Studies on the Mitochondrial Adenosine Triphosphatase System. IV. Purification and Characterization of the Oligomycin Sensitivity Conferring Protein*

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ABSTRACT: A soluble protein of mol wt 18,000, isolated in highly purified form, has been shown to control the sensitivity of the mitochondrial ATPase to oligomycin. The protein also restores energy-linked functions of A particles to which F_1 has been rebound. In these respects the protein appears to correspond to the active component in the crude fraction designated as F_4 by Conover and coworkers (Conover, T. E., Prairie, R. L., and Racker, E. (1963), *J. Biol. Chem. 238*, 2831). The purified protein is, however, at least 240 times more active than the F_4 preparation. The protein has been shown not to be present in the oligomycin-insensitive ATPase (F_1) but is a component of the oligomycin-sensitive ATPase complex. The oligomycin sensitivity conferring protein has been chosen as the designation for the purified enzyme.

In previous communications (Tzagoloff *et al.*, 1968a,-b) we described the isolation of an oligomycin-sensitive ATPase complex (O-S ATPase)[1] from bovine heart mitochondria. The O-S ATPase was shown to be a complex containing multiple protein subunits and about 30% phospholipid by weight. A soluble, oligomycin-insensitive ATPase, similar to F_1 (Pullman *et al.*, 1960), was shown, by isolation, to be part of the complex. We also demonstrated that that portion of the O-S ATPase which was insoluble after extraction with 3.5 M NaBr (O-S ATPase (NaBr)) was devoid of ATPase activity but could bind F_1 and confer oligomycin sensitivity and cold stability upon the bound F_1. These two fractions, F_1 and O-S ATPase (NaBr), accounted for the major protein components of the O-S ATPase complex.

In addition, we showed that when O-S ATPase (NaBr) was extracted at alkaline pH the residue (ATPase (NaBr, NH_4OH)) retained the capacity to bind F_1 but lost the capacity to confer oligomycin sensitivity and cold stability upon bound F_1. In the present communication results of further investigations of this phenomenon are presented. We have found that the alkaline extract of ATPase (NaBr) contains a water-soluble protein, of mol wt 18,000, which when added to ATPase (NaBr, NH_4OH) confers oligomycin sensitivity to bound F_1. This protein has been highly purified and the purified protein has been designated as the oligomycin sensitivity conferring protein (OSCP).

The protein also acts as a coupling factor of oxidative phosphorylation. Evidence is presented that the purified protein is identical with the active component of the F_4 preparation reported by Conover *et al.* (1963); it is, however, considerably more purified and is at least 240 times more active than the preparation reported by these authors.

Materials and Methods

Preparation of the Oligomycin-Sensitive ATPase Complex. The oligomycin-sensitive ATPase complex (O-S ATPase complex) was prepared by a modification of the previously described procedure (Tzagoloff *et al.*, 1968b). Bovine heart mitochondria, suspended in 0.25 M sucrose (Crane *et al.*, 1956), were used instead of submitochondrial particles as the starting material. At step 4, the ammonium sulfate concentration was taken directly to 40% saturation at 4°; the protein fraction precipitating between 0 and 40% corresponded to the O-S ATPase complex.

Preparation of Coupling Factors and Assay Particles. Coupling factor 1 (Pullman *et al.*, 1960) was prepared by procedure B (MacLennan *et al.*, 1968). Coupling factor 4 was prepared from bovine heart mitochondria by the procedure of Conover *et al.* (1963); the last step, consisting of the ammonium sulfate refractionation, was omitted. A particles were prepared by the method of Fessenden and Racker (1966). P particles were prepared by the method of Conover *et al.* (1963).

Assays and Analytical Procedures. ATP-$^{32}P_i$ exchange was measured by the first procedure of Conover *et al.*

* From the Institute for Enzyme Research, University of Wisconsin, Madison Wisconsin 53706. *Received November 28, 1967.* This investigation was supported in part by National Institute of General Medical Sciences' program Project Grant GM-12,847, National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: O-S ATPase, oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff *et al.* (1968a); ATPase (NaBr), O-S ATPase extracted twice with solutions 3.5 M in NaBr; ATPase (NaBr, NH_4OH), O-S ATPase (NaBr) extracted twice with solutions 0.4 M in NH_4OH; F_1, coupling factor 1, (Pullman *et al.*, 1960); F_3, coupling factor 3, (Fessenden and Racker, 1966); F_4, coupling factor 4 (Conover *et al.*, 1963); OSCP, oligomycin sensitivity conferring protein; sucrose-Tris, a solution 0.25 M in sucrose and 0.01 M in Tris-acetate (pH 7.5).

