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Purification of the head-pieces of the elementary particles from beef heart mitochondria: their morphological structure and enzymatic activity*

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Abstract

1. In order to obtain direct evidence for the enzymatic identification of the head-pieces of the elementary particles in the inner mitochondrial membrane, the head-pieces were detached by sonication from the isolated inner membrane of beef heart mitochondria, purified by pursuing the particles with the electron microscope, and analyzed for enzymatic properties. 2. Electron microscope examination revealed that the isolated headpieces are the spherical particles about 90À in diameter which are quite similar in appearance to the head-pieces of the elementary particles lining the inner mitochondrial membranes. 3. The head-pieces are identified as ATPase sensitive to oligomysin when attached by stalks to the membrane, and become insensitive when detached or purified from the membrane. 4. The head-piece is labile to cold with respect to ATPase activity and morphology.

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PURIFICATION OF THE HEAD-PIECES OF THE ELEMENTARY PARTICLES FROM BEEF HEART MITOCHONDRIA: THEIR MORPHOLOGICAL STRUCTURE AND ENZYMATIC ACTIVITY

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The elementary particles associated with the inner mitochondrial membranes or cristae have been demonstrated with the electron microscope in negatively stained preparation of the mitochondria from a wide variety of cell types. Fernández-Morán *et al.* (2) have shown that the elementary particle consists of three parts: head-piece, stalk, and base piece. The head-pieces are connected by stalk to the base pieces, which are fused together to form the membrane. On the nature of the head-pieces of the elementary particles, there was noticeable discrepancy of opinion whether it may contain the electron transfer chain (1), cytochrome *a* (2), cytochrome *b* or c_1 (4), or none of the components of the electron transfer chain (5, 6). Recently, Racker *et al.* (7, 8) have suggested that the head-pieces of the elementary particles correspond to a soluble cold-labile ATP-ase, so called coupling factor F_1 (9), on the basis of the similarity in the properties between the head-pieces and the F_1.

In order to obtain direct evidence of the fine structure and biochemical properties of the head-pieces of the elementary particles in a series of studies on the molecular organization of the electron transfer and oxidative phosphorylation systems in the inner mitochondrial membrane (10, 11), we have isolated the head-pieces by sonication from the inner membranes, purified them by pursuing the particles with the electron microscope, and analyzed their biochemical properties.

MATERIALS AND METHODS

Preparation of the Electron Transfer Particles: Beef heart mitochondria were isolated from fresh tissue by a modification of the method of Crane *et al.* (12). The mitochondrial inner membrane were prepared from the isolated beef heart mitochondria by the following method. The mitochondria suspension stored at −20°C was thawed, centrifuged in a refrigerated Kubota centrifuge (roter No. 3) at 0°C

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for 15 minutes at 10,000×g. The supernatant liquid was discarded. The residue was homogenized in a solution containing 0.25 M sucrose and 1 mM Tris-HCl, pH 7.4, adjusting the final protein concentration to 20 mg per ml and then the homogenate was exposed to sonic oscillation for 50 seconds in 5 ml in the Kaijo Denki ultrasonic oscillator, which was continuously cooled by cold bath at 0°C and adjusted for maximum power output. The sonically treated suspension was centrifuged at 26,000×g in a Spinco Model L centrifuge (rotor No. 30) for 10 minutes. The residue was discarded. The supernatant fraction was centrifuged at 105,000×g for 60 minutes. The supernatant fluid was discarded. The residue was homogenized in 0.25M sucrose-1mM Tris-HCl solution, pH 7.4, in about a 20-fold residue volume, and centrifuged at 105,000×g for 60 minutes. The supernatant fluid was discarded. The residue was subjected to the same procedure once more. The residue resulting from the final centrifugation consists of submitochondrial particles, which is called mildly sonicated electron transport particle (m. s. ETP).

