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Activation and isolation of mitochondrial adenosine triphosphatase by ultrasonic irradiation

Goki Yamamoto* Takuzo Oda†

Hiromichi Tsukamoto‡

*Okayama University,
†Okayama University,
‡Okayama University,

Activation and isolation of mitochondrial adenosine triphosphatase by ultrasonic irradiation*

Goki Yamamoto, Takuzo Oda, and Hiromichi Tsukamoto

Abstract

With the purpose to clarified the mode of localization and mechanisms of activation of ATPase in the mitochondrial membrane, analyses were made on the properties of mitochondrial ATPase from the structural and functional aspects. The activation of ATPase by DNP and Mg++ and the oligomycin sensitivity were investigated in a series of inner membrane fragment samples obtained by ultrasonic irradiation and those samples obtained in the processes of isolation and purification of ATPase from rat liver mitochondria and beef heart mitochondria in parallel with electron microscope observations. As a result it has been found that the membrane fragments obtained from rat liver and beef heart mitochondria by ultrasonication exhibited high respiratory activity and unmasked ATPase activity which was charac· terized by remarkable stimulation by Mg++ and inhibition by oligomycin and azide. Therefore, mitochondrial ATPase seems to be bound fairly closely to the inner mitochondrial membrane. In the membrane fragments prepared by ultrasonication of intact mitochondria, ATPase activity was stimulated by DNP, but in the supernatant fractions was not. On the other hand, the supernatant fraction obtained from BHM and inner membrane fragments by severe sonication exhibits a marked ATPase activity and the activity incresed in each step of the purification on the treatments with acid, protamine and heat. Especially in the case of membrane fragments the protamine treatment can be omitted. Electron microscope observation of the fractions in each step of the purification proved the head pieces to be ATPase. The ATPase activity of solubilized head pieces is insensitive to oligo. mycin and coincides with the soluble ATPase of PULLMAN etat. (8) in the points of its cold labile property and optimum pH, but it shown no accele. ration of ATPase activity by DNP.

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ACTIVATION AND ISOLATION OF MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE BY ULTRASONIC IRRADIATION

Goki YAMAMOTO, Takuzo ODA and Hiromichi TSUKAMOTO

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

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ATPase activity of mitochondria has been extensively studied from the partial reaction of the coupling mechanism in oxidative phosphorylation (1). Uncoupling agents, which stimulate normally latent ATPase activity, not only act to cause the discharge or breakdown of some high-energy intermediate in the coupling reaction of ATP formation (1—3), but also may affect the reaction such as proton and cation translocation across the mitochondrial membrane (4, 5). Mitochondrial ATPase is characteristically stimulated by dinitrophenol (DNP) in both intact mitochondria and phosphorylating submitochondrial fragments and by Mg^{++} in the state of physical or chemical destruction of the membrane structure. However, this does not necessarily mean the existence of different kinds of ATPase in the mitochondria. Such a difference in the reaction of ATPase seems to be rather dependent upon difference in the mode of localization of ATPase in the mitochondrial membrane upon the difference in the action mechanisms of activating agents. For the structural and functional analyses of the compartmentation or masking of ATPase activity and for the clarification of coupling or uncoupling mechanism of oxidative phosphorylation in mitochondrial membrane, it is important to define more clearly the mode of the localization and activation of ATPase in mitochondria.

The fractions isolated as the coupling factor of oxidative phosphorylation often exhibit ATPase activity (6, 7). Recently, PULLMAN and RACKER and their colleagues have described that a soluble ATPase (coupling factor F_1) isolated from beef heart mitochondria can be stimulated by DNP (8). Moreover, they suggested (9, 10) that the soluble ATPase (F_1) corresponds to the head piece of the tripartite form of the elementary particles in mitochondrial membrane from the result that the purified solubule ATPase (F_1) bound to the isolated oligomycin sensitivity conferring factor (CF_0) (14, 15).

In the present experiment, attempts were made to study the localization and activation of ATPase in mitochondrial membrane after destroying the membrane by ultrasonic irradiation, and to isolate ATPase from beef heart mitochondria and submitochondrial particles. The ATPase activity and morphological change were examined at the same time. The results have demonstrated that the head pieces of the elementary particles in isolated form exhibit marked ATPase activity which is insensitive to oligomycin. This communication deals with the results of biochemical study on the activation of mitochondrial ATPase and on the isolation and purification method of ATPase, while morphological observations of mitochondrial ATPase with the electron microscope was described elsewhere (12, 13, 16).