TABLE I: Effect of the Alkaline Extract of ATPase (NaBr) on Conferral of Oligomycin Sensitivity upon F_1 Bound to ATPase (NaBr, NH_4OH).[a]

Additions to ATPase (NaBr, NH_4OH) (μg)	Sp Act. of ATPase (μmoles of ATP hydrolyzed/min per mg of particle)	
	−Rutamycin	+Rutamycin
None	0.00	0.00
F_1 (105)	1.05	0.95
F_1 (105) + extract (20)	0.96	0.79
F_1 (105) + extract (40)	0.96	0.49
F_1 (105) + extract (80)	0.90	0.32
F_1 (105) + extract (160)	0.98	0.31

[a] The alkaline extract of ATPase (NaBr) was a crude preparation of the oligomycin sensitivity conferring protein. It was extracted from ATPase (NaBr) into a solution which was 0.4 M NH_4OH, 0.2 M in KCl, and 1 mM in EDTA. The pH of the extract was then adjusted to 8.0 with 10 N acetic acid, and insoluble protein was removed by centrifugation. The soluble protein was concentrated by precipitation with ammonium sulfate (40% of saturation at 4°), dissolved in a solution which was 0.02 M in Tris-H_2SO_4 (pH 8.0) and 1 mM in EDTA, and clarified by centrifugation. This soluble protein is referred to as the alkaline extract. ATPase (NaBr, NH_4OH) (1 mg) was incubated for 10 min at 23° with the indicated addition of F_1 and of the alkaline extract. The suspending medium was 1.0 ml of sucrose-Tris. The sample was centrifuged in a microcentrifuge, the supernatant was removed, and the pellet was suspended in 0.9 ml of a solution 50 mM in Tris-SO_4 (pH 8.5) and 2 mM in $MgCl_2$. After 2 min at 30°, 0.1 ml of 0.1 M ATP was added. The reaction was stopped after 5 min by the addition of 0.5 ml of 5% TCA and 0.5-ml aliquots were taken for measurement of inorganic phosphate.

(1963). In this method potassium phosphate and magnesium chloride were mixed prior to the addition of the particle and factors to prevent a magnesium-induced aggregation of the particles. DPN^+ reduction by succinate was measured by the method of Fessenden and Racker (1967) except that the temperature of assay was 38°. P:O ratios were determined in the oxygraph assay by the method of MacLennan *et al.* (1966). Pyridine nucleotide transhydrogenation energized by ATP was measured as previously described (MacLennan *et al.*, 1968). ATPase activity was measured by the procedure of Pullman *et al.* (1963).

Disc gel electrophoresis was performed by the method of Takayama *et al.* (1966). Protein was estimated either by the Biuret method (Gornall *et al.*, 1949) or by measuring the absorbancy at 278 mμ.

The molecular weight of the active protein was estimated by the Sephadex retention method described by Andrews (1964). A 3 × 95 cm column of Sephadex G-100 was equilibrated with a solution 0.05 M in Tris-HCl and 0.1 M in KCl. A standard elution curve was determined using 2-mg samples of α-chymotrypsinogen (mol wt 25,000), metmyoglobin (mol wt 17,800), and cytochrome *c* (mol wt 12,400). The alkaline extract (10 mg of protein purified to step 2; see following section) was applied to the column and elution of the active protein was judged by absorbancy at 278 mμ and by the capacity of the fractions to stimulate the ATP-$^{32}P_i$-exchange reaction of A particles.

Purification of the Oligomycin Sensitivity Conferring Protein (OSCP). STEP 1. EXTRACTION OF F_1 FROM THE O-S ATPASE WITH NaBr. The O-S ATPase complex was extracted twice with 3.5 M NaBr as described previously (Tzagoloff *et al.*, 1968b). The extracted residue, designated ATPase (NaBr), was suspended at a protein concentration of 15 mg/ml in a solution 0.3 M in KCl and 1.5 mM in EDTA.

STEP 2. One-half volume of 1.2 N NH_4OH was added to the suspension of ATPase (NaBr). The suspension was incubated 20 min at 0° and centrifuged at 105,000*g* for 15 min. The residue was extracted a second time in the same manner. The extracts were combined and the pH was adjusted to 8.0 by the addition of 10 N acetic acid. The insoluble material was removed by centrifugation at 105,000*g* for 10 min. The clear supernatant fluid was brought to 40% saturation at 4° with respect to ammonium sulfate by the addition of ammonium sulfate solution saturated at 4° and neutralized to pH 7.0. The protein precipitate was collected by centrifugation at 105,000*g* for 10 min and dissolved in a solution which was 20 mM in Tris-SO_4 (pH 8.0) and also 1 mM in EDTA. Insoluble material was removed by centrifugation at 105,000*g* for 10 min. The concentration of protein in the supernatant fluid was 3–5 mg/ml.

STEP 3. FRACTIONATION ON CM-CELLULOSE. The protein solution obtained in step 2 was diluted with ten volumes of cold distilled water and applied to a 2.0 × 2.5 cm column of CM-cellulose previously equilibrated with 2 mM Tris-acetate (pH 7.5). The column was washed sequentially with 20 ml of a solution 2 mM in Tris-acetate (pH 7.5), 20 ml of a solution 20 mM in Tris-acetate (pH 7.5) which was also 50 mM in NaCl or KCl (this step is optional when the source of OSCP is O-S ATPase NaBr), and finally with 50 ml of a solution, 20 mM in Tris-acetate (pH 7.5) in which the concentration of NaCl or KCl was varied linearly from 50 to 300 mM. The volume of each fraction collected was 3 ml. Active material, designated as the oligomycin sensitivity conferring protein, was eluted in a broad peak after the salt concentration reached about 0.1 M.

