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Goki Yamamoto*

*Okayama University,

Isolation and some properties of oligomycin-sensitive adenosine triphosphatase from beef heart mitochondria and its morphological study*

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Abstract

1. To have a rapid isolation of oligomycin-sensitive ATPase particles (OSA particles), 0.1 mg DOC per mg of protein and 72 g potassium chlo. ride per I were added to mitochondria suspended in a tris.sucrose-histidine solution, which was followed by addition of 2-fold volume of chilled water, and fractionated by a discontinuous sucrose density gradient centrifugation. As a result, it was possible to reveal the OSA particle structure, composed of the head piece, stalk and thread-like structure of a superficial portion of the base pieces, stripped off from the mitochondrial inner membrane, in a layer of density.l.lO. This fraction exhibited a remarkable activity of ATPase sensitive to oligomycin, approximately 15 ,lJ.moles Pi released per mg of protein per minute at pH 8.6 at 37° in a non-ATP regenerating assay system, and contained almost no cytochromes. 2. When the OSA particles thus isolated were heated in water bath at 65° for 2 minutes, the head pieces were detached with a concomitant loss of oligomycin-sensitivity and were purified from the supernatant by precipitation with ammonium sulfate. 3. Trypsin in low concentration slightly induced a rise in the ATPase activity of OSA particles but in higher concentration it inhibited the activity. 4. OSA particles were resistant to the treatment of urea, and it was difficult to detach the head pieces by this treatment. 5. The some fraction obtained by solubilization of thc crude OSA particles with cholate and fractionation with ammonium sulfate exhibited ATPase activity in a masked form, and the ATPase activity with oligomycin. sensitivity was restored on addition of phospholipid. 6. A discussion was made on the mode of assembly of the head pieces and associated components and biochemical properties of OSA particles.

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ISOLATION AND SOME PROPERTIES OF OLIGOMYCIN-SENSITIVE ADENOSINE TRIPHOSPHATASE FROM BEEF HEART MITOCHONDRIA AND ITS MORPHOLOGICAL STUDY

Goki YAMAMOTO

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama. Japan (Director: Prof. T. Oda)

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It has been demonstrated that the isolated head pieces of the tripartite form (1—3) of the elementary particles from mitochondrial inner membrane exhibit a remarkable oligomycin-insensitive ATPase activity (2, 4—11), while the head pieces bound to the membrane show oligomycin-sensitive ATPase activity (2, 5, 8, 12). On the other hand, factors conferring oligomycin-sensitivity, F_0 (5, 6, 13—15) and CF_0 (5, 8, 16), were isolated in RACKER's laboratory. As reported in the previous paper (17), the head pieces attached by stalks to a superficial portion of the base pieces were successfully isolated from beef heart mitochondria by the systematic and stepwise microdissection of the inner membrane by treating with bile salt and salts (2, 9, 17, 18). These particles with almost free of membrane structure exhibited oligomycin-sensitive ATPase activity and thus these particles were arbitrarily designated as oligomycin-sensitive ATPase particles (OSA particles) (2, 9, 11, 17).

The present communication reports a rapid and improved method of isolating highly active oligomycin-sensitive ATPase particles from beef heart mitochondria by a sucrose density gradient centrifugation method, and describes some biochemical properties of the OSA particles and the correlation of their fine structure to their function as observed with electron microscope.

MATERIALS AND METHODS

ATP was obtained from Sigma Chemical Co. Deoxycholate (DOC) and cholate obtained from Difco Laboratories were recrystallized with active charcoal, and used as a 10 % solution adjusted to pH 8.0 by potassium hydroxide. Oligomycin was donated by Dr. D. E. GREEN, Institute for Enzyme Research, University of Wisconsin, and used by dissolving in ethanol. Asolectine (purified soya bean phosphatide) from Associated Concentrates Inc. was dissolved by the method of LESTER and SMITH (19). Trypsin (EC 3, 4, 4, 4) and trypsin inhibitor

(from soya bean) were obtained from Sigma Chemical Co. and Difco Laboratories, respectively, and urea from Katayama Kagaku Co. Other chemicals were of special reagent grade, and distilled deionized water was used throughout the experiment.

Beef heart mitochondria were prepared by the routine method (20) of our laboratory, which is a modification of CRANE *et al.* (21). Mitochondria finally suspended in 0.25 M sucrose were stocked in a freezer at −20°. Prior to the experiment the stocked mitochondria were thawed at room temperature and resuspended in 10-fold volume of the chilled tris-sucrose-histidine solution (TSH solution: 0.05 M tris (hydroxymethyl) aminomethane, 0.66 M sucrose, 0.001 M histidine, pH adjusted to 8.0 by hydrogen chloride), and centrifuged at 10,000×g for 10 minutes. The sediment was suspended in TSH solution to a final protein concentration of 23 mg mitochondria per ml, and this suspension served as original mitochondria.

ATPase activity was measured in the medium containing 50 μmoles tris-chloride buffer, pH 7.4 or 8.6, 3 μmoles magnesium chloride, and less than 0.1 mg of sample protein in 0.1 ml of TSH solution. Oligomycin and asolectine in dose of 25 μg and 100 μg per system, respectively, were added. Aliquot (0.9 ml) of the medium containing the sample was preincubated 2 to 5 minutes at 30° or 37°, and then the reaction was started by addition of 0.1 ml of 0.05M ATP (pH adjusted with tris) making the final volume 1.0 ml. After 2 to 5 minutes incubation at 30° or 37° the reaction was stopped by addition of 1.0 ml of 16 % chilled perchloric acid. After centrifugation for deproteinization, the released inorganic phosphate (Pi) was determined by the method of TAKAHASHI (22).