Isolation of the Head-Pieces of the Elementary Particles: The isolation of the head pieces of elementary particles from m. s. ETP was done by the following procedure: m. s. ETP was homogenized in 0.25M sucrose-1 mM Tris-HCl solution, pH 7.4, and diluted to a final protein concentration of 10 mg per ml. The sample, 4ml suspension was exposed to severe sonic oscillation for 5 minutes, keeping at 15～20°C by continuous cooling of the sample tube. This sonically-treated suspension was called severely sonicated electron transport paticles (s. s. ETP). The isolation of the heud-piece particles from s. s ETP was made by a modification of ATPase by PULLMAN *et al.* (9). A sample of s. s. ETP was centrifuged at 105,000×g in a Spinco Model L centrifuge (rotor No. 30) for 30 minutes. The supernatant fluid was adjusted with 1 N acetic acid to pH 5.4 at 4°C. After the solution was centrifuged in a refrigerated Kubota centrifuge (rotor No. 3) at 0°C for 15 minutes at 18,000×g and the precipitate was discarded. The pH of the supernatant solution was slowly adjusted to 6.7 by the addition of 2M Tris, pH 11.0. For 10 mg each of protein recovered from this adjusted solution 0.2 ml of the 0.5% protamine sulfate solution (pH 7,0) was added at 4°C with gentle mechanical stirring, which was continued for an additional 15 minutes at 4°C. The mixture was centrifuged for 15 minutes at 10,000×g. The residue was dissolved at room temperture in a solution containing 0.4M ammonium sulfate pH 7.4, 0.25M sucrose, 0.01M Tris pH 7.4, and 0.001 M EDTA pH 7.4 to give a protein concentration of about 3 to 4 mg of protein per ml. An insoluble residue was centrifuged for 15 minutes at 18,000×g and discarded. To the clear supernatant solution, an equal volume of saturated ammonium sulfate solution, pH 5.5, was added with gentle stirring. The solution was kept at 4°C for about 15 minutes until complete precipitation occurred. The head-pieces were collected by centrifugation of the precipitated sample for 15 minutes at 105,000×g. In order to investigate the changes in the structure and enzymatic activity of the sample induced by heat treatment, the precipitate was adjusted to 15 mg of protein per ml., and for each milliliter of this solution 0.02 ml of 0.2 M sodium

ATP, pH 7.4 was added and the mixture was placed in a hot water bath at 55°C for 2 minutes. After cooling in a water bath to 25°C, the denatured protein was removed by centrifugation at 18,000×g for 15 minutes at room temperature. The enzyme was kept at 4°C in 50% ammonium sulfate solution. For the assay of ATPase activity, the ammonium sulfate suspention was centrifuged, and the enzyme was dissolved at room temperature in sucrose-Tris-EDTA solution.

Enzyme assay: ATPase activity was determined by the following method. The reaction medium contained, in a final volume of 1.0 ml, 50 μ moles of Tris-HCl (pH 7.4), 6 μ moles of sodium ATP (pH 7.4)., 3 μ moles of $MgCl_2$. After a 5-minute equilibration at 30°C, the reaction was initiated by the addition of an appropriate amount of enzyme (0.2—0.5 mg of protein) and after incubation for 10 minutes at 30°C, the reaction was terminated by the addition of 1 ml of 16% perchloric acid. After centrifugation, the supernatant fluid was analyzed for Pi by the method of TAKAHASHI (13). When present, the concentration of dinitrophenol and oligomycin was 0.0001 M and 2.5 μg/ml respectively. ATPase activity was also determined in the presence of an ATP regenerating system using phosphoenol-pyruvate (5 μ moles) and pyruvate kinase (30 μg). Protein was measured by a modified biuret (14) and Lowry *et al.* method (15). Solutions of crystalline bovine serum alubumin were used as standards.

Electron microscopy: Electron microscopic observations by negative staining techniques were carried out in each step of the purification on samples suspended in distilled water and mixed with an equal volume of 1—2% aqueous solution of phosphotungstate or uranyl acetate. The mixture was placed on 150-mesh copper grids, which were covered with a 4% collodion supporting membrane and coated with carbon. Micrographs were taken at an original magnification of 20,000 or 40,000 employing a Hitachi HU-11P electron microscope operating at 75 KV.