Purification of the Active Component in F_4. STEP 1. REMOVAL OF F_1 PROTEIN WITH PROTAMINE SULFATE. F_4 was prepared as described by Conover *et al.* (1963) except that the ammonium sulfate refractionation was omitted. F_4 (950 mg) was dissolved in 50 ml of a solution 0.25 M in sucrose and 0.01 M in Tris-acetate (pH 7.5). To this solution was added 100 ml of 0.5% protamine sulfate (Pullman *et al.*, 1960). The mixture was incubated at 4° for 10 min and centrifuged at 105,000*g*

TABLE II: Effect of OSCP on Binding of F_1 to ATPase (NaBr, NH_4OH) and on Conferral of Oligomycin Sensitivity.[a]

Addn to Extracted O-S ATPase Complex (μg)	Sp Act. of ATPase (μmoles of ATP hydrolyzed/min per mg of particle)	
	−Rutamycin	+Rutamycin
None	0.00	0.00
F_1 (100)	0.72	
F_1 (150)	1.39	1.30
F_1 (200)	1.39	1.30
F_1 (200) + OSCP (3)	1.30	0.92
F_1 (200) + OSCP (6)	1.34	0.70
F_1 (200) + OSCP (12)	1.40	0.70

[a] ATPase (500 μg) (NaBr, NH_4OH) was incubated for 10 min at 23° with the indicated levels of F_1 and OSCP. The reaction mixture was 0.3 ml of a solution which was 0.25 M in sucrose, 0.01 M in Tris-acetate (pH 7.5), 4 mM in ATP, and 2 mM in EDTA. Sucrose-Tris (0.7 ml) was then added to the mixture and the insoluble pellet was collected by centrifugation and resuspended in 1.0 ml of the ATPase reaction mix (minus ATP) described in Table I and samples were assayed for activity in the presence and absence of rutamycin. The ATPase reaction was initiated by the addition of ATP.

for 10 min. The supernatant fluid was brought to 30% saturation with respect to ammonium sulfate by the addition of an ammonium sulfate solution saturated at 4°. The precipitate was collected by centrifugation and dissolved in 8.5 ml of a solution that was 20 mM in Tris-acetate (pH 8.0) and 1 mM in EDTA.

STEP 2. FRACTIONATION ON CM-CELLULOSE. The solution from step 1 (2 ml) was diluted with nine volumes of cold distilled water and the diluted sample was applied to a 2 × 2.5 cm column of Cellex-CM (Tris form) which had previously been equilibrated with a solution 2 mM in Tris-acetate (pH 7.5). The column was washed successively with 15 ml of a solution 2 mM in Tris-acetate (pH 7.5), 30 ml of a solution that was both 20 mM in Tris-acetate (pH 7.5) and 0.05 M in KCl, and finally with 50 ml of a solution 20 mM in Tris-acetate (pH 7.5), and in which the concentration of KCl was varied linearly from 50 to 300 mM. The active protein was eluted when the KCl concentration reached about 0.1 M.

Results

Effect of Alkaline Extract on Oligomycin Sensitivity of Reconstituted ATPase Complexes. The O-S ATPase complex after extraction with 3.5 M NaBr has been shown to be devoid of ATPase activity but to retain the

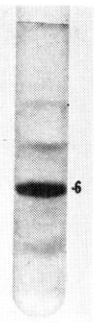

FIGURE 1: Profile obtained by electrophoresis on polyacrylamide gel of the oligomycin sensitivity conferring protein purified from Cellex-CM

capacity to bind F_1, with formation of an oligomycin-sensitive ATPase (Tzagoloff *et al.*, 1968b). When ATPase (NaBr) was further extracted with ammonia the lipoprotein residue could still bind F_1 but could no longer confer oligomycin sensitivity or cold stability upon the bound F_1. This phenomenon is documented in Table I. The ATPase activity of the reconstituted complex could be made sensitive to oligomycin by the addition of protein partially purified from the alkaline extract of O-S ATPase (NaBr). This phenomenon is also documented in Table I. The addition of 80 μg of protein (purified to step 2) induced more than 65% sensitivity to oligomycin.