Amount of protein was determined by the method of LOWRY *et al.* (23) comparing with bovine serum albumin obtained from Armour Laboratories (Fraction V) as standard.

Spectrophotometric measurements were carried out using Shimadzu photoelectric spectrophotometer (QR-50) and Hitachi recording spectrophotometer (EPS-3T) for estimating mitochondrial cytochrome components. The components were estimated by the method described by SEKI *et al.* (18, 20).

Electron microscope observation was carried out by negative staining with 1 % phosphotungstate (pH 7.2, Merk) (PTA) using the floating method (18) and micrographs were taken at an electron optical magnification of 40,000 or 80,000 by Hitachi electron microscope (HU-11B-S).

RESULTS

Effect of DOC in the presence of potassium chloride on ATPase activity of mitochondria:

OSA particles were stripped off from mitochondrial membrane by treating with DOC in the presence of potassium chloride in ice-bath, but the mode of their solubilization from membrane varied according to different concentrations of DOC (17, 18). The treatment with DOC at low

concentration (0.1 mg per mg of protein) accelerated the ATPase activity of mitochondria in the absence of potassium chloride (Fig. 1). In the presence of potassium chloride (72 g per l) only, the ATPase activity of the

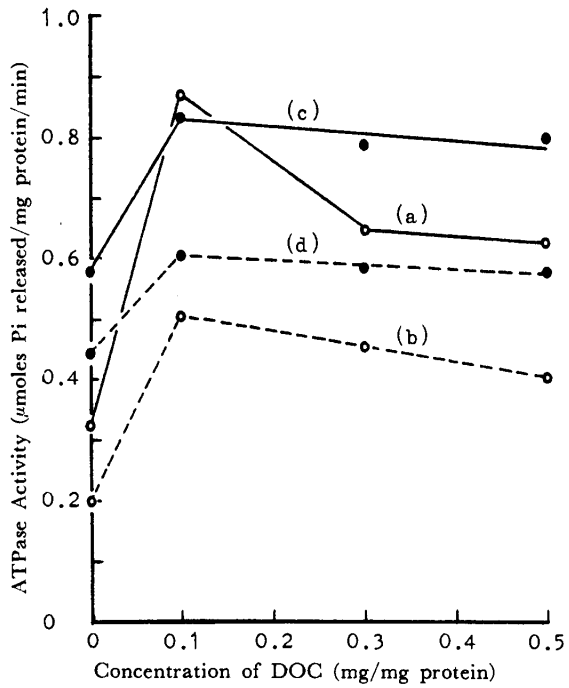


Fig. 1. Effects of different concentrations of DOC in the presence or absence of KCl (72g/l) on the ATPase activity of beef heart mitochondria. The incubation medium contained ATP (5mM), $MgCl_2$ (3mM), tris-Cl (0.05M) buffer and the treated mitochondrial suspension. The final volume was 1 ml. The experiment at 30° was performed on the conditions as follows: Effects of the assay system (pH 7.4) on the mitochondria after 1 hour incubation with DOC in the absence (a) or in the presence (b) of KCl at 0°. Effects of assay system (pH 8.6) on the mitochondria after 1 hour (c) and 4 hour (d) incubation with DOC in the presence of KCl at 0°.

original mitochondria was inhibited and in the presence of potassium chloride and DOC the ATPase activity was accelerated. As shown in Fig. 1, DOC at the concentration of 0.1 mg per mg of protein enhanced the ATPase activity more than at any concentration in the presence of potassium chloride, although the activity decreased in proportion to aging after the treatment. On the other hand, oligomycin practically inhibited the ATPase activity in the present condition. The electron microscope observation revealed that the concentration of DOC in 0.1 mg per mg of protein in the presence of potassium chloride was suitable for isolation of

OSA particles (17) as well as for enhancing the ATPase activity.

Isolation of OSA particles from mitochondria :

