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ISOLATION, PURIFICATION AND CHARACTERIZATION OF ADENOSINE TRIPHOSPHATASE FROM MITOCHONDRIA OF RICE SEEDLINGS

AMITABHA BASU and SUBHENDU MUKHERJI

Plant Physiology Laboratory, Department of Botany, University of Calcutta, Calcutta 700019, India

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Key Word Index—*Oryza sativa*; Gramineae; rice; F_1 ATPase; mitochondria.

Abstract—A new and improved procedure is described for the isolation and purification of adenosine triphosphatase (F_1 ATPase) from rice seedling mitochondria. The enzyme has a multi-subunit structure, as revealed by electron microscopy and SDS-mercaptoethanol gel electrophoresis. Oligomycin sensitivity as well as cold lability of this enzyme clearly prove that the mitochondria of monocotyledons have an ATPase similar to the F_1 ATPases from other sources.

INTRODUCTION

The universal presence of Racker factor 1 protein, which catalyses both ATP synthesis and its hydrolysis in the inner mitochondrial membrane of eukaryotic systems, is now well established. Higher plants have been shown to contain a similar enzyme system of mitochondrial origin. In earlier times, there had been a dispute regarding the nature of this enzyme system in monocotyledonous plants, but it has now been proved that monocots also contain an F_1 ATPase system similar to that found in other eukaryotes [1, 2]. In a previous paper, we described a method of purification of this enzyme from rice seedling mitochondria [3]. We have recently been able to improve the purification procedure described earlier and to obtain a purified enzyme retaining high activity. We now report a new and rapid method of purification of an enzyme catalysing ATP hydrolysis from rice seedling mitochondria. Some of the physicochemical properties of this enzyme are also described.

RESULTS AND DISCUSSION

The sonicated and washed mitochondrial vesicles were negatively stained with 2% phosphotungstic acid at pH 7.1 and mounted as a whole on a 400 mesh carbon-coated grid without sectioning. These were examined under an electron microscope to see whether or not these inverted vesicles were actually covered with the characteristic 90 Å projections. It was found that the inverted vesicles possessed the usual 90 Å projected particles looking like dots similar to those from other sources (Fig. 1).

The purification profile and specific activity at the different steps are presented in Table 1. The enzyme activity is expressed in units/mg protein where 1 unit of enzyme activity corresponds to μmol of Pi liberated in 1 min. Some interesting variations of specific activity were noted at different steps of purification. For instance, the specific activity of step 2 (washed membrane) was about half of the first one (whole sonicate). It was further noticed that the soluble supernatant fraction of the whole sonicate which was devoid of membrane contained a high ATPase activity which was neither cold-labile nor oligomycin-sensitive. The specific activity of this fraction (soluble) was *ca* 10–11 units/mg protein. This result was consistent with that of the maize system [4]. Thus about half of the total activity of the whole sonicate might be attributed to this unknown soluble component. The decrease in specific activity after Triton X-100 solubilization was also observed in rat liver mitochondrial enzyme [5], but no satisfactory explanation was given by these authors for this unusual behaviour. In our system, even after Triton X-100 solubilization of the ATPase from the washed membrane, we detected another ATPase activity in the remaining insoluble membrane portion (unpublished observation). This enzyme could be solubilized with prolonged sonication and, unlike the heat-activated Triton fraction, it was cold-stable. Thus the Triton X-100 solubilized fraction probably exhibits only a part of the total enzyme activity of the parent membrane itself.

The final purification of the enzyme on 4–12% discontinuous glycerol gradient showed that the highest enzyme activity was at the centre of the gradient. The sedimentation rate of the enzyme was compared with that of catalase (MW 58 000) as standard. The distribution of catalase in the gradient under the same conditions was detected from the third to the eighth fractions where the fourth one was the peak. Thus the central orientation of the enzyme clearly indicates that this protein has a high MW comparable with the F_1 ATPase from other sources like yeast [6].

