PREPARATION AND CHARACTERIZATION OF HOMOGENEOUS COUPLING FACTOR 6 FROM BOVINE HEART MITOCHONDRIA

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SUMMARY: Coupling factor 6 (F_6) and mitochondrial ATPase inhibitor were isolated from the rutamycin-sensitive ATPase complex of bovine heart mitochondria by heating and fractionation with ethanol. F_6 appeared in acrylamide gel electrophoresis in the presence of sodium dodecylsulfate and urea as a single band corresponding to a molecular weight of 8,000. This protein which is required for the $^{32}P_i$-ATP exchange in submitochondrial particles treated with silicotungstate was very sensitive to trypsin.

Several proteins, called coupling factors, are required for the phosphorylation process associated with oxidations in mitochondria. Four of them are firmly established (1) as distinct components required for the uncoupler-sensitive $^{32}P_i$-ATP exchange: F_1[2] (2), F_2 or factor B (3), F_4 or OSCP (4,5) and F_6 or Fc_2 (6,7). The latter was shown to be required, together with OSCP, for the firm binding of F_1 to the membrane (7). In the past, F_6 was isolated by a complex procedure (6) which yielded multiple bands in acrylamide gels. We have recently described a rapid procedure for the isolation of a very active ATPase complex from submitochondrial particles of bovine heart which is completed in 4 hours (8). We now report on a simple procedure which is completed in about 2 hours, for the isolation of virtually homogenous preparations of F_6 and mitochondrial inhibitor from the ATPase complex of bovine mitochondria.

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[2]Abbreviations: F_1, F_2 and F_6, coupling factors 1 (2), 2 (3) and 6 (6,7), respectively; OSCP, oligomycin sensitivity conferral protein (5); A-particles and AS-particles, depleted submitochondrial particles derived from bovine heart mitochondria by sonic oscillation in the presence of ammonia before and after passage through a Sephadex G-50 column, respectively (9); STA-particles, particles derived from A-particles after treatment with silicotungstate (12); TUA-STA particles, particles prepared by exposure of light layer submitochondrial particles to trypsin, sequentually treated with 2 M urea, sonic oscillation in the presence of ammonia and silicotungstate (7); STA, silicotungstic acid; Buffer A, 250 mM sucrose, 0.25 mM EDTA and 10 mM Tris-SO_4, pH 8.0; DCCD, N,N'-dicyclohexylcarbodiimide; SDS, sodium dodecylsulfate; TEMED, N,N,N'N'-Tetramethylethylenediamine; BSA, defatted bovine serum albumin.

Materials and Methods

The ATPase complex (38-45 p fraction) (8), AS-particles (9), F_1 (10), F_2 or factor B (3), partially purified OSCP (11) and pure OSCP (5) were prepared according to the published methods. STA-particles (12) and TUA-STA particles (7) were prepared as described, and stored under liquid nitrogen.

$^{32}P_i$-ATP exchange was measured as follows: STA-particles, coupling factors and BSA (4 mg) were incubated in 0.5 ml of buffer A for 20 min at 30°. The reaction was started by the addition of an equal volume of a mixture containing 40 mM KP_i, 500 mM sucrose, 20 mM $MgSO_4$, 20 mM sodium ATP and 40 mM Tris-SO_4, pH 8.0. After 10 min at 30° the reaction was terminated and esterified phosphate was measured as described (13). ATPase activity and its DCCD sensitivity were measured at 30° in a volume of 0.5 ml containing 60 mM Tris-SO_4 (pH 7.7), 5 mM $MgSO_4$, 5 mM phosphoenolpyruvate, 16 µg of pyruvate kinase, 0.5 mg BSA, 200 mM sucrose, 0.2 mM EDTA as well as TUA-STA particles (200 µg), F_1 (4 µg), partially purified or purified OSCP (10 µg) and purified F_6 (0.5 - 5 µg). After 10 min preincubation, the reaction was started with 2.5 µmoles sodium ATP and the reaction was terminated after 10 min and liberated inorganic phosphate was determined (14). Whenever slight turbidity was present, it was clarified after color development with SDS (1% final concentration). The F_1 inhibitor was assayed as described (10). Binding of F_1 to TUA-STA particles was determined according to procedure B of ref. 7.

