ATPase activity was assayed. The percentage inhibition of the ATPase activity by oligomycin was calculated as described in Table I.

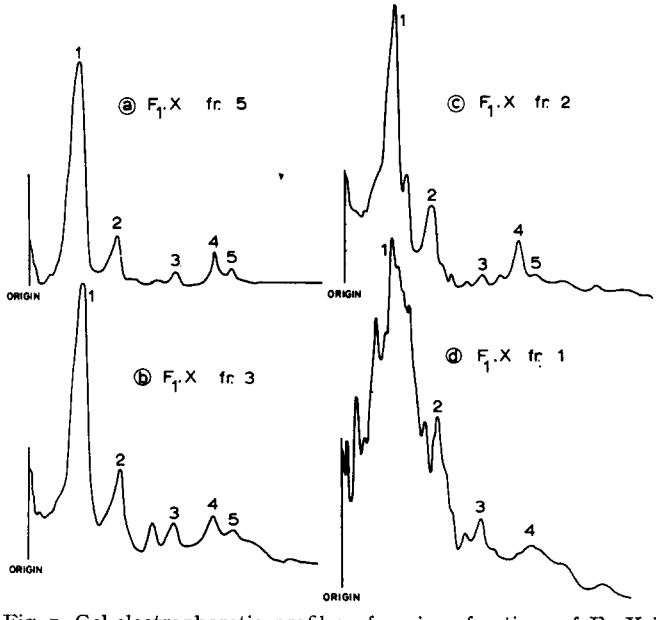

Fig. 7. Gel-electrophoretic profiles of various fractions of $F_1 \cdot X$ in the polyacrylamide–dodecyl sulphate system. (a) 100 μg Fraction 5, (b) 100 μg Fraction 3, (c) 125 μg Fraction 2 and (d) 150 μg Fraction 1.

of $F_1 \cdot X$ (Table I) is equal to the percentage free F_1 in $F_1 \cdot X$. On the basis of the reported molecular weights for F_1 and OSCP of 360000 (ref. 7) and 18000 (ref. 18), respectively, molar ratios of OSCP: F_1 can be calculated for any percentage inhibition in Fig. 6. From this the molar ratio of OSCP: F_1 in $F_1 \cdot X$ can be deduced and when one assumes that OSCP is associated with F_1 in a 1:1 ratio, the percentage of free F_1 in $F_1 \cdot X$ can be calculated (Table I) to be approximately 30 % (see also ref. 33).

Gel electrophoresis of $F_1 \cdot X$, F_1, OSCP and the ATPase inhibitor

In Fig. 7 the gel electrophoresis profiles are shown of the different preparations of $F_1 \cdot X$, listed in Table I. The electrophoresis profile of the purest fraction

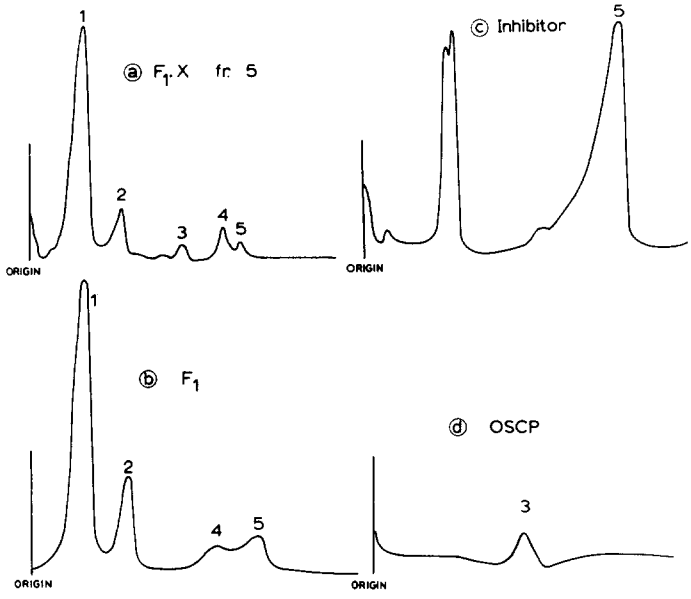

Fig. 8. Comparison of the gel electrophoretic profiles of $F_1 \cdot X$, F_1, OSCP and the ATPase inhibitor in the polyacrylamide–dodecyl sulphate system. (a) 100 μg $F_1 \cdot X$ (Fraction 5); (b) 100 μg F_1; (c) 100 μg inhibitor and (d) 20 μg OSCP.