The special problem of purification of this enzyme from monocotyledonous material may be attributed to its high lability under changing environmental conditions during processing and handling. This fact was tested by exposing the enzyme to different stringent conditions under polyacrylamide gel electrophoresis (PAGE). It was found that not only the recovered proteins but also the submitochondrial particles themselves were very delicate and prone to denaturation into smaller irregular fragments, unlike the animal counterparts. Even precipitation of this protein by cold acetone from the Tris–phosphate buffer (pH 7.4) at $-10 \pm 2°$ damaged the polypeptides so severely that it gave a high background on electrophoresis in comparison with a non-precipitated one. Typical Weber and Osborn

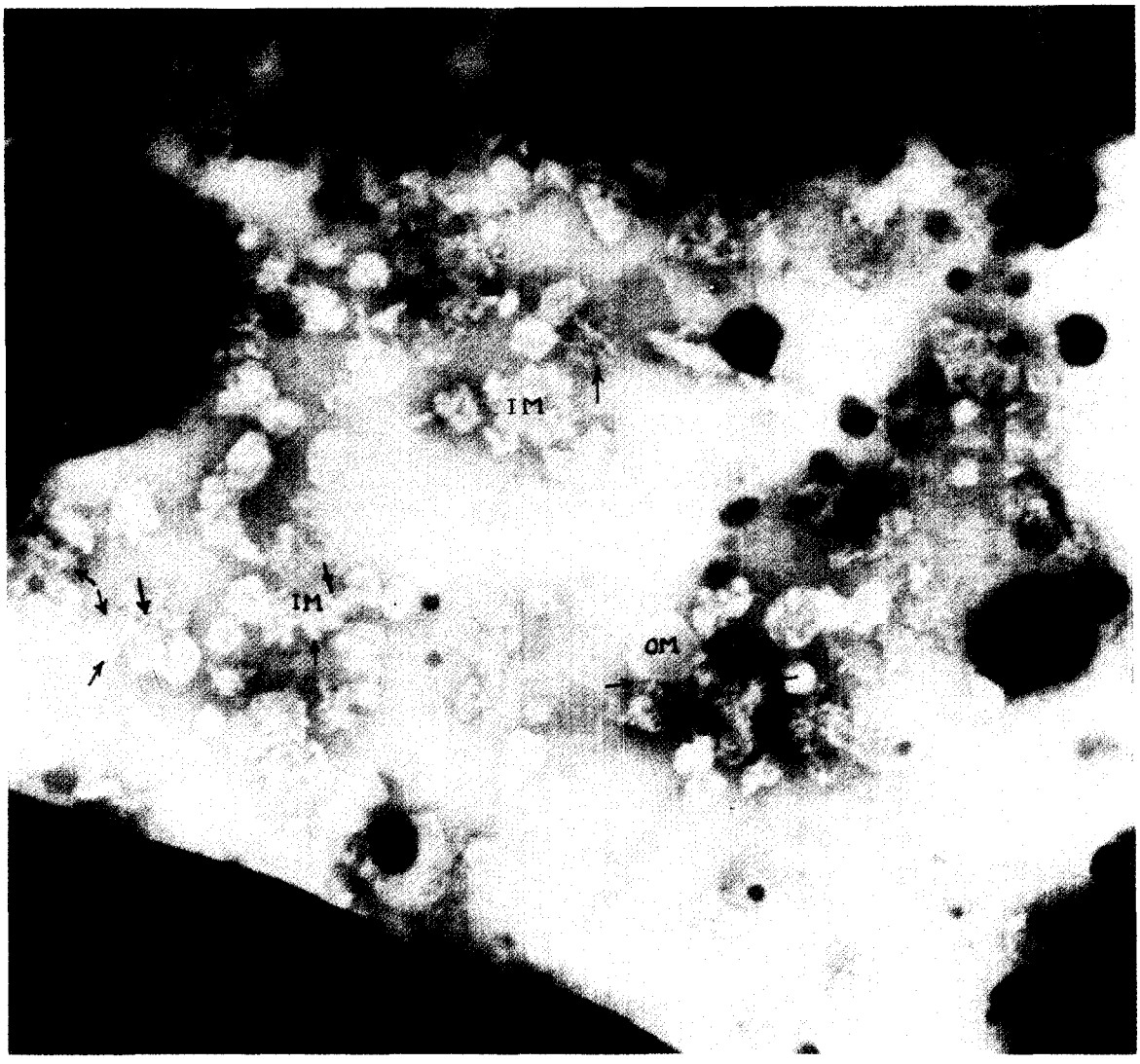

Fig. 1. Transmission electron micrograph of rice seedling mitochondrial inner membrane vesicle preparation, negatively stained with 2% phosphotungstic acid at pH 7.1. The inner membrane vesicle preparation was done as described in the Experimental, mounted on a carbon-coated grid as a whole, and electron microscopy was carried out as 80 000 × at 80 kV. The 90 Å particles of the inner membrane (IM) vesicles appeared as white dots (shown by arrows) covering the surface. OM, Outer membrane.

Table 1. Specific activities and yields of ATPases from rice seedling mitochondria using 200 mM Tris–HCl (buffer A) and 200 mM KH_2PO_4–Tris (buffer B)

Fraction	Tris–HCl (buffer A) Sp. act. (units/mg protein)	Yield (%)	KH_2PO_4–Tris (buffer B) Sp. act. (units/mg protein)	Yield (%)
Whole sonicate	0.17	100	25	100
Washed membrane	0.10	75	12	81
Soluble supernatant	0.15	34	11.5	45
Triton X-100 supernatant	0.21	25	8.2	41
Heat-purified supernatant	0.86	12	30	15
Tenth fraction from gradient	1.79	7	52	7.5

The specific activities of the different fractions were determined under standard assay conditions of the purified ATPase as described in the text.

[7] method of SDS-PAGE of the purified protein revealed a series of four to five detectable bands, some of which may arise from mere contamination. The apparent MWs of the three major polypeptides from the top of the gel were 55 000, 49 000 and 36 000, respectively. However, the MWs of the lower bands were not calculated from this preparation. There is a striking similarity of the MWs of the major subunits as well as the gel electrophoretic pattern of rice mitochondrial ATPase with those from other sources reported by various workers [7–15].