MATERIALS AND METHODS

Preparation of mitochondria: Rat liver mitochondria (RLM) were prepared by the modified method of HOGEBOOM (17) and beef heart mitochondria (BHM) by the method of CRANE *et al.* (18) as described previously (19). Both of these mitochondrial fractions were washed in 0.25 M sucrose and centrifugated at 7,000×g for 10 minutes. After removing the fluffy layer, the washing was repeated. Final mitochondrial residue was suspended in 0.25 M sucrose to the concentration of approximately 30 mg of protein per ml. RLM were used in intact state, and BHM were stocked at −20° and thawed at room temperature before use.

Ultrasonic irradiation: Ultrasonic irradiation was carried out using 20 Kc Ultrasonic Oscillator (Kaijo Denki Co. Ltd.; Type 4251 7φ conical step chip). Mitochondria in an experimental tube (1.5 cm in diameter) kept in ice-bath were irradiated at maximum out-put. Fig. 1 shows an example of the destruction of BHM during the period of ultrasonic irradiation.

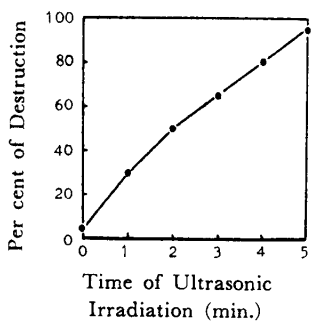

Fig. 1. Destruction of beef heart mitochondria due to ultrasonic irradiation for varying time. Ultrasonication set at 20 Kc maximum out-put was carried out to the mitochondria suspended in 5 ml of 0.25 M sucrose to final concentration of 20 mg of protein per ml keeping in ice-water bath. Per cent of destruction was expressed as yield of protein in the supernatant after centrifugation at 26,000×g for 10 minutes.

Measurement of ATPase activity: ATPase activity of RLM was measured in the medium containing 0.15M KCl, 0.02M tris-Cl, 0.003M ATP, pH 7.4, as standard system. $MgCl_2$ and DNP when added were 0.003M and 0.5mM respectively. Total volume was 2.0 ml, and the reaction was carried out at 25° for 10 minutes. ATPase activity of BHM was measured in the medium containing 0.05M tris-Cl, 0.003M $MgCl_2$, 0.006M ATP, pH 7.4. Total volume was 1.0 ml, and the reaction was allowed to proceed at 30° for 5 minutes. Oligomycin added was 2.5 μg per system. Reaction mixture containing mitochondrial preparation was preincubated for 5 minutes at reaction temperature, and then reaction was started by addition of ATP (pH 7.4) and stopped by adding chilled perchloric acid to make the final concentration 8 per cent. After deproteinizing by centrifugation, released inorganic phosphate (Pi) was estimated by the method of TAKAHASHI (20). Pi released during the priod of incubation as a result of ATPase reaction on submitochondrial particles is as shown in Fig. 2 and ATPase activity of submitochondrial particles is almost linear up to 10 minutes under the assay conditions.

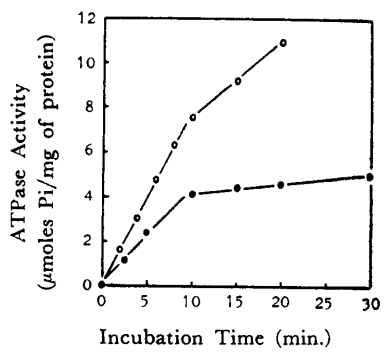

Fig. 2. Curve of mitochondrial ATPase activity during the period of incubation. ATPase activity (●—●) of rat liver submitochondrial particles was measured at 25° in the medium containing 0.15M KCl, 0.02M Tris-Cl, 0.003M $MgCl_2$, 0.003M ATP, pH 7.4, total volume 2 ml. The ATPase actvity (○—○) of beef heart submitochondrial particles was measured at 30° in the medium contaninig 0.05M Tris-Cl, 0.003M $MgCl_2$, 0.005M ATP, pH 7.4, total volume 1 ml.

Measurement of succinate- and NADH-oxidase activities: Both succinate- and NADH-oxidase activities were measured in 2 ml of reaction mixture containing 0.01 M phosphate buffer, pH 7.4, 0.1 mM EDTA at 37° by the method of rotating platinum electrode reported previously (21). Sodium succinate and NADH added as substrate were in the concentration of 0.02M and 0.002M respectively, and cytochrome *c* was 0.05 per cent in its final concentration.