TABLE II

MOLECULAR WEIGHTS OF THE SUBUNITS PRESENT IN $F_1 \cdot X$, F_1, OSCP AND THE ATPase INHIBITOR
The molecular weights were estimated from the migration velocity in the polyacrylamide–sodium dodecyl sulphate gel-electrophoresis system, with the relative mobility of standard proteins as a reference.

Component	*$F_1 \cdot X$ (Fraction 3)**	*$F_1 \cdot X$ (Fraction 5)***	*F_1****	*OSCP*	*ATPase inhibitor*
1	56 000	55 000	56 000		
2	39 000	40 000	39 000		
3	24 000	23 000		24 000	
4	16 000	16 000	18 000		
5	12 000	12 000	12 000		10 500

* Specific activity, 49 units/mg protein.
** Specific activity, 59 units/mg protein.
*** Specific activity, 73 units/mg protein.

(No. 5) contains five components. Under the same conditions F_1 gives four bands and OSCP one (Fig. 8). The impure preparation of ATPase inhibitor used, contains a major rapidly migrating component (indicated as 5 in Fig. 8). The molecular weights of the subunits calculated from the calibration curve obtained with standard proteins, are given in Table II.

Subunits 1, 2 and 5 are present in both $F_1 \cdot X$ and F_1. Subunit 4 present in $F_1 \cdot X$, with mol. wt. 16 000, may be identical with Subunit 4 present in F_1 (mol. wt., 18 000). Subunit 3, present in $F_1 \cdot X$, is not present in F_1 and has the same molecular weight as OSCP (24 000). Subunit 5 (mol. wt., 12 000) present in both $F_1 \cdot X$ and F_1 is probably identical with the ATPase inhibitor, despite the fact that the isolated protein has a higher mobility (mol. wt., 10 500). It has been shown that the F_1 used here contains endogenous inhibitor[5,7]. Judging from the method of preparation of $F_1 \cdot X$ it is not surprising that it contains some ATPase inhibitor.

The electrophoresis profile of pure $F_1 \cdot X$ gives no support to the suggestion of Sani *et al.*[23] that it contains a protein in addition to F_1 and OSCP.

DISCUSSION

The experiments reported clearly demonstrate that F_1 and OSCP isolated from beef-heart mitochondria associate with each other. This is not surprising in view of the strong effect of OSCP on the binding of F_1 to the mitochondrial membrane[12,18,31]. The association is so strong that a complex of the two proteins, previously termed $F_1 \cdot X$, may be isolated by aqueous extraction of an acetone powder of beef-heart mitochondria, followed by ammonium sulphate fractionation[21]. Surprisingly, according to Tzagoloff[31], F_1 and OSCP from yeast do not associate.

The fact that both $(NH_4)_2SO_4$ and deoxycholate are required to dissociate the complex suggests that both ionic and hydrophobic interactions are involved in the complex formation. MacLennan and Tzagoloff[18] have reported that F_1 bears a net negative charge and OSCP a net positive charge at neutral pH.

It is known from the literature that the ATPase inhibitor stabilizes F_1 against cold inactivation[11]. This is not found with OSCP[18], but Vallejos *et al.*[21] have shown that F_1 partially protects cold-treated $F_1 \cdot X$ (*i.e.* OSCP) against inactivation at 37°. Whether the binding of the inhibitor or of OSCP to F_1 influences the conformation of F_1 is currently being investigated. Preliminary experiments in this laboratory with ammonia–Sephadex particles, which are virtually devoid of ATPase inhibitor, have shown that the binding of the inhibitor alters neither the K_m for ATP nor the K_i for ADP when the ATPase activity is measured with a sensitive pH electrode. Similar results were reported by Juntti *et al.*[34].

That pure $F_1 \cdot X$ resembles in many aspects the complex between F_1 and OSCP was further confirmed by comparison of the electrophoretic profiles in dodecyl sulphate of $F_1 \cdot X$ with F_1, OSCP and the ATPase inhibitor. The molecular weights of the components seen in $F_1 \cdot X$ and F_1 are somewhat different from those reported in other studies[6,10]. This could be caused by the different conditions used in the gel-electrophoresis system.

From the capacity of $F_1 \cdot X$ to induce F_0 activity in ASUA particles, the molar ratio of OSCP and F_1 in $F_1 \cdot X$ was estimated. Probably about 30 % of the F_1 in $F_1 \cdot X$ is free and 70 % is associated with OSCP in a 1:1 ratio.