Effect of Purified OSCP on Oligomycin Sensitivity of Reconstituted ATPase Complex. Table II shows that the oligomycin sensitivity conferring protein, purified by fractionation on CM-cellulose, restored at least 50% oligomycin sensitivity to F_1 bound to ATPase (NaBr, NH_4OH). The level required for maximal oligomycin sensitivity, however, was only about 6 μg of OSCP/mg of ATPase (NaBr, NH_4OH). Increased ATPase activity in the residue was not observed upon addition of OSCP. Nevertheless OSCP did induce a greater loss of ATPase

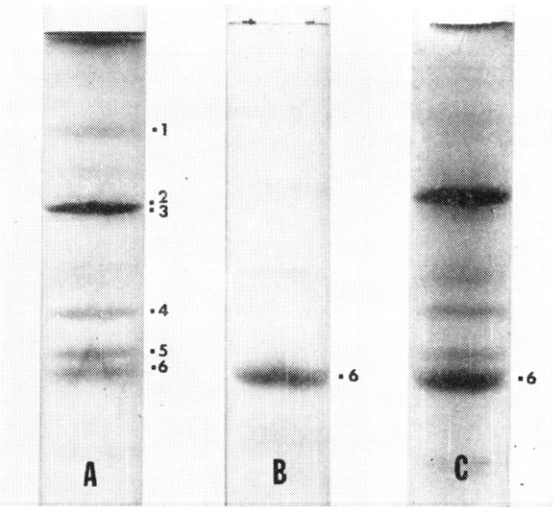

FIGURE 2: Profiles obtained by electrophoresis on polyacrylamide gel. (A) Oligomycin-sensitive ATPase complex; (B) alkaline extract from O-S ATPase (NaBr) purified to step 2; and (C) cochromatography of sample A and sample B.

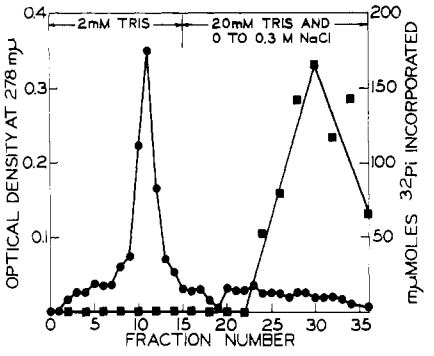

FIGURE 3: Elution from a Cellex-CM column. Protein (●-●); activity in stimulating ATP-$^{32}P_i$ exchange (■-■). The protein applied to the column was the alkaline extract of ATPase (NaBr) purified to step 2.

from the supernatant. The lack of quantitative recovery was due to the fact that ATPase activity was depressed when F_1 was bound in an oligomycin-sensitive as opposed to insensitive fashion. For this reason it is not possible to accurately measure OSCP-induced binding of F_1. We have observed OSCP-dependent increases in particle-bound ATPase activity when the extracted particle was ETP_H instead of the O-S ATPase complex.

Electrophoretic Profiles of Purified OSCP. Figure 1 shows the protein profile following electrophoresis of OSCP, purified by fractionation on CM-cellulose. One major and three minor bands can be observed. The minor bands represent contamination levels of only a few per cent. The relationship of the purified protein to the protein subunits of the O-S ATPase complex has been determined by coelectrophoresis of OSCP with the depolymerized O-S ATPase complex. Figure 2 demonstrates that OSCP is electrophoretically identical with protein band 6 of the O-S ATPase complex. O-S ATPase (NaBr, NH_4OH), not shown here, still contained a large amount of OSCP as judged by gel electrophoresis.

TABLE III: Comparison between the Specific Activity of OSCP and of F_4 in Stimulating the ATP-$^{32}P_i$-Exchange Reaction of A Particles.[a]

Addn to A Particle (μg)	ATP-$^{32}P_i$ Exchange (mμmoles/min per mg)
None	9
F_1 (40)	31
F_1 (40) + OSCP (0.4)	80
F_1 (40) + OSCP (1.0)	138
F_1 (40) + OSCP (2.0)	161
F_1 (40) + F_4 (120)	62
F_1 (40) + F_4 (240)	137

[a] The ATP-$^{32}P_i$-exchange reaction was measured by the method of Conover *et al.* (1963). A particle (1 mg) was used for assay.

TABLE IV: Specific Activity of the Oligomycin Sensitivity Conferring Protein (OSCP) in Stimulating Energy-Linked Reactions of A Particles.

Addn (μg)	P:O	DPN+ Reduction[a] by Succinate	Transhydrogenation[b]
None	0.14	7	80
F_1 (40)	0.16		
F_1 (60)		77	124
F_1[c] + OSCP (0.2)	0.14	96	136
F_1 + OSCP (0.4)	0.21	110	155
F_1 + OSCP (1.0)	0.29	121	165
F_1 + OSCP (2.0)	0.33	121	155
OSCP (1.0)		9	74
OSCP (2.0)	0.07		

[a] DPN^+ (mμmoles) reduced by succinate per minute per milligram of A particle at 38° in presence of ATP. [b] TPN^+ (mμmoles) reduced by DPNH per minute per milligram of A particle at 38° in presence of ATP. [c] F_1 (40 μg) was used in the measurement of P:O ratios; 60 μg of F_1 was used in the assay of reversed electron transfer and of ATP-energized transhydrogenation.