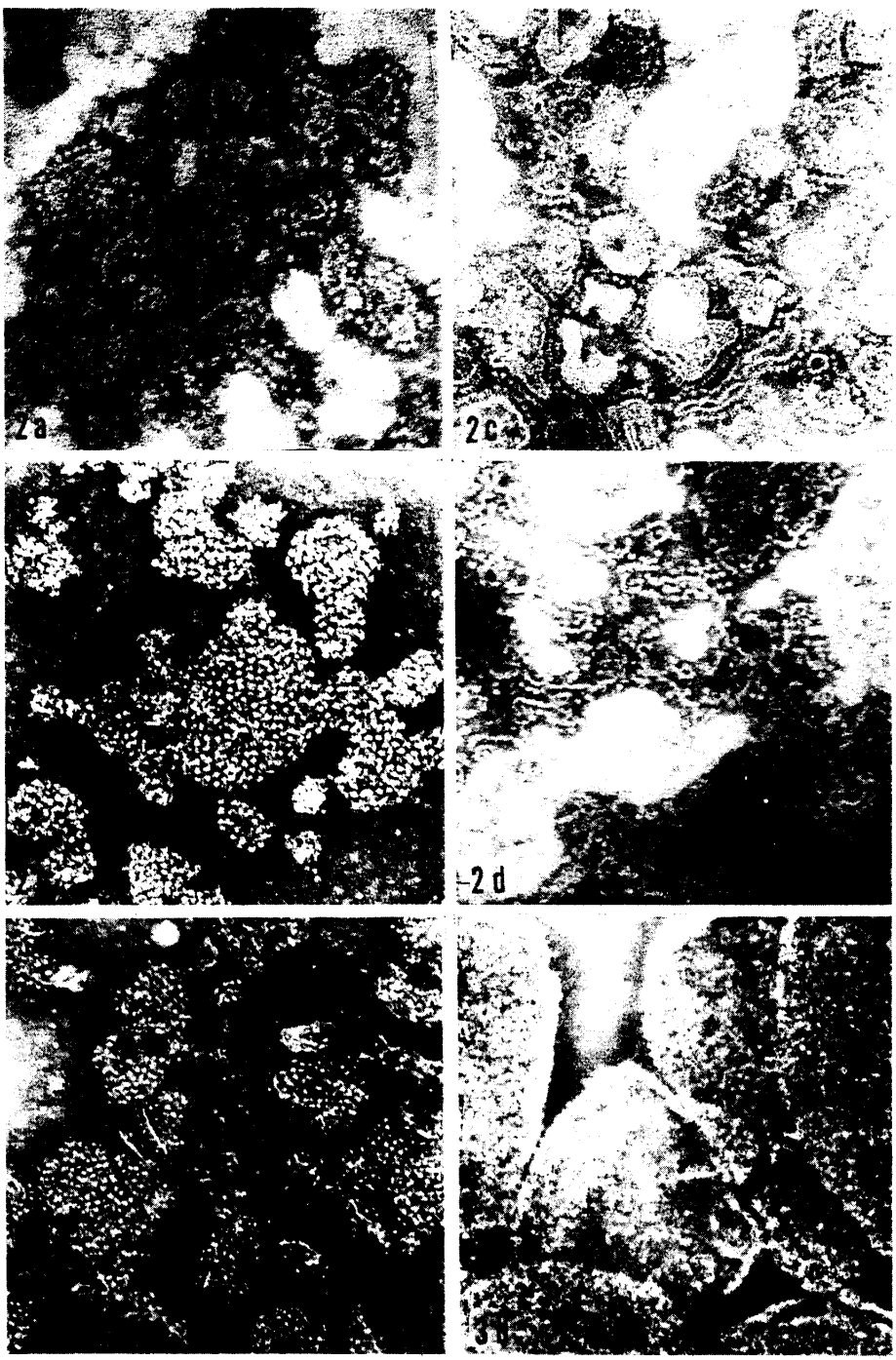
In the trials with various methods applied to the isolation of OSA particles, the following method proved to be most suitable. All manipulations were carried out within the range of 0° to 4°, unless otherwise stated. Beef heart mitochondria (suspended in TSH solution at the cencentration of 23 mg of protein per ml) were added with DOC at the concentration of 0.1 mg per mg of protein, followed by the addition of solid potassium chloride (72 g per 1). After dissolution of added potassium chloride, 2-fold volume of chilled water was added to the mitochondrial suspension with gentle shaking. The diluted mitochondrial suspension was layered promptly on the discontinuous sucrose density gradient solution composed of 1.10 and 1.14 in density, and was centrifuged at 105,000×g for 60 minutes by using the rotor 39 (SW) or 30 of Beckman ultracentrifuge. model L2. ATPase activities of each centrifuged fraction are illustrated in Table 1. The sucrose density-1.10 layer fraction exhibited more marked

Table 1. THE ATPASE ACTIVITY OF THE PREPARATION OF OLIGOMYCIN-SENSITIVE ATPASE BY SUCROSE DENSITY GRADIENT CENTRIFUGATION FROM BEEF HEART MITOCHONDRIA. MITOCHONDRIA (23 mg OF PROTEIN/ml IN TSH SOLUTION), TREATED WITH DOC (0.1mg/mg OF PROTEIN) AND KCl (72g/l) FOLLOWED BY ADDITION OF TWO-FOLD VOLUME OF DISTILLED WATER, WERE LAYERED ON THE DISCONTINUOUS SUCROSE DENSITY GRADIENT SOLUTION AND CENTRIFUGED AT 105,000×g FOR 60 MINUTES. SPECIFIC ACTIVITY IS EXPRESSED AS μMOLES OF PI RELEASED PER mg OF PROTEIN PER MINUTE IN THE ASSAY SYSTEM AT pH 8.6 AT 37°.

Fractions	Protein recovery (%)	ATPase activity Specific activity	ATPase activity Total activity (%)	Oligomycin inhibition (%)
BHM	100	1.94	100	95
Treated BHM	100	1.77	91.3	94
Sample layer	24.3	0.52	6.5	70
d-1.10 layer	5.2	15.7	42.1	87
d-1.14 layer	17.5	4.73	43.6	93
Precipitate	49.1	0.84	21.3	95

Fig. 2. Eelectron micrographs, negatively stained with phosphotungustate, of oligomycin-sensitive ATPase particles prepared by sucrose density centrifugation method from beef heart mitochondria treated with DOC and KCl as described in the text. Structures were observed in which the head pieces were arranged on the thread-like base pieces by stalks (a), to sheet-like or bulk phase (b), and in regular array around the ring-like structure (c). The Fig. 2 (b) structure contained the associated components as observed after the treatment with phospholipid (d). Magnification 120,000.