Estimation of protein concentration: Concentrations of protein of mitochondria and other fractions were determined by the method of GORNALL *et al.* (22) or LOWRY *et al.* (23) and calculated from bovine serum albumin (Sigma Chemical Co., Fraction V) as a standard,

RESULTS

Ultrasonic irradiation of RLM: RLM (20 mg of protein per ml in 0.25 M sucrose) were mildly sonicated for 1 minute per 5 ml and followed by the differential centrifugation as given in Table 1. In this instance, the supernatant (140, 000 ×g, 30 min.) contained 15 per cent of protein, and most of the mitochondrial layer (residue fraction, 26, 000 ×g, 10 min.) proved to be unbroken mitochondria. ATPase activity of each fraction is as illustrated in Table 1. Mg^{++}-stimulated ATPase activity was accelerated by

Table 1. ATPase activity of rat liver mitochondria and submitochondrial fractions separated by differential centrifugation as indicated in the table after ultrasonic irradiation. The conditions of irradiation are as described in the text.

Fractions	Mildly sonicated				Severely sonicated				
	Protein recovery %	ATPase activity None S. A.	Mg^{++} S. A.	DNP S. A.	Protein recovery %	ATPase activity Mg^{++} S. A.	T. A.	Mg^{++}+DNP S. A.	T. A.
RLM		3	6	73		0		118	
RLM (sonicated)	100	8	43	30	100	245	100	289	100
Residue-1 (26,000×g, 10min.)	77	12	33	23	15	132	8	191	11
Residue-2 (140,000×g, 30min.)	6	14	201	20	9	546	20	612	26
Supernatant-2	15	0	20	0	72	182	54	167	45

S. A.: specific activity expressed as mμmoles Pi released per mg of protein per minute
T. A.: total activity expressed as per cent of yield

ultrasonication, but the DNP-stimulated one was decreased. Submitochondrial fraction exhibited a marked specific activity of Mg^{++}-stimulated ATPase and only slightly of DNP-stimulated one. In the case of fractionation after 5 minutes ultrasonication of RLM most of the mitochondria were destroyed making the concentration of protein in the supernatant fraction markedly high as shown in Table 1. In this instance, specific activity of Mg^{++}-stimulated ATPase in submitochondrial fraction was much higher than in any other fractions and also it showed a slight increase in DNP-stimulated ATPase activity. In contrast to this, there could be observed no DNP-stimulated ATPase activity in the supernatant fraction.

After the freezing-thawing treatment of RLM at −20°, RLM showed a rise in Mg^{++}-stimulated ATPase activity and the inactivation of DNP-stimulated ATPase activity, but in the case with ultrasonication of RLM there could be observed marked activation of Mg^{++}-stimulated ATPase

activity (Table 2). The activation in intact RLM was slightly higher than that treated by the freezing-thawing method, and this tendency could also be seen in the submitochondrial fraction. However, in the presence of oligomycin, an inhibitor of phosphorylation in mitochondrial oxidative phosphorylation, over 70 to 80 per cent of the inhibition of ATPase activity could be observed both in mitochondria and submitochondrial fragments.

Table 2. Effect of oligomycin on ATPase activities in the presence and absence of DNP of rat liver mitochondria, ultrasonic irradiated one, and submitochondrial particles separated after ultrasonic irradiation.

Fractions	ATPase activity (μmoles Pi released/mg of protein/min.)					
	Mg^{++}	Mg^{++} +oligomycin	% of inhibition	Mg^{++}+DNP	Mg^{++}+DNP +oligomycin	% of inhibition
RLM	0.018	0.002	70	0.057	0.012	80
RLM (sonicated)	0.142	0.034	76	0.143	0.034	76
Submitochondrial particles	0.229	0.042	82	0.220	0.051	75
RLM (freezing-thawing)	0.036	0.008	78	0.038	0.008	79
RLM (sonicated)	0.121	0.015	80	0.126	0.017	86
Submitochondrial particles	0.168	0.026	84	0.170	0.031	82

The yield of submitochondrial fragments from RLM after ultrasonication was significantly less than that from BHM as to be described later. Since mitochondrial protein was collected mostly in the supernatant, further differential centrifugation was attempted (Table 3). Judging from the protein yield, with exception of 10 per cent unbroken mitochondria, 25 per cent of them was harvested as membrane fragments as shown in Table 3, and the specific ATPase activity of these submitochondrial fractions was higher than that of soluble supernatant fraction. There was also a slight rise in the DNP-stimulated ATPase activity. On the other hand, since the total recovery of ATPase activity was less after the fractionation, an inactivation of ATPase activity coud be considered, but the majority of the activity was seen in the membrane fragments. On the basis of respiratory enzyme activity (Table 3), the membrane fragments seem to be so-called electron transfer particles (ETP). In this instance, the succinate oxidase was recovered more in larger membrane fragments and that of NADH oxidase more in smaller membrane fragments, showing slight difference. These results, however, indicate that, with exception of the supernatant, the distrubution of ATPase and respiratory enzymes is in a parallel relationship and both of them are closely bound to each other, suggesting a possibility that ATPase in the supernatant is free of respiratory

Table 3. Distribution and activities of ATPase, succinate- and NADH-oxidase in rat liver mitochondria and submitochondrial fractions separated by the differential centrifugation as indicated in the table after severe ultrasonic irradiation. The condition of the irradiation and the assay systems of enzyme activities were detailed in the text.