Effect of OSCP on ATP-$^{32}P_i$ Exchange of Submitochondrial Particles. The oligomycin sensitivity conferring protein has also been found to stimulate the ATP-$^{32}P_i$-exchange reaction of A particles and of P particles. Table III documents the stimulation of A particles. In the same table a comparison is made between the specific activity of F_4 (Conover *et al.*, 1963) and of OSCP in stimulating the ATP-$^{32}P_i$ exchange of A particles. Both proteins reactivated the exchange reaction but OSCP had a specific activity 240-fold greater than that of F_4. OSCP, like F_4, was incapable of stimulating ATP-$^{32}P_i$ exchange in A particles in absence of added F_1. OSCP also stimulated the ATP-$^{32}P_i$-exchange reaction in P particles.

Effect of OSCP on Energy-Linked Reaction of A Particles. The oligomycin sensitivity conferring protein, in the presence of F_1, was found to stimulate other energy-linked reactions of A particles. Table IV shows that the P:O ratio, the rate of DPN reduction by succinate (in presence of ATP), and the rate of pyridine nucleotide transhydrogenation (in presence of ATP) were all enhanced by purified OSCP. Approximately 1 μg of OSCP/mg of A particle was sufficient for maximal stimulation of all of these reactions.

Other Properties of OSCP. A. ADSORPTION TO CM-CELLULOSE. Stimulation of the ATP-$^{32}P_i$-exchange reaction of A particles has been found to be the most sensitive assay for the presence of OSCP. Consequently, we have routinely used this assay in studies of the purification and properties of OSCP. Figure 3 shows the results of an experiment in which stimulation of the ATP-$^{32}P_i$-exchange reaction was used as an assay for elution

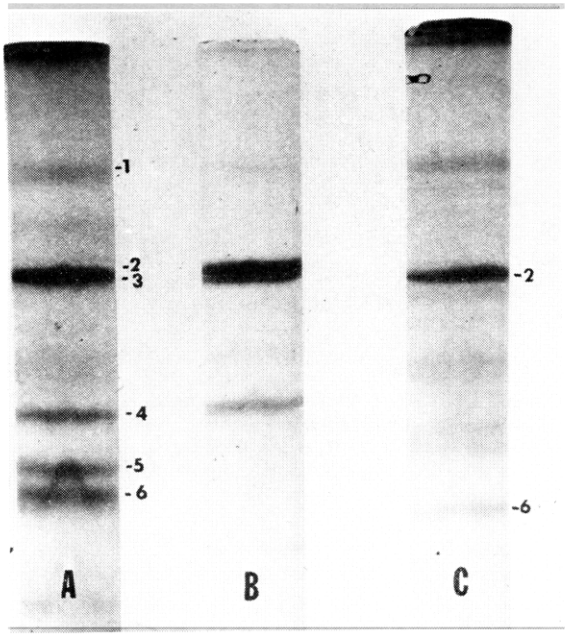

FIGURE 4: Profiles obtained by electrophoresis on polyacrylamide gel. (A) Oligomycin-sensitive ATPase complex; (B) F_1; and (C) F_4 from light-layer bovine heart mitochondria (Conover *et al.*, 1963).

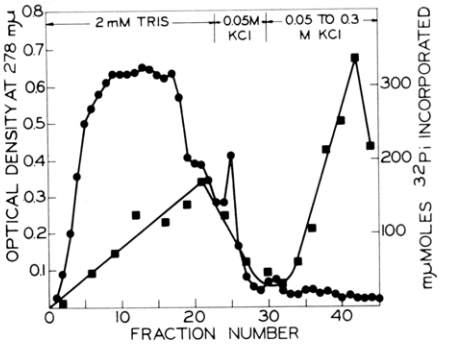

FIGURE 5: Elution from a Cellex-CM column: protein (●—●) and activity in stimulating ATP–$^{32}P_i$ exchange (■—■) from Cellex-CM column. The protein applied was F_4 (from mitochondria which was partially purified by protamine sulfate and ammonium sulfate precipitation; see text).

of OSCP from a column Cellex-CM (step 3 of the purification schedule). The active fraction was adsorbed to CM and was only eluted with NaCl concentrations in excess of 0.1 M. The fact that the protein has a strong net positive charge should be useful in distinguishing it from coupling factor preparations such as F_1 (Pullman *et al.*, 1960), F_3 (Fessenden and Racker, 1967), factor A (Andreoli *et al.*, 1965), and the transphosphorylase (Beyer, 1968), since all of these have a net negative charge.

B. MOLECULAR WEIGHT. The molecular weight of OSCP has been determined by comparing its elution from Sephadex G-100 with the elution of α-chymotrypsinogen, metmyoglobin, and cytochrome *c* (Andrews, 1964). The fraction which was most active in stimulating ATP–$^{32}P_i$ exchange corresponded to the elution point of the standard protein, metmyoglobin. From these data the molecular weight has been estimated to be 18,000.