Fig. 3. (a) An electron micrograph of oligomycin-sensitive ATPase prepared by sucrose density gradient centrifugation method from S-2 fraction previously reported (17). Negatively stained with PTA, magnification 120,000. (b) An electron micrograph of reddish membrane precipitated by the sucrose density gradient centrifugation method from S-2 fraction (17). Negatively stained with PTA, magnification 120,000.


2a
2c
2d

ATPase activity with sensitivity to oligomycin than any other fractions; namely, Pi released approximately 15 μmoles per mg of protein per minute in non-ATP regenerationg system. The electron micrograph of this fraction is shown in Fig. 2. The head pieces attached by stalk to a thread-like structure were observable as previously described (17) (Fig. 2a). The sheet-like arrangement or bulk phase of head pieces (Fig. 2b) and head pieces regularly arranged around the ring-like structure by associated components, stalks and a thread-like structure were also revealed (Fig. 2c). The associated components of the former (Fig. 2b) were disclosed by addition of phospholipid (Fig. 2d). By this method, the OSA particles are easily collected in a broad band of 1.10-sucrose density layer. This fraction, by spectrophotometery, contains very low concentration of cytochrome (*b* and *c*, less than 10% of the original inner membrane) but no cytochrome *a*. Electron transfer particles (ETP) were obtained in the density-1.14 sucrose layer and brownish membrane (17, 18) in the precipitate at bottom.

OSA particles could also be prepared from S-2 fraction (17, 18) by treating with 0.3 mg DOC per mg of protein and potassium chloride using the present discontinuous sucrose density gradient centrifugation after the dilution of S-2 fraction with chilled water, but better results could be obtained, in this instance, provided that the volume of water for dilution was 3-fold to S-2 sample. The ATPase activity in the case of the S-2 fraction is illustrated in Table 2. The OSA particle fraction in layer of density-1.10 thus obtained was shorter fragments than the ones shown in Fig. 2a and also revealed the sheet-like arrangement of head pieces (Fig. 3a). The bottom precipitate (reddish membrane) is shown in Fig. 3b. This

Table 2. THE ATPASE ACTIVITY OF THE PREPARATION OF OLIGOMYCIN-SENSITIVE ATPASE BY SUCROSE DENSITY GRADIENT CENTRIFUGATION FROM S-2 FRACTION DESCRIBED PREVIOUSLY (17). S-2 FRACTION WAS OBTAINED FROM BEEF HEART MITOCHONDRIA (23 mg OF PROTEIN/ml IN TSH SOLUTION) TREATED WITH DOC (0.3 mg/mg OF PROTEIN) AND KCl (72 g/l) FOLLOWED BY ADDITION OF 0.25 VOLUME OF WATER (17), AND WAS DILUTED AGAIN BY ADDITION OF 2-FOLD VOLUME OF DISTILLED WATER, LAYERED ON THE DISCONTINUOUS SUCROSE DENSITY GRADIENT SOLUTION AND CENTRIFUGED AT 105,000×g FOR 60 MINUTES. SPECIFIC ACTIVITY IS EXPRESSED AS μMOLES PI RELEASED PER mg OF PROTEIN PER MINUTE IN THE ASSAY SYSTEM AT pH 8.6 AT 37°.

Fractions	Protein recovery (%)	ATPase activity Specific activity	ATPase activity Total activity (%)	ATPase activity Oligomycin inhibition (%)
BHM	100	1.98	100	93
Treated BHM	100	1.84	92.9	87
S-2 fraction	55.3	1.63	45.6	73
Sample layer	21.8	0.86	9.5	61
d-1.10 layer	6.1	12.8	39.4	82
d-1.14 layer	2.3	6.30	7.2	96
Precipitate	22.6	3.19	36.5	98

is probably reconstituted membrane fragments but no head pieces could be seen attached around the vesicles.

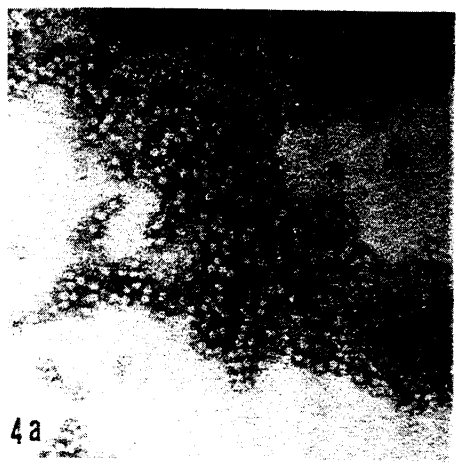
Effect of heat treatment on OSA particles:

The OSA particle fraction of the density-1.10 layer was precipitated by centrifugation at 105, 000×g for 60 minutes after dilution with chilled water to about 0.25 M in sucrose concentration. The sediment was suspended in TSH solution to the concentration of 5 mg of protein per ml. OSA particles in glass tube were kept for 2 minutes in the water bath at 65° in the presence or absence of ATP (final 3 mM) and cooled at room temperature. The ATPase activity of OSA particles after such heat treatment decreased slightly in the absence of ATP, but in the presence of ATP there was no decrease in the ATPase activity, showing a loss in oligomycin-sensitivity as illustrated in Table 3. Electron micrograph of