Fractions	Protein recovery %	ATPase activity						Succinate-oxidase			NADH-oxidase		
		None	Mg^{++}		DNP	Mg^{++} + DNP		None	Cyt. c		None	Cyt. c	
		S. A. *	S. A. *	T. A.***	S. A. *	S. A. *	T. A.***	S. A.**	S. A.**	T. A. ***	S. A.**	S. A.**	T. A. ***
RLM		0.007	0.017		0.050	0.042		0.02	0.13		0.04	0.12	
RLM (sonicated)	100	0.023	0.118	100	0.026	0.129	100	0.06	0.14	100	0.05	0.22	100
Residue-1 (26,000×g, 10min.)	8	0.023	0.118	8	0.025	0.118	7	0.04	0.19	11	0.08	0.12	4
Residue-2 (100,000×g, 30min.)	12	0.029	0.117	17	0.039	0.179	16	0.25	0.45	38	0.44	0.58	31
Residue-3 (150,000×g, 45min.)	9	0.027	0.158	11	0.039	0.182	12	0.06	0.23	14	0.29	0.62	24
Residue-4 (198,000×g, 60min.)	5	0.026	0.193	8	0.049	0.255	9	0.05	0.16	5	0.18	0.85	18
Fluffy-4	12	0.014	0.038	4	0.015	0.062	6	0.02	0.03	2	0.07	0.29	16
Supernatant-4	33	0.011	0.035	10	0.008	0.026	7	0.00	0.00	1	0.00	0.02	3

S. A.*: specific activity expressed as μmoles Pi released per mg of protein per minute

S. A.**: specific activity expressed as μatoms oxygen consumed per mg of protein per minute

T. A.**: total activity expressed as per cent of yield

enzymes and is in a soluble state. Oxidase activities were markedly increased in the presence of cytochrome *c*. The recovery of the oxidase activities was much sooner than that of ATPase activity, so that the less recovery of ATPase activity seems to be due to cold-labile property similar to the soluble ATPase reported by PULLMAN *et al.* (8, 24). These findings indicate that Mg^{++}-stimulated ATPase is either bound to or is located close to ETP and most of it is distributed in the inner mitochondrial membrane system. As shown in Fig. 3, the optimal pH for the ATPase activity of ETP fractionated after ultrasonication is around 9.0. Table 4 illustrates the

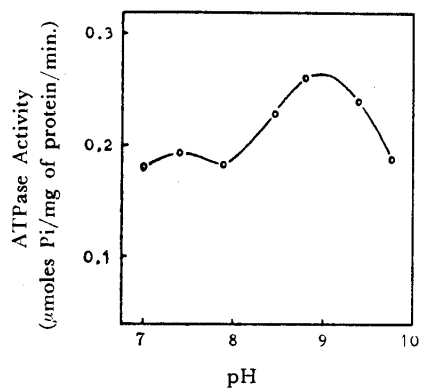

Fig. 3. Effect of pH on ATPase activity of rat liver submitochondrial particles. The reaction mixture contained 0.15M KCl, 0.003M $MgCl_2$, 0.003M ATP and 0.02M Tris-Cl buffer, and was measured at varying pH at 25°.

Table 4. Effects of various inhibitors on ATPase activity of rat liver mitochondria, the ultrasonicated one, and submitochondrial particles separated after ultrasonic irradiation. Concentrations of added inhibitors were described in the table as final in the 2 ml assay system.

Inhibitors	Mitochondria	Ultrasonicated mitochondria		Submitochondrial particles	
	S. A.	S. A.	% I	S. A.	% I
None	0	0.193		0.218	
Oligomycin (5μg)	0	0.015	92	0.002	99
Azide (1 mM)	0	0.042	88	0.065	70
ACMB (0.06mM)	0	0.193	0	0.190	13
DNP (0.05 mM)	0.082	0.155	19	0.187	14
Amytal (1 mM)	0.006	0.133	30	0.165	24
Antimycin (10μg)	0.045	0.105	45	0.174	20
KCN (1 mM)	0.005	0.151	21	0.187	14

S. A.: specific activity expressed as μmoles Pi released per mg of protein per minute
% I: percentage of inhibition

effect of various inhibitors on RLM, sonicated one, and ETP. As is obvious from the table, ATPase activity is inhibited remarkably by oligomycin and azide and slightly by other respiratory inhibitors.