C. EFFECT ON OTHER SYSTEMS. OSCP had no intrinsic ATPase activity and it did not stimulate the ATPase activity of either A particles or of F_1. The protein did not affect the oligomycin sensitivity or the cold stability of F_1 when the two were mixed in a soluble system.

D. EVIDENCE THAT THE ACTIVE COMPONENT OF OSCP IS A PROTEIN. The purified factor showed a typical protein absorption spectrum with maximal absorption at 278 mμ. The ratio between the absorbancies at 260 and 280 mμ indicated that no significant amount of nucleotide was present. Also, the fact that the preparation was devoid of phosphorus confirmed the absence of both nucleotides and phospholipid. The active component has been found to be precipitable with ammonium sulfate and with trichloroacetic acid. It is completely inactivated by tryptic digestion and by heat treatment (see Table V).

Comparison of OSCP with F_4. The fact that OSCP restores the capacity to confer oligomycin sensitivity to ammonia-extracted particles and the fact that it can act as a coupling factor of oxidative phosphorylation indicate a similarity between the purified protein and F_4 since both of these properties have been reported for F_4 (Conover *et al.*, 1963; Kagawa and Racker, 1966; Pullman and Schatz, 1967). The physical properties reported for F_4 and the levels required for activity, however, indicate differences between these two preparations.

Figure 4 is a comparison of the protein components of F_4 with the components of the O-S ATPase complex and of F_1. The electrophoretic profile of F_4 indicated that it was a relatively homogeneous preparation of the protein designated band 2. Band 2 is a major component of both F_1 and of the O-S ATPase complex (MacLennan *et al.*, 1968; Tzagoloff *et al.*, 1968a). In addition,

TABLE V: Stability of the Oligomycin Sensitivity Conferring Protein.

Treatment	ATP–$^{32}P_i$ Exchange (mμmoles/min mg
None	70
Trypsin,[a] 5 min	2
Trypsin, 10 min	1
Trypsin + inhibitor[b]	61
Heated 2 min at 60°	11
Heated 2 min at 80°	0

[a] OSCP (6 μg) was digested with 0.2 μg of three-times-recrystallized trypsin for the indicated times at 30° in a total volume of 0.3 ml. The reaction was stopped with 2 μg of trypsin inhibitor. For assay of ATP–$^{32}P_i$ exchange 1 mg of A particle, 40 μg of F_1, and 1 μg of the treated OSCP were used. [b] Inhibitor was added prior to addition of trypsin and the mixture was kept at 0°.

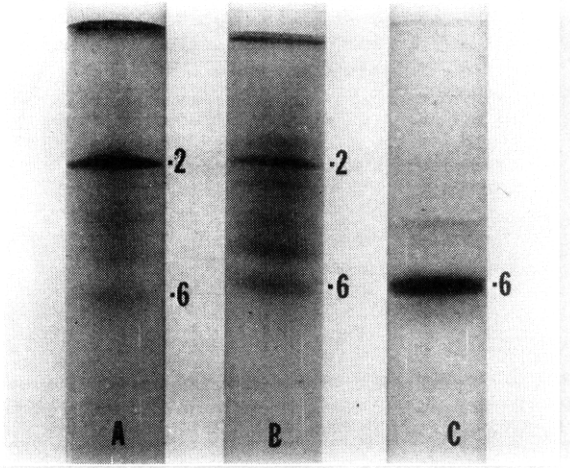

FIGURE 6: Profiles obtained by electrophoresis on polyacrylamide gel. (A) F_4 from light mitochondria; (B) F_4 after purification of activity by fractionation with protamine sulfate and ammonium sulfate; and (C) F_4 after purification of activity on Cellex-CM.

F_4 contained a small amount of band 6 which we have identified with OSCP. In view of this result, the active species of F_4 could be the major species (the F_1 subunit) or the minor species which corresponded to OSCP. That the source of the active component in the F_4 preparation is F_1 is contradicted by experimental evidence. Attempts to prepare F_4 from F_1 have proved unsuccessful.

To determine whether the active component of F_4 was identical with OSCP we have carried the Conover preparation through two additional purification steps described in the section on Materials and Methods. Purification step 2, the elution from CM-cellulose of protein and activity (stimulation of ATP–$^{32}P_i$ exchange in A particles), is illustrated in Figure 5.

Electrophoretic comparisons of F_4 with the preparation after purification steps 1 and 2 indicate that the protamine sulfate procedure removed protein corresponding to band 2, the major component of F_1 (*cf.* Figure 6a,b). A further removal of F_1 protein was achieved by fractionation on Cellex-CM since F_1 protein was not adsorbed. The active fraction which was eluted from Cellex-CM by the Tris-plus-KCl eluent consisted almost exclusively of OSCP protein which corresponds to band 6 (see 6c). The specific activity of this material was increased approximately 60 times over that of the initial F_4 preparation (see Figure 7).