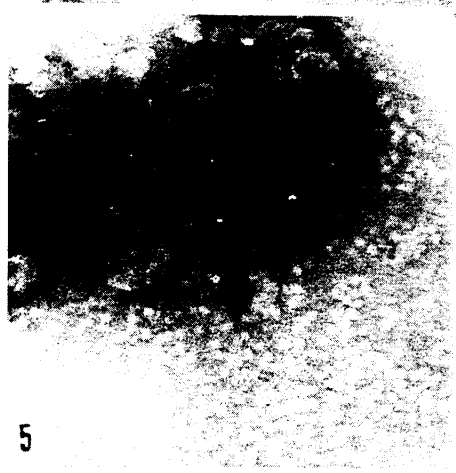
Table 3. EFFECT OF HEAT TREATMENT ON THE ACTIVITY OF OLIGOMYCIN-SENSITIVE ATPASE. ACTIVITY WAS MEASURED IN THE ASSAY SYSTEM AT pH 8.6 AT 37° AND IS EXPRESSED AS μMOLES Pi RELEASED PER mg OF PROTEIN PER MINUTE. DETAILS IN THE TEXT.

	Protein recovery (%)	ATPase activity					
		−Asolectine			+Asolectine		
		−Oligomycin	+Oligomycin	Inhibition (%)	−Oligomycin	+Oligomycin	Inhibition (%)
Control	100	14.2	2.0	86	14.0	4.8	66
Heat treated (−ATP)	100	9.5	8.9	6	9.4	8.6	9
Sediment	57.2	8.0	7.0	13	7.7	6.9	10
Supernatant	11.2	17.7	14.7	13	16.4	15.1	8
Heat treated (+ATP)	100	14.8	12.0	13	12.7	12.5	2
Sediment	41.9	9.2	7.0	24	8.5	8.2	4
Supernatant	22.5	22.1	21.7	2	22.9	21.3	7

the heat-treated particles revealed the detachment of head pieces (Fig. 4a) from the OSA structure. Such detached head pieces were purified in the supernatant by the centrifugation at 105, 000×g for 30 minutes at 15° and collected by precipitation with ammonium sulfate (50 per cent saturation), as shown in Fig. 4b. Specific activity of ATPase of purified head pieces is accelerated to approximately 2-fold of that of OSA structure, suggesting that the protein of head pieces has half that of OSA particles. On the addition of asolectine, there was observed no restoration of oligomycin-sensitivity to the ATPase activity nor reconstitution of OSA structure, but rather a decrease in ATPase activity. The identical heat treatment on electron transfer particles (ETP) which obtained by ultrasonic irradaition (9) and on mitochondria resulted in the detachment of the head pieces from membrane (Fig. 5), but the ATPase activity was inhibited and



4a



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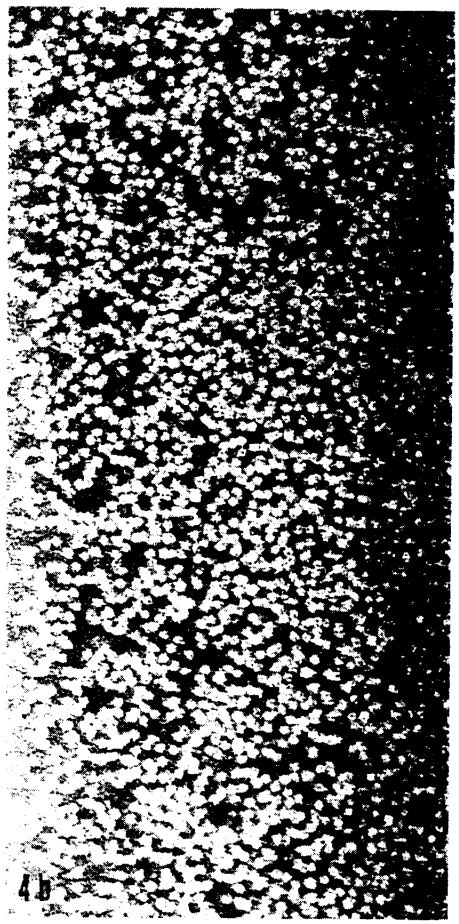
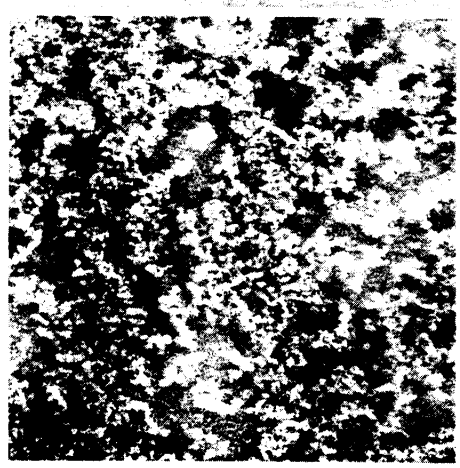
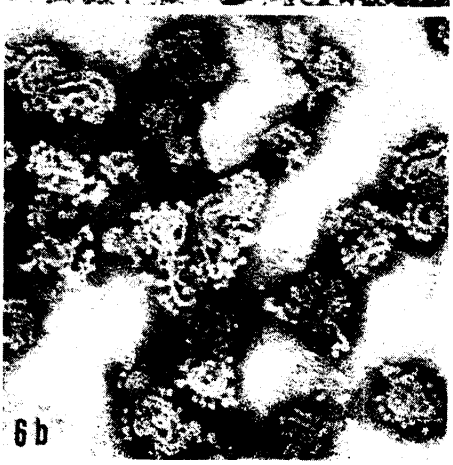


4b





6b

hardly protected by addition of ATP, and became insensitive to oligomycin (Table 4).