Electron microscope observation of the thin sectioned specimens fixed with potassium permanganate and the specimens negatively stained with phosphotungstate revealed that the ETP obtained by ultrasonication were consisted of membrane structures, each measuring approximately 0.1 μ in diameter. There can be observed tripartite form of the elementary particles in the negatively stained specimens.

Ultrasonic irradiation of BHM: RLM are not suitable for electron microscope observation by negatively staining method, because they have less cristae than BHM. In contrast, BHM have more abundant and firm cristae and have been used for the study of submitochondrial structure and function by many investigators (9—13, 25—27). Therefore, the morphological changes of BHM were observed simultaneously with changes in the activity of ATPase (12, 16).

BHM were severely sonicated for 5 minutes under the identical conditions as in the case of RLM, then fractionated by centrifugation as shown in Table 5, which summarizes the distribution of protein, ATPase activity,

Table 5. Distribution and activities of ATPase, succinate- and NADH-oxidase in beef heart mitochondria and submitochondrial fractions separated by differential centrifugation as indicated in the table after severe ultrasonic irradiation. Details were given in the text.

Fractions	Protein recovery	ATPase activity		Succinate-oxidase activity			NADH-oxidase activiy		
	%	S. A. *	T. A. ***	S. A. **		T. A. ***	S. A. **		T. A.***
				−Cyt.c	+Cyt.c	+Cyt.c	−Cyt.c	+Cyt.c	+Cyt.c
BHM		0.13		0.06	0.06		0.70	1.20	
BHM (sonicated)	100	0.17	100	0.04	0.04	100	1.38	2.76	100
Residue-1 (26,000×g, 10min.t)	12.9	0.13	9.9	0.09	0.09	29	1.05	2.28	10.7
Residue-2 (100,000×g, 30min.)	50	0.13	38.3	0.10	0.10	125	1.47	2.00	36.3
Residue-3 (150,000×g, 45min.)	7.8	0.41	18.7	0.04	0.14	22	1.73	1.76	5.0
Residue-4 (198,000×g, 60min.)	1.7	0.84	8.5	0	0	0	0.06	0.14	0.9
Supernatant-4	9.5	0.49	27.1	0	0	0	0.02	0.02	0.7

S. A. *: specific activity as expressed μmoles Pi released per mg of protein per minute
S. A. **: specific activity as expressed μatoms oxygen consumed per mg of protein per minute
T. A. ***: total activity expressed as per cent of yield

succinate- and NADH-oxidase activities. Protein was abundantly recovered in membrane fragments and less in supernatant as compared with that in the case of RLM. Besides, the characteristically high specific activity of ATPase appeared in the residue of 198, 000 ×g from the supernatant of 150, 000×g. This fraction exhibited no activity of succinate oxidase and NADH oxidase was negligible. ATPase activity was recovered by 65 per cent in membrane fragments as residue so that ATPase coexisted in ETP with respiratory enzymes similarly to the case of RLM, but the ATPase activity was also recovered by 27 per cent in the supernatant which exhibited a high specific activity. Differing from RLM, BHM exhibit a lower activity of succinate oxidase and a higher NADH oxidase, and the yield of ATPase activity was satisfactory. The observations of negatively stained specimens of the fractions revealed that the residue-2 proved to be membrane fragments and the residue-3 also smaller fragments than the residue-2. Both of these fragments were idetified as the inner membrane. However, the residue-4 showed mostly free or aggregates of particles and the supernatant also revealed particles. Some of the head pieces of the elementary particles of the membrane fragments were being detached from the residue-2 rather that from the residue-3. Judging from the fact that the particles in the residue-4 have marked specific ATPase activity and the membrane fragments with the head pieces of the residue-2 and -3 contain the majority of total ATPase activity, it seems that the head pieces are ATPase and the particles in the residue-4 are mostly the head pieces detached from the inner membrane by ultrasonic irradiation.

Concentration of cytochrome components of ETP from BHM obtained by ultrasonication was 0.91 for cyt. *b*, 0.68 for cyt. $c+c_1$, and 0.93 for cyt. *a* as compared with 0.43 for cyt. *b*, 0.40 for cyt. $c+c_1$, and 0.54 for cyt. *a* in BHM, all units being represented in mμ moles per mg of protein, estimated by the differential spectrophotometry (19).