RESULTS

Negatively stained membrane fractions derived from mitochondria by mild sonic treatments show the presence of a vesicular membrane ranging in size from 30 to 1000 mμ. This fraction is illustrated in Fig. 1. The membrane fragments show the presence of membrane-bound head-pieces, each one being supported by a stalk and projecting from the periphery of the membrane. The membrane fragments contain these units, not only in the periphery but also on the whole surface. The m. s. ETP is illustrated in Fig. 2. This fraction shows predominantly a concentration of membrane fragments measuring about 50 mμ-300 mμ in diameter. The membrane fragments show the presence of head-pieces supported by stalks and projecting from the periphery of the membrane, and the head-pieces appear on the entire surface of the membrane. This fraction also exhibits partial loss of the head-pieces from some of the membrane fragments, showing a discontinuous pattern of the elementary particles. It is apparent that sonic

treatment is capable of removing some of the head-pieces from membrane fragments. For this reason, the m. s. ETP were treated with severe sonic oscillation, which is illustrated in Fig. 3. The negatively stained fraction shows the presence of isolated head-pieces and denuded membrane fragments, which do not bear head-pieces on both the periphery and the surface of the membranes. Apparently, by severe sonic treatment of the m. s. ETP, most of the head-pieces of the elementary particle were removed from the membrane. This fraction was centrifuged at 105, 000×g for 30 minutes. The supernatant fraction is illustrated in Fig. 4, showing a large number of isolated head-pieces and a few contaminated small membrane fragments. None of the stalks are observed in this fraction. Fig. 5 illustrates uranyl acetate stained head-pieces in a supernatant fluid resulting from adjusting with 1 N acetic acid to pH 5.4. The head-pieces measuring about 90 Å in diameter are recovered in the supernatant fraction without contamination of membrane fragments. The residue shows aggregation of head-pieces and membrane fragments, which is illustrated in Fig. 6. The head-piece fractions after treatment with protamine and ammonium sulfate show highly purified uniform particles, measuring approximately 90 Å in diameter. Fig. 7 illustrates the head-pieces after the ammonium sulfate fractionation. By the heat treatment, the head-piece did not show a structural change, as is illustrated in Fig. 8.

ATPase activity in beef heart mitochondria and submitochondrial particles obtained by sonic irradiation is shown in Table 1. ATPase activity was relatively high in the mildly sonicated mitochondria and the fraction sedimenting between 26, 000×g and 105, 000×g. The addition of oligomycin inhibited the ATPase activity in these membrane fractions, while it had less effect on ATPase activity in the supernatant fraction at

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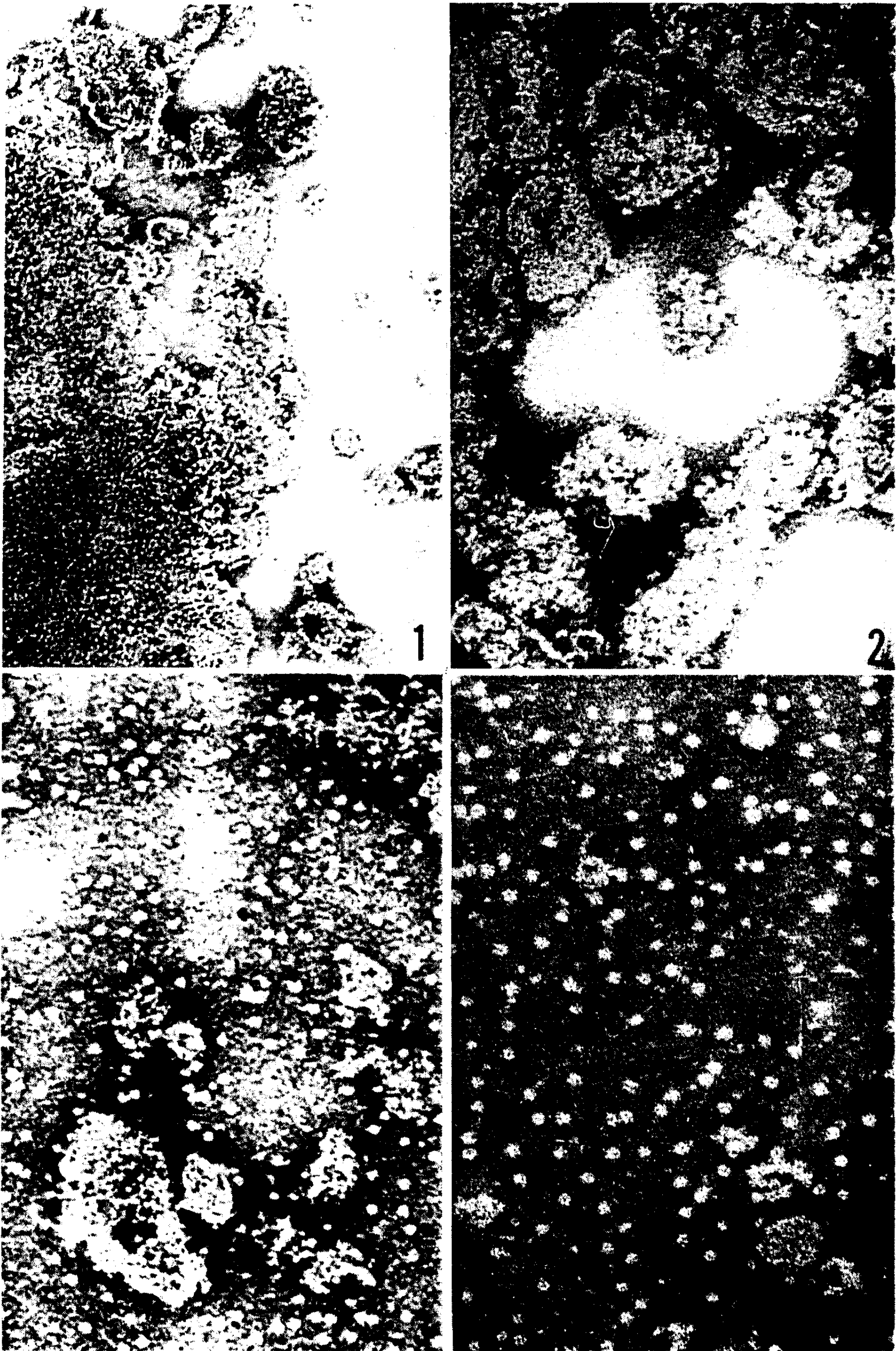
Fig. 1 Membrane fragments derived from beef heart mitochondria by sonic treatment, negatively stained with 2% sodium phosphotungstate. The fragments range in size from 30 to 1000 mμ. Not only the periphery but also the whole surface of the membrane fragments present head-pieces. (×50, 000)