Table 4. EFFECT OF HEAT TREATMENT ON THE ATPASE ACTIVITIES OF MITOCHONDRIA AND ETP. ACTIVITY WAS MEASURED IN THE ASSAY SYSTEM AT pH 8.6 AT 37° AND IS EXPRESSED AS μMOLES PI RELEASED PER mg OF PROTEIN PER MINUTE.

	ATPase activity		
	−Oligomycin	+Oligomycin	Inhibition (%)
Mitochondria	1.10	0.16	85
Heat treated −ATP	0.19	0.15	21
+ATP	0.43	0.39	9
ETP	3.14	0.61	81
Heat treated −ATP	0.44	0.40	9
+ATP	1.24	0.94	24

Effect of trypsin on OSA particles:

OSA particles suspended in TSH solution were treated with trypsin at 37° for 45 minutes and the reaction was stopped by the adding trypsin inhibitor in the concentration 3-fold to the added trypsin. For the control similar procedures were carried out without trypsin treatment. As shown in Table 5, ATPase activity of OSA particles is slightly accelerated by

Table 5. EFFECT OF TRYPSIN ON THE ATPASE ACTIVITIES OF OSA PARTICLES AND ETP. ACTIVITY WAS MEASURED IN THE ASSAY SYSTEM AT pH 8.6 AT 37° AND IS EXPRESSED AS μMOLES PI RELEASED PER mg OF PROTEIN PER MINUTE.

	ATPase activity					
	OSA particles			ETP		
	−Oligomycin	+Oligomycin	Inhibition (%)	−Oligomycin	+Oligomycin	Inhibition (%)
Original	13.5	0.41	97	3.14	0.03	99
Control	13.4	0.94	93	4.64	0.16	97
Trypsin treated						
0.01mg/mg prot.	14.2	2.56	82	4.62	0.32	93
0.05mg/mg prot.	14.0	4.76	66	5.19	0.72	86
0.1mg/mg prot.	10.6	6.45	39	4.23	1.79	58
0.3mg/mg prot.	1.72	1.20	30			

Fig. 4. (a) An electron micrograph of the heat-treated OSA particles, negatively stained with PTA. Magnification 120,000. (b) An electron micrograph of the purified head pieces from the heat-treated OSA particles. Negatively stained with PTA, magnification 160,000.

Fig. 5. An electron micrograph of the heat-treated ETP, negatively stained with PTA. The head pieces detached from mitochondrial inner membrane. Magnification 120,000.

Fig. 6. Electron micrographs of R-3 fraction (in Table 6) obtained from crude OSA particles by ammonium sulfate fractionation in the presence of cholate. Negatively stained with PTA in the absence (a) of or in the presence (b) of asolectine. Magnification 120,000.

trypsin of low concentration, but it became more insensitive to oligomycin on being treated with trypsin at high concentration, accompanied by the decrease in the ATPase activity. Trypsin digestion of ETP also tended to yield similar results (Table 5). The fall in the oligomycin-sensitivity by trypsin treatment parallels with detachment of head pieces as observed in electron micrograph.

Effect of urea on OSA particles:

In the treatment of purified OSA particles with 2M urea at 0° for 60 minutes there could be observed no detachment of the head pieces from OSA structure nor any change in the ATPase activity. In the case of ETP treated with urea in similar manner the head pieces were readily detached and ATPase activity was lowered, but the detached head pieces seemed to be altered to fibrous structure (24) in electron micrograph.

Effect of ammonium sulfate on OSA particles in the presence of potassium cholate:

Fractionation of OSA particles by a large scale centrifugation with rotor 30 (Spinco) resulted in the contamination of small fragments of ETP-like structure. Therefore, an attempt was made to fractionate such a crude OSA particle fraction further with ammonium sulfate in the presence of potassium cholate as indicated in Table 6. Namely, OSA particle fraction was suspended in TSH solution to make its concentration

Table 6. FRACTIONATION WITH AMMONIUM SULFATE IN THE PRESENCE OF POTASSIUM CHOLATE ON THE OLIGOMYCIN-SENSITIVE ATPASE FRACTION. ATPASE ACTIVITY WAS MEASURED IN THE ASSAY SYSTEM AT pH 8.6 AT 37° AND IS EXPRESSED AS μMOLES PI RELEASED PER mg OF PROTEIN PER MINUTE.