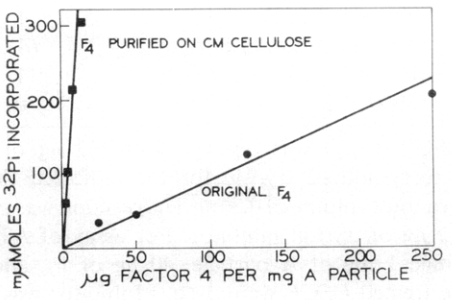

FIGURE 7: Specific activity of F_4 in stimulating the ATP–$^{32}P_i$ exchange of F_4 before purification (●—●) and after purification (■—■).

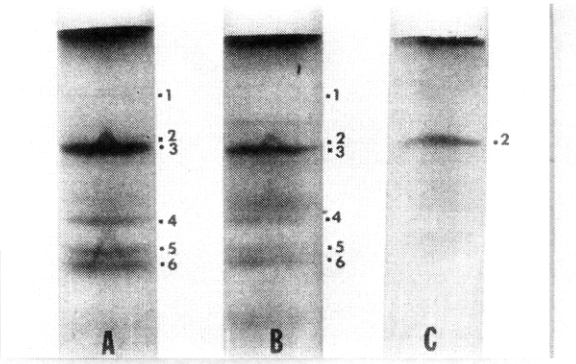

FIGURE 8: Profiles obtained by electrophoresis on polyacrylamide gel. (A) Oligomycin-sensitive ATPase complex; (B) structural protein of Richardson *et al.* (1964); and (C) structural protein after purification by the method of Lenaz *et al.* (1967).

Comparison of OSCP with Structural Protein. Zalkin and Racker (1965) reported that structural protein prepared by the method of Richardson *et al.* (1964) could also stimulate energy-linked reactions in resolved particles. In this respect it could replace F_4. This observation can be explained by the fact that the Richardson *et al.* (1964) preparation of structural protein is not homogeneous. In Figure 8 structural protein, as prepared by Richardson *et al.* (1964), is compared electrophoretically with the O-S ATPase complex. Not only did this preparation of structural protein contain OSCP (band 6) but it also contained all of the other protein components of the O-S ATPase complex. Structural protein has been highly purified by Lenaz *et al.* (1967) using a urea-extraction method. When their scheme of purification was applied to bovine heart mitochondria, the protein species corresponding to band 2 was highly purified (see Figure 8c). This band, which is a component of the F_1 portion of the O-S ATPase complex (MacLennan *et al.*, 1968; Tzagoloff *et al.*, 1968a) is also the major component of the F_4 preparation of Conover *et al.* (1963) (*cf.* Figures 4 and 8).

Discussion

In this paper we have reported the purification of a protein component of the oligomycin-sensitive ATPase complex to the point at which it is virtually free of contaminating protein as judged by gel electrophoresis. The protein restores to certain NH_4OH-extracted lipoproteins the capacity for conferring oligomycin sensitivity upon bound F_1. The protein also acts as a coupling factor in that it stimulates energy-linked reactions in resolved particles.

We propose that this highly purified protein be designated the oligomycin sensitivity conferring protein (OSCP). This designation pinpoints the site of action of the protein (the oligomycin-sensitive site) and defines an assay by which the protein can be identified.

Experiments with the highly purified protein provide additional insight into the molecular architecture of the mitochondrial adenosine triphosphatase system. After extraction with sodium bromide the O-S ATPase com-

plex has been shown to be largely devoid of the protein subunits of F_1 but is purified with respect to those factors which bind F_1 and confer oligomycin sensitivity upon bound F_1. This lipoprotein complex contains only two major protein subunits. This material is the source of the purified oligomycin sensitivity conferring protein. Analysis by electrophoresis on polyacrylamide gel has enabled us to identify OSCP with one of the two major protein components of O-S ATPase (NaBr).

The isolated oligomycin-sensitive ATPase complex (Tzagoloff *et al.*, 1968a) contains F_1, as well as phospholipid, and additional proteins which are directly involved with binding of F_1 and with the site of action of oligomycin. The evidence that F_1, in a bound form, is identifiable with the 90-Å headpiece of the inner membrane is fairly conclusive (Racker and Horstman, 1967). Since the headpiece is bound to the inner membrane through the stalk (Fernández-Moran *et al.*, 1964; Kopaczyk *et al.*, 1967), it follows that a preparation capable of binding F_1 must contain elements of the stalk. Whether the stalk is also sufficient to confer oligomycin sensitivity to bound F_1, or whether this property is determined by additional structural elements, is unresolved at present.

The lipoprotein fraction designated ATPase (NaBr, NH_4OH), from which OSCP was extracted, retained the capacity to bind F_1 but was no longer capable of conferring oligomycin sensitivity upon the F_1 which was bound. Upon the addition of OSCP, partial sensitivity to oligomycin was restored. From these considerations it would appear that OSCP was not concerned with binding of F_1 but was concerned with conferral of oligomycin sensitivity upon bound F_1. Two other observations must be considered, however. ATPase (NaBr, NH_4OH) still contains OSCP as judged by gel electrophoresis. The residual OSCP may be capable of binding but may have been modified so that it no longer is capable of conferring oligomycin sensitivity. Secondly, when F_1 was bound to particles in an oligomycin-sensitive fashion a depression in total activity was observed but this was not seen when F_1 was bound in an oligomycin-insensitive fashion. Therefore the addition of OSCP could result in increased binding without detectable increase in the activity of the residue. These observations make it possible that OSCP is in fact the stalk portion which binds F_1 to the residue. Further studies to clarify this point are in progress.