Fig. 2 Mildly sonicated electron transfer particles (m. s. ETP), negatively stained with 2% sodium phosphotungstate. The fragments range in size from 50 mμ to 300 mμ. The whole surface of the membrane fragments contains head-pieces. The head-pieces are lost from some parts of the membrane fragments. (×200, 000)

Fig. 3 The severely sonicated m. s. ETP, negatively stained with 2% sodium phosphotungstate, showing isolated head-pieces and membrane fragments which do not bear head-pieces. (×180, 000)

Fig. 4 The supernatant fraction after centrifugation of the sample in Fig. 3 at 105, 000×g for 30 minutes, negatively stained with 2% sodium phosphotungstate. In this fraction isolated uniform particles quite similar to the head-pieces and small membrane fragments can be seen. (×200, 000)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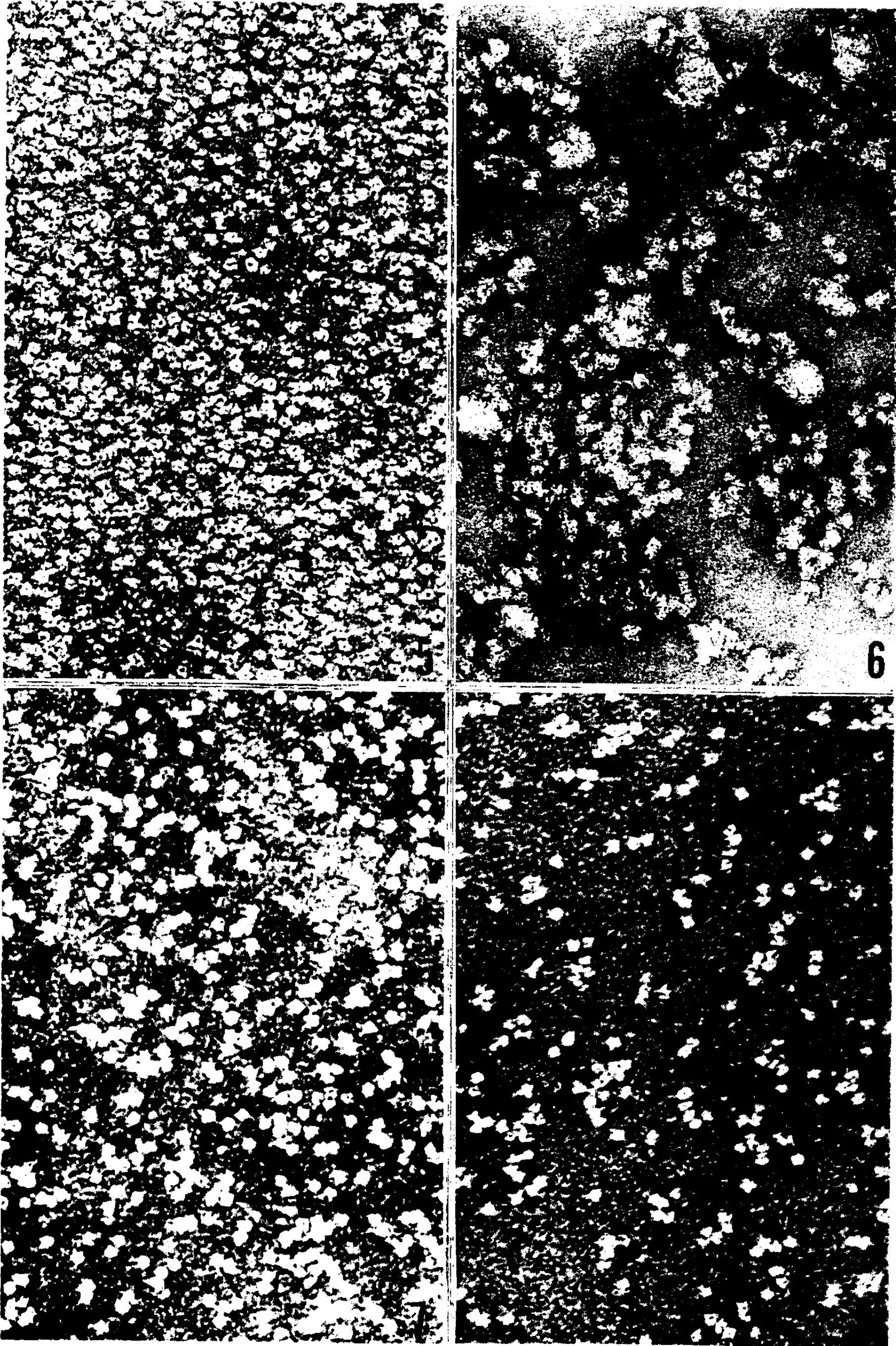
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Table 1 ATPase activity in beef heart mitochondria and submitochondrial particles obtained by sonic irradiation

Fractions	Protein (mg)	ATPase activity: Specific activity (μmoles Pi released /mg protein/min.)	ATPase activity: Total Activity (μmoles)	ATPase activity: Oligomycin inhibition (%)
Mitochondria	800	0.13	1,040	93
Mildly sonicated* mitochondria	800	0.70	5,600	86
26,000×g residue	379	0.59	2,220	85
105,000×g residue	276	0.89	2,450	90