	Concentration of ammonium sulfate	Frotein recovery (%)	ATPase activity −Asolectine −Oligomycin	−Asolectine +Oligomycin	−Asolectine Inhibition (%)	+Asolectine −Oligomycin	+Asolectine +Oligomycin	+Asolectine Inhibition (%)
OSA particles		100	12.8	0.29	98	10.3	0.74	93
Residue-1	5 %	28.4	3.89	0.24	94	6.65	0.28	96
Residue-2	35 %	4.4	0.37	0.01	97	0.28	0.13	54
Residue-3	50 %	28.0	1.66	1.19	28	8.55	2.60	70

10 mg protein per ml, and then solubilized with neutral potassium cholate (0.5 mg per mg of protein) followed by fractionation with cold saturated neutralized ammonium sulfate in the concentration as described in Table 6. As a result, the residue-3 fraction between thc concentrations of 35 and 50 per cent of ammonium sulfate did not change appreciably ATPase activity but in the presence of asolectine a high activity with oligomycin-sensitivity. This residue-3 fraction in electron micrograph showed a uniform but weakly contrast structure (Fig. 6a), while in the presence of

asolectine it exhibited an OSA particle structure as shown in Fig. 6b. Therefore, the residue-3 fraction contains the head pieces and associated components of OSA, and its ATPase activity is in a masked state.

DISCUSSION

The electron micrograph of the inner membrane of mitochondria reveals uniform spherical particles (25) in regular array on the surface. Many comments have been made on these inner membrane spherical particles (1—3, 5—7, 10, 25, 26). RACKER *et al.* (4—8) maintain that the spherical particles represent soluble ATPase (F_1). Judging from the aspects of the tripartite form of the mitochondria (1, 2, 11), the sphere is a head piece that is a part of the tripartite form composed of head piece, stalk and base piece. The author isolated previously (9—11) the head pieces from beef heart mitochondria by systematic extraction by the two-step ultrasonic irradiation and a modified method of PULLMAN *et al.* (4). It is obvious that the purified head pieces exhibit a marked ATPase activity which is insensitive to oligomycin (2, 9—11).

Isolated OSA particles, that reveal a structure in which head pieces are regularly linked by way of stalks to a thread-like structure derived from a superficial portion of base pieces, exhibit a remarkable ATPase activity which is sensitive to oligomycin. This is the smallest structural unit of ATPase known to show oligomycin-sensitivity up to the present(17). When the head pieces are detached from such a structure, for example by the heat treatment, ATPase activity turns to oligomycin-insensitive type. From these results, it is suggested that the factor conferring oligomycin-sensitivity may exist in the stalks and/or the thread-like portion of base pieces of the OSA structure, without requiring cytochromes or membrane structure (17).

The factors that confer oligomycin-sensitivity to mitochondrial ATPase have been isolated in RACKER's laboratory in the form of fractions F_0 (5, 6, 13—15) and CF_0 (5, 8, 12, 16). However, in electron micrographs F_0 proves to be a fragment of membrane (8, 12) and CF_0, prepared by solubilization of submitochondrial particles with 2 per cent cholate and fractionation with ammonium sulfate, appears as an amorphous mass (5, 8, 12, 27). CF_0 is the fraction depleted of phospholipid and respiratory enzymes, but the particles reconstituted with CF_0 and F_1 by addition of phospholipid for unmasking of ATPase activity are morphologically difficult to distinguish from functional submitochondrial particles with a full component of respiratory enzymes (5, 8, 27). Thus the correlation between the factor conferr-

ing oligomycin-sensitivity to head pieces and the membrane components remains obscure. BYINGTON *et al.* (28) have reported the properties of an oligomycin-sensitive ATPase from mitochondria, but not about the fine structure.

An improved method for isolating OSA particles in the present paper seems to be easier and more rapid than the method described in the previous paper (17), and ATPase activity can be restored without significant loss. It is true that there is a possibility of the reconstitution of OSA particles with solubilized mitochondrial lipids in the process of diluting the DOC-KCl-treated mitochondrial membrane suspension with distilled water, but under the condition of the present method no reconstitution of head pieces to vesicles is observed. In other words, the OSA particles have been observed denuded of the superficial portion of mitochondrial inner membrane.

The head pieces can be purified from OSA particles by heat treatment, but it is not possible to prepare purified head pieces from ETP, though head pieces can be detached from the membrane. ATPase activity of OSA fraction losses its oligomycin-sensitivity after heat treatment and it cannot be restored on the addition of phospholipid. This seems to suggest that the factor conferring oligomycin-sensitivity, if present, is heat labile, and ETP may contain ATPase inhibitor that is activated by heat treatment, but not present in OSA particles. F_0 and CF_0 contain no ATPase inhibitor (8, 12, 15), as reported by PULLMAN and MONROY (29), which is heat stable and sensitive to trypsin (12, 29). From the fact that trypsin in low concentration has not affected the ATPase activity of OSA particles, the OSA particles seem to contain no such ATPase inhibitor (29) and the full ATPase activity of head pieces is likely exhibited. However, RACKER (13) reported marked elevation in the ATPase activity after trypsin treatment of ETP.

Effect of urea on ETP has been reported by several investigators (3, 5, 7, 8, 13, 27, 30). KAGAWA states (8, 27) that F_1 is inactivated by treating it with 0.8 M urea for 30 minutes at 0° and that of the membrane ATPase activity is lost by treating with 2 M urea for 2 hours at 0°. The purified OSA particles in the present experiment can better sustain their structure and activity against 2 M urea than ETP. The reason for this is obscure, and the attempt to isolate an oligomycin-sensitive factor from OSA particles using urea resulted in failure.