In order to clarify whether the head pieces are detached from membrane by ultrasonication and to determine the correlation between ATPase and the head pieces, the ETP were subjected to severe ultrasonic irradiation and fractionated by differential centrifugation. BHM suspension was prepared with the medium containing 0.25 M sucrose and 0.001 M tris-Cl, pH 7.4, to make its protein concentration 20 mg per ml and mildly sonicated for 50 seconds per 5 ml, and followed by the cetrifugal fractionation. The fractionation in Exp. 1 was carried out at 4° and in Exp. 2 at 15°. As shown in Table 6, the protein recovery of ETP thus obtained (abbreviated as ms-ETP) is less and that of unbroken mitochondrial fraction is more than those shown in Table 5, but the ms-ETP show a higher activity

of ATPase which amount to 50 per cent of the total activity. The total activity in the supernatant is less but its activity is hardly inhibited by oligomycin. Since the soluble ATPase of BHM is labile at low temperature (23), the difference in the ATPase activities between Exp. 1 and Exp. 2 may be due to the inactivation, but the distribution of ATPase shows a similar tendency in both fractions. In order to eliminate the contamination of soluble protein in the ms-ETP, the ETP prepared by mild sonication were washed twice by the sucrose solution and these were suspended in the medium to the concentration of 10 mg of protein per ml adjusting pH to 7.4 with tris buffer, and again ultrasonicated for 5 minutes per 5 ml. The ATPase activities of the sediment (ss-ETP) and its supernatant (105,000 ×g, 60 min.) are as shown in Table 6. The supernatant protein was over

Table 6. Effect of temperature on the ATPase activity of the electron transfer particles and the supernatant obtained in the process of isolation procedure after ultrasonic irradiation and differential centrifugation. Experiment-1 shown in the procedure as indicated in the table was carried out at 4° and Experiment-2 at 15°. Condition of ultrasonic irradiation and other details were given in the text.

Fractions	Protein recovery	ATPase activity				
		Eeperiment-1		Experiment-2		% inhibition
	%	S. A.	T. A.	S. A.	T. A.	of oligomycin
BHM		0.13		0.13		86
BHM (mildly sonicated)	100	0.24	100	0.70	100	93
Rsidue-1 (26,000×g, 10min.)	47	0.27	54	0.59	40	85
Residue-2(105,000×g, 60min.) (ms-ETP)	35	0.37	54	0.89	44	90
Supernatant-2	15	0.13	8	0.26	6	57
ms-ETP (washed)		0.37		0.97		90
ms-ETP (severely sonicated)	100	0.19	100	1.13	100	60
Residue (105,000×g, 60min.) (ss-ETP)	80	0.19	80	1.88	133	83
Supernatant	12	1.12	7	3.15	33	10

S. A.: specific activity expressed as μmoles Pi released per mg of protein per minute
T. A.: total activity expressed as per cent of yield

10 per cent and its ATPase activity was high but the activity of the supernatant obtained after the re-sonication was labile at low temperature. The ATPase activity of ms-ETP is inhibited by oligomycin by about 90 per cent whereas that of after sonication is down by 60 per cent, but the activity in the supernatant is inhibited by oligomycin only 10 per cent. By the negatively stained electron micrograph this supernatant fraction contained the detached head pieces with a slight contamination of small membrane

fragments (16).

Isolation of ATPase: PULLMAN *et al.* purified soluble ATPase (F_1) from BHM treated by Nossal shaker (8). Following their method, the purification of ATPase was attempted from the supernatant obtained after severe ultrasonication of BHM and of ms-ETP. Namely, each supernatant was adjusted to pH 5.4 at 0° by adding 1N acetic acid, then the precipitate was removed by cetrifugation. Each supernatant was immediately readjusted to pH 6.7 by adding 2M trisaminomethane and the protamine sulfate solution (8) was added to make its final concentration to 0.2 ml per 10 mg of protein of the supernatant, while stirring with magnetic shaker for 15 minutes at 4°. After centrifugation supernatant was discarded, and the residue was dissolved in the solution containing 0.4M ammonium sulfate, 0.25M sucrose, 0.01M tris-Cl, 0.001M EDTA, pH 7.4, at room temperature. Insoluble substance was removed by centrifugation and equal volume of ammonium sulfate saturated at 4° was added to the solution and kept for 15 minutes at 4°. The precipitate obtained by centrifugation was dissolved in the sucrose solution without ammonium sulfate to the protein concentration of 5 mg per ml, then treated at 65° for 2 minutes in the presence of 1 mM ATP (pH 7.4), and chilled at room temperature. After centrifugation, denatured protein was removed.