The complex $F_1 \cdot$OSCP is easily extracted by sonication of an acetone powder of mitochondria in water. OSCP is extracted from membrane fragments only at a very high pH[18] and sonication of untreated mitochondria only solubilizes F_1 (ref. 5). This suggests that phospholipids are important for the binding of OSCP to the membrane system. This is analogous to the binding of F_1 to CF_0 (ref. 12), where added phospholipids are required to render the ATPase activity sensitive to oligomycin.

ACKNOWLEDGEMENTS

We thank Mrs. A. Ruizendaal-de Koning and Mr. H. van Ballegoy for their technical assistance. The helpful discussions with Professor Slater are gratefully acknowledged. We also thank Dr. G. S. P. Groot for his help with the gel-electrophoresis studies. This work was in part supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES

1 M. E. Pullman, H. S. Penefsky, A. Datta and E. Racker, *J. Biol. Chem.*, 235 (1960) 3322.
2 G. Schatz, H. S. Penefsky and E. Racker, *J. Biol. Chem.*, 242 (1967) 2552.
3 W. A. Catterall and P. L. Pedersen, *J. Biol. Chem.*, 246 (1971) 4987.
4 H. S. Penefsky and R. C. Warner, *J. Biol. Chem.*, 240 (1965) 4694.
5 L. L. Horstman and E. Racker, *J. Biol. Chem.*, 245 (1970) 1336.
6 A. E. Senior and J. C. Brooks, *Arch. Biochem. Biophys.*, 140 (1970) 257.
7 D. O. Lambeth, H. A. Lardy, A. E. Senior and J. C. Brooks, *FEBS Lett.*, 17 (1971) 330.
8 A. L. Shapiro, E. Viñuela and J. V. Maizel, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
9 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
10 A. E. Senior and J. C. Brooks, *FEBS Lett.*, 17 (1971) 327.
11 M. E. Pullman and G. C. Monroy, *J. Biol. Chem.*, 238 (1963) 3762.
12 Y. Kagawa and E. Racker, *J. Biol. Chem.*, 241 (1966) 2461, 2467.
13 E. Racker and L. L. Horstman, *J. Biol. Chem.*, 242 (1967) 2547.
14 J. M. Fessenden-Raden, *J. Biol. Chem.*, 244 (1969) 6662.
15 H. S. Penefsky, M. E. Pullman, A. Datta and E. Racker, *J. Biol. Chem.*, 235 (1960) 3330.
16 E. Racker, L. L. Horstman, D. Kling and J. M. Fessenden-Raden, *J. Biol. Chem.*, 244 (1969) 6668.
17 A. Tzagoloff, D. H. MacLennan and K. H. Byington, *Biochemistry*, 7 (1968) 1596.
18 D. H. MacLennan and A. Tzagoloff, *Biochemistry*, 7 (1968) 1603.
19 B. Bulos and E. Racker, *J. Biol. Chem.*, 243 (1968) 3891, 3901.
20 A. F. Knowles, R. J. Guillory and E. Racker, *J. Biol. Chem.*, 246 (1971) 2672.
21 R. H. Vallejos, S. G. van den Bergh and E. C. Slater, *Biochim. Biophys. Acta*, 153 (1968) 509.
22 G. S. P. Groot and M. Meyer, *Biochim. Biophys. Acta*, 180 (1969) 575.
23 B. P. Sani, K. W. Lam and D. R. Sanadi, *Biochem. Biophys. Res. Commun.*, 39 (1970) 444.
24 F. L. Crane, J. L. Glenn and D. E. Green, *Biochim. Biophys. Acta*, 22 (1956) 475.
25 J. M. Fessenden and E. Racker, *J. Biol. Chem.*, 241 (1966) 2483.
26 S. O. Nielsen and A. L. Lehninger, *J. Biol. Chem.*, 215 (1955) 555.
27 G. S. P. Groot, W. Rouslin and G. Schatz, *J. Biol. Chem.*, in the press.
28 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
29 J. B. Sumner, *Science*, 100 (1944) 413.
30 K. W. Cleland and E. C. Slater, *Biochem. J.*, 53 (1953) 547.
31 A. Tzagoloff, *J. Biol. Chem.*, 245 (1970) 1545.
32 K. Takayama, D. H. MacLennan, A. Tzagoloff and C. D. Stoner, *Arch. Biochem. Biophys.*, 114 (1966) 223.
33 G. S. P. Groot, *Enige Aspecten van de Mitochondriale Energiehuishouding*, Ph. D. Thesis, Amsterdam, Mondeel, Amsterdam, 1970.
34 K. Juntti, K. Asami and L. Ernster, *Abstr. No. 660, 7th FEBS Meet., Varna*, 1971.