Protein was determined according to a modification of the Lowry method (15). Electrophoresis in gels of polyacrylamide with SDS and urea was performed as described (16) with 12.5% acrylamide and 0.82% or 1.25% methylenebisacrylamide. Urea (4 M) added to the reservoir solutions, improved the resolution. Samples were prepared by treating for 10 min at 85° at 0.1 to 1.3 mg protein per ml in 10% sucrose, 0.01% bromophenol blue, 2% SDS, 8 M urea, 0.1 M DTT, 1 mM EDTA and 10 mM H_3PO_4, adjusted to pH 7.0 with Tris. The samples containing 4-30 µg protei were applied per gel and occasionally sperm whale myoglobin (3 µg) was added along with the sample. Chymotrypsin, myoglobin, cytochrome c and the three

cyanogenbromide fragments of cytochrome c were applied to a standard gel. When the logarithm of the molecular weights of these standards were plotted against their relative mobility to myoglobin, a highly reproducible straight line was obtained which was used to calculate the molecular weights of the proteins in the samples. The gels were incubated with staining solution (16) for 3-4 hours at 37° and destained with acetic acid (7%, v/v) - methanol (30%, v/v). Gels were scanned at 550 nm in a Gilford spectrophotometer equipped with a linear scanner.

Cyrstalline bovine serum albumin from Sigma was defatted as described (17). DCCD was obtained from Schwarz-Mann, pyruvate kinase from Boehringer, Trypsin and Trypsin inhibitor from Worthington, STA from Hopkins and Williams, Ltd. (Chadwell Heath, Essex, England), $^{32}P_i$ from ICN (Irvine, Ca.). The latter was diluted to about 2 x 10^{10} cpm/ml in 2 N HCl and boiled for 1-2 hours.

Results and Discussion

During efforts to characterize the ATPase complex from bovine heart mitochondria (8), we observed that upon heating of the preparation at 75° two polypeptide chains were released. When small amounts of this preparation were added to STA particles in the presence of F_1, F_2 and OSCP, the $^{32}P_i$-ATP exchange reaction catalyzed by these particles was greatly enhanced. This observation suggested that one of the polypeptide chains released by heating is F_6, and the following procedure was developed to obtain the factor in virtually homogeneous form.

The ATPase complex (38-45 p fraction) at a protein concentration of about 25-30 mg/ml was heated in a test tube for 4 min at 75° in a heating block with occasional shaking. The denatured proteins were removed by centrifugation at 4° for 10 min at 12,000 x g. The supernatant (S_1) was removed with a Pasteur pippette and 2.8 ml of ice cold 95% ethanol was added per ml of S_1 (final ethanol concentration 70%, v/v). The mixture was agitated for 20-30 sec on a vortex mixer and centrifuged as above. The pellet was resuspended in buffer

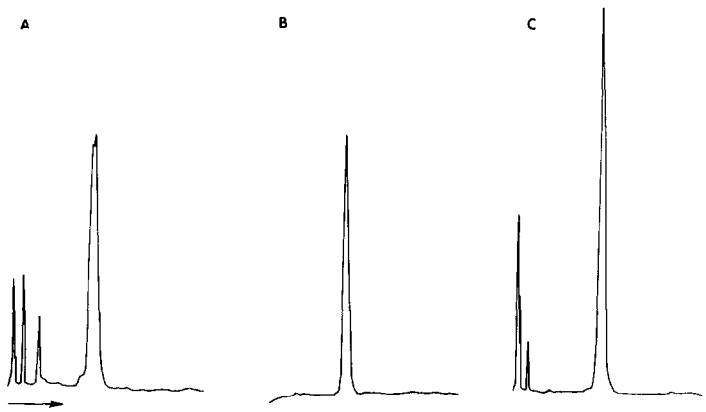

Fig. 1. Polypeptide composition of fractions derived from heated ATPase complex. Densitometric traces of S_1 (a), F_6 (b) and P_2 (c). Electrophoresis, staining, destaining and scanning were performed as described under "Materials and Methods". The gels contained 0.82% methylenebisacrylamide. Urea (4 M) was included in the reservoir solutions. The amount of protein applied was 5.2 µg (A), 4.7 µg (B) and 6.7 µg (C).

A at 0.5 - 1.0 mg protein per ml (P_2). The supernatant (S_2) was taken to dryness under reduced pressure. The residue was taken up in 0.5 ml of buffer A for each ml of starting material, and insoluble material was discarded after centrifugation as described above (final F_6).

Scans of polyacrylamide gels of S_1, P_2 and the final F_6 preparation, run in the presence of SDS and urea, are presented in Fig. 1, A-C. The F_6 preparation stimulated about 8 to 10 fold the $^{32}P_i$-ATP exchange of STA particles in the presence of F_1, F_2 and OSCP (Fig. 2). Under our experimental conditions, a half maximal stimulation was obtained with about 0.4 µg of the purified coupling factor. This stimulation by the coupling factor was abolished by exposure of F_6 to trypsin (Fig. 2), a property also exhibited by crude F_6 preparations (6,7). The highly purified coupling factor was also effective in conferring DCCD sensitivity to F_1 with TUA-STA particles in the presence of OSCP (7) and in the binding of F_1 to the latter particles (7)[3] (not shown).