The fraction (residue-3 in Table 6), obtained by solubilization of crude OSA particles with cholate and fractionation with ammonium sulfate, hardly shows any ATPase activity, but the activity is increased

on the addition of phospholipid. This tendency is similar to CF_0F_1 (8, 15) in that its completely masked ATPase activity is fully restored on the addition of lipid. Fine structure of the residue-3 fraction remains unexplained, but the interaction between the fraction and phospholipid makes the fine structure to be very similar to that of OSA particles. F_1 contains no lipid (31) and its ATPase activity is not affected by lipid. The ATPase activity of the purified head pieces after heat treatment is not affected on the addition of phospholipid. The residue-3 fraction, if head pieces are detached, would show a marked ATPase activity, suggesting the head pieces to be bound by stalks to the associated components of OSA structure. In addition, the masking of ATPase activity of the fraction is not due to mere aggregation of enzymes.

In the case of the OSA particles isolated from R-10 fraction (by the previous method) (9, 17, 18), a minute increase in the concentration of cholate and temperature during the fractionation can readily detach the head pieces from the OSA particles, making more dense aggregate (17). However, even such aggregate does exhibit oligomycin-insensitive ATPase activity under the reaction system and oligomycin-sensitivity is fully restored on addition of phospholipid in an appropriate quantity (Fig. 7). This may be due to difference in the fractionation method between the

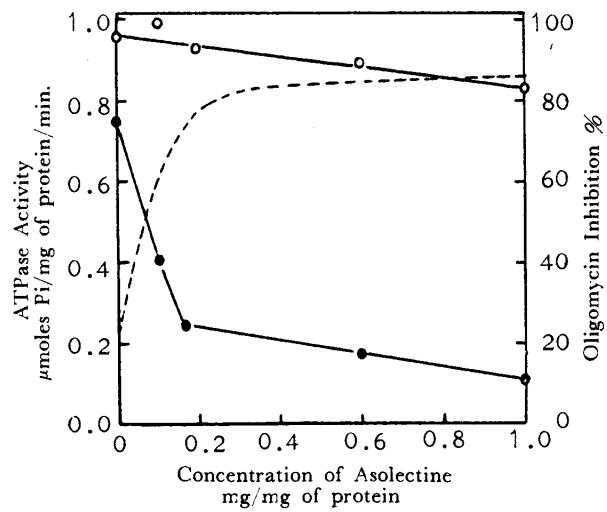


Fig. 7. Restoration of oligomycin-sensitivity with asolectine on the ATPase activity of the OSA particles, obtained by the previous method (17), converted to oligomycin-insensitive type after the treatment with cholate and ammonium sulfate. ATPase activity was measured in the assay system at pH 7.4 at 30° in the absence of (○—○) and in the presence of (●—●) oligomycin. Dotted line shows per cent of inhibition of oligomycin.

present method (0.1 mg DOC treating) and the previous method (0.3 mg DOC treating) (17). The findings suggest that at least for the OSA particles to exhibit ATPase activity and make their structural components clear, the presence of phospholipid must be taken into consideration. However, the mechanism of masking on ATPase activity and the relationship between the ATPase activity of masked type and phospholipid remain obscure. FLEISHER *et al.* (32) have reported that the membrane bound elementary particles persist even when 80 % of the lipid is removed and it is only when more than 95 % of lipid is removed that the detachment of head pieces occurs.

The mode of assembly of the head pieces and associated components in OSA particles in a thread-like structure, in a sheet, in a bulk phase, or in an inactive form seems to depend upon the associated components and the medium conditions, especially on the concentrations of phospholipid, detergents and salts.

SUMMARY

1. To have a rapid isolation of oligomycin-sensitive ATPase particles (OSA particles), 0.1 mg DOC per mg of protein and 72 g potassium chloride per l were added to mitochondria suspended in a tris-sucrose-histidine solution, which was followed by addition of 2-fold volume of chilled water, and fractionated by a discontinuous sucrose density gradient centrifugation. As a result, it was possible to reveal the OSA particle structure, composed of the head piece, stalk and thread-like structure of a superficial portion of the base pieces, stripped off from the mitochondrial inner membrane, in a layer of density-1.10. This fraction exhibited a remarkable activity of ATPase sensitive to oligomycin, approximately 15 μmoles Pi released per mg of protein per minute at pH 8.6 at 37° in a non-ATP regenerating assay system, and contained almost no cytochromes.

2. When the OSA particles thus isolated were heated in water bath at 65° for 2 minutes, the head pieces were detached with a concomitant loss of oligomycin-sensitivity and were purified from the supernatant by precipitation with ammonium sulfate.

3. Trypsin in low concentration slightly induced a rise in the ATPase activity of OSA particles but in higher concentration it inhibited the activity.

4. OSA particles were resistant to the treatment of urea, and it was difficult to detach the head pieces by this treatment.

5. The some fraction obtained by solubilization of thc crude OSA

particles with cholate and fractionation with ammonium sulfate exhibited ATPase activity in a masked form, and the ATPase activity with oligomycin-sensitivity was restored on addition of phospholipid.

6. A discussion was made on the mode of assembly of the head pieces and associated components and biochemical properties of OSA particles.

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