Recovery of protein during the process of the purification and ATPase activities of fractions at each step are illustrated in Table 7. Specific activity of ATPase of the final fraction was significantly high, whereas the final fraction from ms-ETP was observed as uniform particles, 85 to 97 Å in diameter, by negatively stained electron micrograph (16), but the final yields of protein and activity were not satisfactory. On the other hand, the specific activity of ATPase of the final fraction prepared from severely sonicated BHM was lower than that from ETP, and the activity increased proportionately to the process of the purification procedure. In contrast, in the case of the purification from ETP, uniform particles were already observed in a fraction after the acid treatment. Therefore, it did not require the next treatment with protamine. However, a slight inhibition by oligomycin on ATPase activity was observed in the fractions except the final fraction. This fact suggests that the oligomycin-sensitivity conferring factor may exist in the samples and is destroyed by the heat treatment. It was demonstrated that the particles of final fraction from ETP are head pieces of the elementary particles from morphological study of electron microscopy in each step of the purification procedures (12, 16). ATPase activity of head pieces was increased to 2-fold in the assay system of ATP-regenerating, but found no distinct stimulation by DNP (16). The effect

Table 7. Changes in ATPase activity during the process of purification of ATPase from ultrasonic irradiated beef heart mitochondria and electron transfer particles. Purification procedure was detailed in the text.

Step from BHM	Protein recovery	ATPase activity			Step from ETP	Protein recovery	ATPase activity		
	%	S. A.	T. A.	% I		%	S. A.	T. A.	% I
BHM		0.12		85	ms-ETP		1.25		97
BHM (severely sonicated)	100	0.21	100	80	ms-ETP (severely sonicated)	100	0.86	100	33
Residue-1 (26,000×g, 10min.)	3.7	0.28	5	81	—	—	—	—	—
Residue-2 (105,000×g, 60min.)	44.8	0.41	87	93	ss-ETP	71	0.68	56	94
Supernatant-2	34	0.24	39	48	ss-Supernatant	15	2.50	44	8
Supernatant after acid treatment	13.1	0.19	14	35		2.6	11.00	33	22
Precipitate after protamine treatment	0.8	1.08	5	6		—	—	—	—
Precipitate with ammonium sulfate	0.6	2.16	7	7		0.5	11.07	7	12
Supernatant after heat treatment	0.2	7.00	8	0		0.3	16.90	6	0

S. A.: specific activity expressed as μmoles Pi released per mg of protein per minute
T. A.: total activity expressed as per cent of yield
% I : percentage of inhibition with oligomycin

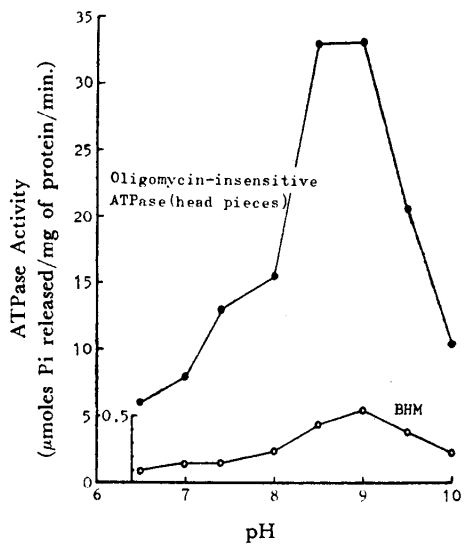

Fig. 4. Effect of pH on the ATPase activities of head pieces and mitochondria from beef heart. The incubation medium contained 0.05 M Tris-Cl buffer, 0.005 M ATP and 0.003 M $MgCl_2$ at varying pH at 30°.

of various pH's on the ATPase activity of head pieces was similar in BHM, RLM, and their ETP's, and the optimum range of activity was attained near pH 9 as shown in Fig. 4.

DISCUSSION

Among a number of applications of sonication on mitochondria, ANDREOLI *et al.* have reported the purification of coupling factor for oxidative phosphorylation by sonication (7). PULLMAN *et al.* (8) have purified soluble ATPase from BHM using Nossal shaker but unsatisfactorily using sonic oscillation. On the other hand, there are quite many observation on the properties of the membrane fragments separated by sonication (6, 8, 28, 29). The present results indicate that fairly uniform membrane fragments can be separated by mean of ultrasonication of RLM and BHM. These membrane fragments possess clearly ATPase and respiratory enzymes, and the capacity of phosphorylating respiration under certain conditions of ultrasonication (30). Intact mitochondria exhibited ATPase activity by ultrasonication and the ATPase was mainly recovered in their ETP fraction. It is clarified that ATPase is distributed in the inner membrane, and this result is supported by the histochemical ATPase analysis (31, 32). ATPase activity in the membrane fragments was inhibited by oligomycin, and the activity in the supernatant after ultrasonication was hardly inhibited by oligomycin. Soluble ATPase purified by PULLMAN *et al.* is insensitive to oligomycin (8), suggesting that the ATPase may be solubilized by ultrasonic irradiation.