105,000×g	119	0.26	310	57

* Condition of mild sonic irradiation: 20 Kc, 50 sec./5 ml, 20 mg protein/ml

Table 2 Summary of purification procedures

Step	Protein (%)	ATPase activity (specific activity)*: Complete system	+Oligomycin	+DNP	+PEP, +PK
m. s. ETP	100	1.25	0.04	1.13	2.30
Severely sonicated m. s. ETP	100	0.86	0.53	0.84	1.98
s. s. ETP	71	0.68	0.04	0.63	1.14
s. s. ETP-supernatant	15	2.50	2.30	2.38	4.20
Supernatant of acid precipitate	2.6	11.00	8.60	8.67	19.40
Fractionation with protamine	0.5	11.07	9.10	8.67	19.40
Heating at 65°C	0.28	16.90	17.35	17.35	33.70

* Specific activity is expressed as μ moles of inorganic phosphate formed per mg of protein per minutes.
Assay conditions are given in "Materials and Methods"

105,000×g. ATPase activity increased in each step of purification of the head-pieces of the elementary particles, as shown in Table 2. ATPase activity of the m. s. ETP was inhibited in the presence of oligomycin, and

Fig. 5 The supernatant fluid obtained by adjusting the sample in Fig. 4 with 1 N acetic acid to pH 5.4, negatively stained with 2% uranyl acetate. The isolated head-piece particles measure about 90 Å in diameter. (×200,000)

Fig. 6 The residue fraction obtained by the treatment shown in Fig. 5, negatively stained with 2% uranyl acetate, showing aggregation of head-pieces and small membrane fragments (×200,000)