A comparison between OSCP and F_4 has been made in this study. The conclusion that the active component in F_4 is identical with OSCP is based on several lines of evidence. (1) Both stimulate ATP–$^{32}P_i$ exchange in P particles and in A particles. (2) Both increase the P:O ratio in A particles. (3) Both restore the ability to confer oligomycin sensitivity to preparations of the ATPase complex which have been extracted with ammonia. (4) OSCP can be purified from the F_4 preparation of Conover *et al.* (1963). If the identification of OSCP with the active component of F_4 is accepted then several misconceptions concerning F_4 can be clarified.

The preparation of F_4 reported by Conover *et al.* (1963) has been considered to be highly purified. Because the physical properties as well as the electrophoretic profile of this preparation were similar to those of structural protein and also because preparations of structural protein were found to have F_4 activity (Zalkin and Racker, 1965), it was thought that F_4 and structural protein were identical. Our evidence confirms that the major protein species in F_4 is "structural protein" but suggests that the active component is not "structural protein," (that is protein band 2 of the ATPase complex) but, rather, is the minor component (protein band 6) which is identical with OSCP.

Because very large additions of F_4 were required for coupling activity and because structural protein was known to replace F_4, the concept evolved that the coupling capacity of F_4 resided in its ability to restore structural lesions (Zalkin and Racker, 1965). In view of the fact that we have shown that low levels of highly purified OSCP are sufficient for these restorations and since we have shown that structural protein is not a component of OSCP, a structural repair function on a massive scale is made unlikely.

In a recent review Pullman and Schatz (1967) discussed evidence that F_4 is a carrier of F_3 but, in addition, is unique in inducing oligomycin sensitivity in a preparation of F_0 extracted with ammonia. The data presented in this paper show that a soluble protein of mol wt 18,000 can restore coupling function at levels of 1 μg/mg of A particle and that the same protein at levels of 6 μg/mg of O-S ATPase (NaBr, NH_4OH) protein can restore oligomycin sensitivity in the reconstituted ATPase complex. These data provide clear evidence that at least one component of F_4 is not only an authentic coupling factor but is involved in coupling at the level of the oligomycin-sensitive site.

Acknowledgments

We thank Dr. David E. Green for his encouragement and helpful discussions during the course of this work; Dr. William H. Orme-Johnson for aid in the molecular weight determination; Dr. Carlton Paulson for assistance in the course of this study; Dr. Mary V. Buell for aid in the preparation of the manuscript; and Dr. Giovanna Lenaz, Mrs. Elke Reagan, and Mrs. Fay Yang for assistance in experimentation. Meat by-products were generously supplied by Oscar Mayer and Co., Madison, Wis.

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CORRECTIONS

In the paper "The Deoxycytidylate Deaminase Found in *Bacillus subtilis* Infected with Phage SP8," by Mutsuko Nishihara, Andreas Chrambach, and H. Vasken Aposhian, Volume 6, July 1967, p 1877, the following corrections should be made.

On p 1878, the second paragraph, second sentence under Experimental Procedure should read "The pooled fractions from the Dowex 1-formate column were identified as dHMUMP by the ratios of absorbancies at 250 and 280 to that at 260 mμ (280/260 = 0.55 and 250/260 = 0.68 at pH2)." The change is "Dowex 50-formate" to "Dowex 1-formate" and the addition of "and 280" to the sentence.

On p 1880, the last two lines on the right side should read ". . . . .in 0.2 M Tris-HCl buffer, pH 8.0 (w/v)." rather than "(pH 8.0, w/v)."

In the paper "Streptococcal Nucleases. III. Kinetics of Action and Inhibition by Transfer Ribonucleic Acid," by Walid G. Yasmineh, Ernest D. Gray, and Lewis W. Wannamaker, Volume 7, January 1968, p 91, the following correction should be made.

In the abstract a line was omitted. The correct version should read "(values are 6.5×10^{-4}, 1.8×10^{-3}, and 1.4×10^{-4}, respectively, by viscosimetry, and 2.1×10^{-4}, 6.8×10^{-4}, and 4.3×10^{-5}, respectively, by pH-Stat titration)."

In the paper "Soybean Inhibitors. I. Separation and Some Properties of Three Inhibitors from Commercial Crude Soybean Trypsin Inhibitor," by V. Frattali and R. F. Steiner, Volume 7, February 1968, p 521, Table III on p 526 should be corrected as follows: under Reference, the fourth entry should read "Rackis *et al.* (1962)," instead of "this paper."