RACKER *et al.* (9, 10) proposed that the soluble ATPase would be elementary particles as reported by GREEN *et al.* (11) from the structural similar of the inner membrane with the elementary particles to the membrane fragments formed by reconstruction of the soluble ATPase (8) with oligomycin-sensitivity conferring factor (14, 15). Head pieces of the elementary particles in the tripartite form (12, 13) is skilfully observed in negatively staining method by electron microscopy, but the direct evidence that the soluble ATPase is identical with head pieces was not obtained from the results by RACKER *et al.* (9, 10). Membrane fragments obtained by ultrasonication show ATPase activity and head pieces in the structure. In order to obtain a direct evidence of the correlation between head pieces and ATPase, ATPase was purified by the modified method of PULLMAN *et al.* (8) from the supernatant of ETP after repeated ultrasonication. As a result, the electron micrographs of negatively stained specimens revealed that the final ATPase fraction is undoubtedly head pieces (12, 16). Since the purified

head pieces exhibit high ATPase activity insensitive to oligomycin and cold-labile property, this head piece fraction seems to be identical with the soluble ATPase (F_1) reported by PULLMAN *et al.* (8). The ATPase (F_1) is said to be accelerated by DNP, but the activity of the head pieces prepared by ultrasonication is not so clearly enhanced (16). The acceleration of ATPase by DNP is observed more markedly in the intact mitochondria but such an acceleration can hardly be observed in the mitochondria undergoing treatments like freezing-thawing. From the facts that DNP binds with mitochondrial protein (33) and induces the breakdown of the high-energy intermediate in coupling mechanism of oxidative phosphorylation (1, 3), it is not certain whether there is a correlation between the activation by DNP and the membrane structure and/or head pieces. The finding that membrane fragments prepared from intact mitochondria by ultrasonication exhibit some ATPase activity stimulated by DNP probably means the existence of membrane fragments capable of phosphorylating respiration.

The supernatant after centrifugation of ultrasonicated ETP still contains some membrane fragments (16). These fragments can be removed by precipitation with the acid treatment, and the subsequent protamine treatment is not reqired in the procedure of ATPase purification from ms-ETP, but not so in the purification from sonicated BHM. ATPase from the supernatant of severely ultrasonicated BHM is less active than that from severely ultrasonicated ms-ETP. The cold-labile property of the solubilized ATPase may be taken into consideration during the purification procedure, but there is also a possibility that ATPase from BHM may still contain some impure proteins. In the purification either from BHM or from ETP according to the method of PULLMAN *et al.* (8), the final yield of ATPase proves to be unsatisfactory. In the purification from ETP, however, ATPase already exhibits high activity after the treatment of acid precipitation. In fact, the supernatant after the acid treatment is consisted of uniform particles in the observation of electron micrograph (16). We isolated (25) the oligomycin-sensitive ATPase from BHM, which was composed of the head piece, stalk and a part of base piece, so that stalk and a part of base piece were related closely to oligomycin-sensitivity. But the isolation of the stalk or a superficial portion of base piece was not successful, and the individual free fine structures could not be observed in soluble state. It the supernatant after acid treatment contained oligomycin-sensitive part not distinguishable by electron microscopy, such a substance was removed with denaturated head pieces after the heat treatment (34).

Besides, the ATPase activity of mitochondria requires lipid under some conditions (14, 34), but the ATPase activity of head pieces purified

by ultrasonic irradiation is not affected by the added phospholipid.

SUMMARY

With the purpose to clarified the mode of localization and mechanisms of activation of ATPase in the mitochondrial membrane, analyses were made on the properties of mitochondrial ATPase from the structural and functional aspects. The activation of ATPase by DNP and Mg^{++} and the oligomycin sensitivity were investigated in a series of inner membrane fragment samples obtained by ultrasonic irradiation and those samples obtained in the processes of isolation and purification of ATPase from rat liver mitochondria and beef heart mitochondria in parallel with electron microscope observations.

As a result it has been found that the membrane fragments obtained from rat liver and beef heart mitochondria by ultrasonication exhibited high respiratory activity and unmasked ATPase activity which was characterized by remarkable stimulation by Mg^{++} and inhibition by oligomycin and azide. Therefore, mitochondrial ATPase seems to be bound fairly closely to the inner mitochondrial membrane.

In the membrane fragments prepared by ultrasonication of intact mitochondria, ATPase activity was stimulated by DNP, but in the supernatant fractions was not.

On the other hand, the supernatant fraction obtained from BHM and inner membrane fragments by severe sonication exhibits a marked ATPase activity and the activity incresed in each step of the purification on the treatments with acid, protamine and heat. Especially in the case of membrane fragments the protamine treatment can be omitted. Electron microscope observation of the fractions in each step of the purification proved the head pieces to be ATPase.

The ATPase activity of solubilized head pieces is insensitive to oligomycin and coincides with the soluble ATPase of PULLMAN *et al.* (8) in the points of its cold labile property and optimum pH, but it shown no acceleration of ATPase activity by DNP.

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