Fig. 7 The head-pieces obtained by ammonium sulfate fractionation from the sample in Fig. 5, showing highly purified uniform particles, measuring about 90 Å in diameter, negatively stained with 2% sodium phosphotungstate. (×180,000)

Fig. 8 The head-pieces after heating to 65°C, negatively stained with 2% sodium phosphotungstate. Note the head-pieces have not undergone a structural change. (×200,000)

the addition of dinitrophenol had little effect. The oligomycin sensitivity was lost in the supernatant fraction of the severely sonicated m. s. ETP. By further purification of this supernatant fluid by acid treatment, oligomycin-insensitive ATPase activity was markedly increased. In this step, the fraction contained highly uniform particles. Further increase in ATPase activity was observed by treatment with either protamine sulfate or with ammonium sulfate. ATPase activity increased by exposure to heat and decreased by keeping in the cold. The ATPase activity in the fractions of each step of purification was approximately two times higher when assayed in ATP-generating system containing phosphoenol pyruvate and pyruvate kinase. Detailed description on the biochemical properties of the isolated head-pieces will be published elsewere (16).

DISCUSSION

FERNÁNDEZ-MORÁN, GREEN and ODA (1∼3) have shown that the elementary particle consists of head-pieces, stalks and base pieces. Up to the present time, many works have been carried out to clarify the biochemical nature of these particles. Membrane derivatives from mitochondria have been isolated by addition of surface active agents (17∼20), by sonication (17, 18, 21), and by mechanical treatment (22, 23). In any case, negative staining was not employed, and stalked particles were not detected. GREVILLI *et al.* (24). have sonicated *Calliphora* mitochondria in water, and observed with the electron microscope by negative staining that the stalk particles were firmly attached to cristae and vesicles. However, detachment of the head-pieces from the inner membrane is frequently observed in the mitochondria or submitochondrial particles negatively stained with phosphotungstic acid. This observation suggests that the head-pieces may be removed during exposure to sonic irradiation. We have attempted to isolate the head-pieces from the inner membrane of beef heart mitochondria by sonic treatment and to obtain direct evidence on their properties. The data presented in this paper show that the head-pieces of the elementary particles can be isolated by severe sonic treatment of the isolated inner membrane of beef heart mitochondria obtained by mild sonication. The isolated head-pieces can be purified by pursuing the particles with the electron microscope. The purified particles measured about 90 Å in diameter and were quite similar in appearance to the head-pieces lining the mitochondrial inner membrane. Recently, RACKER *et al.* (7, 8, 25). have suggested that the head-pieces may correspond to a so-called coupling factor F_1 ATPase which are isolated by treating the beef heart mitochondria

with Nossal Shaker (9). ANDREOLI and SANADI (26) have also reported evidence suggesting that their soluble factor contains a heat stimulated ATPase activity similar to that of PULLMAN *et al.* (9)., but they have not observed it with an electron microscope.

In the present experiment, the purified head-pieces have high oligomycin-insensitive ATPase activity. When exposed to cold, the head-pieces are depolymerized to form an irregular aggregates with a concomitant loss of ATPase activity. The purified head-pieces are very similar in their oligomysin-insensitivity and cold-lability of ATPase activity to the ATPase or coupling factor F_1 originally described by PULLMAN *et al.* (9)., and may be functionally identical with the soluble factor described by ANDREOLI *et al.* (26).

In view of the above factors the most reasonable conclusion to be drawn from available data is that the head-pieces are same as the ATPase descrived by PULLMAN *et al.* (9).

SUMMARY

1. In order to obtain direct evidence for the enzymatic identification of the head-pieces of the elementary particles in the inner mitochondrial membrane, the head-pieces were detached by sonication from the isolated inner membrane of beef heart mitochondria, purified by pursuing the particles with the electron microscope, and analyzed for enzymatic properties.

2. Electron microscope examination revealed that the isolated head-pieces are the spherical particles about 90 Å in diameter which are quite similar in appearance to the head-pieces of the elementary particles lining the inner mitochondrial membranes.

3. The head-pieces are identified as ATPase sensitive to oligomysin when attached by stalks to the membrane, and become insensitive when detached or purified from the membrane.

4. The head-piece is labile to cold with respect to ATPase activity and morphology.

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