[3]Recently Slater also demonstrated that both OSCP and F_6 are required for the binding of F_1 to the membrane (personal communication).

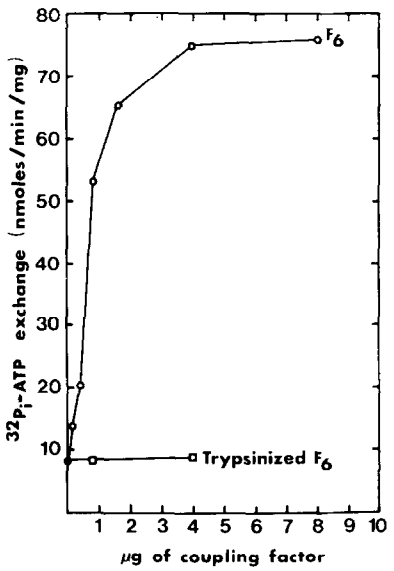

Fig. 2. Effect of F_6 on the $^{32}P_i$-ATP exchange reaction of STA particles. $^{32}P_i$-ATP exchange was measured as described under "Materials and Methods" with 225 μg of STA particles, 120 μg of F_1, 20 μg of partially purified OSCP and 25 μg of F_2. Trypsin treatment of F_6 was performed as follows: 40 μl of a Trypsin solution (0.5 mg/ml in 1 mM H_2SO_4) was added to 60 μg F_6 in 25 mM Tris SO_4, pH 8.0, 75 mM sucrose and 0.08 mM EDTA (volume 0.26 ml), and the mixture was incubated for 5 min at 30°. Then a five-fold excess of Trypsin inhibitor was added and the mixture was diluted to 0.6 ml. F_6 (0 —— 0) and Trypsinised F_6 (□—— □)

The F_6 preparation appears to be virtually homogeneous (Fig. 1B). F_6 extracted from some batches of the ATPase complex contained a small amount of contaminant with an apparent molecular weight of 30,000. The molecular weight of F_6 was estimated to be 8,000.

The additional band present in the S_1 fraction was effectively removed by the ethanol precipitation step (Fig. 1, A-C). This band was identified as the F_1 inhibitor (Fig. 3). The level of contamination was variable; usually the preparations were purer than shown in this figure. The mobility of this polypeptide chain on polyacrylamide gels in the presence of SDS and urea was found to be identical with that of F_1 inhibitor isolated by an alternative procedure (18). A molecular weight of 6800 was estimated. This is at variance with the value of 10,000 obtained with polyacrylamide electrophoresis in sodium dodecyl sulfate,

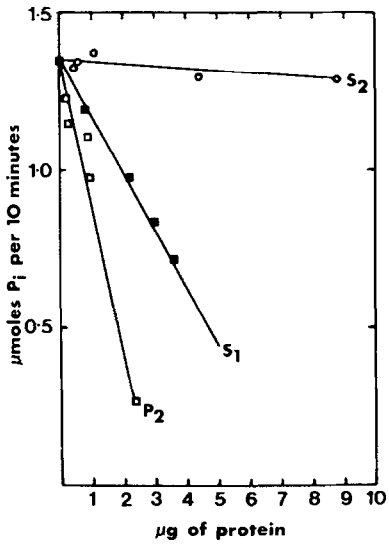

Fig. 3. F_1 inhibitor activity of fractions derived from heated ATPase complex. AS particles containing 3.4 units of ATPase activity (1 unit = 1 μmole P_i liberated at 30°) were incubated with those amounts of the various fractions indicated on the abscissa, exactly as described (10). After 20 min at room temperature, 20 μl aliquots were assayed for ATPase activity as described. When S_2 was used, aliquots of this fraction were dried down in the preincubation tube under a stream of nitrogen gas. Subsequently all the other components of the incubation mixture were added. P_2 (□——□), S_1 (■——■) and S_2 (○ —— ○).

without urea present (18,19). The latter value has also been derived from calculations based on the amino acid composition of the inhibitor protein (19,20). The inhibitor activity in the S_1 fraction was found to be 40-50% of that of P_2 (Fig. 3), in fair agreement with the gel scans (Fig. 1A). Virtually no inhibitor activity could be detected in the highly purified F_6 preparation.

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