The complex subunit composition of purified mitochondrial ATPase creates a need for the provision of direct evidence for the existence of a similar complex in mitochondrial membranes. Comparison of the specific activities of purified mitochondrial ATPase and of the initial mitochondrial membrane fraction suggests that a small part of the total protein of the membrane fraction was mitochondrial ATPase (Table 1). Thus the A and B subunits of mitochondrial ATPase seemed to be two of the major membrane components detectable on gel electrophoretic analysis of mitochondrial membranes. Analysis of mitochondrial membrane fraction at the three stages of purification and of the heat-fractionated soluble ATPase part showed polypeptide chains which co-migrated with the A and B subunits of the purified mitochondrial ATPase. Subunit C appeared as an indistinct band in all the membrane fractions and became detectable after heat fractionation only. The gel electrophoretic patterns showed that there were mitochondrial membrane components corresponding to subunits A and B and probably C, suggesting that these subunits of purified mitochondrial ATPase were not artefacts generated during the purification process.

Mitochondrial ATPase complex is known to catalyse the hydrolysis of ATP to ADP and Pi in the presence of divalent metal ions [16]. The kinetic constants of the ATPase activity under standard conditions (50 mM Tris–HCl buffer at pH 7.65, 10 mM magnesium chloride and an enzyme concentration of 2.5 μg/ml at 25°) were characterized by a K_m (ATP) of 0.192 mM and V_{max} of 58.8 μmol per min and mg of protein. The results presented here also showed typical Michaelis–Menten kinetics at relatively low ATP concentrations. The divalent cation dependent ATPase velocity as a function of $MgATP^{2-}$ concentrations serves to indicate substrate inhibition at high ATP concentrations. Although most investigators have generally obtained typical Michaelis–Menten kinetics for the hydrolysis of ATP catalysed by purified F_1 ATPase, there are reports that reciprocal plots of initial velocity data with varying $MgATP^{2-}$ concentrations are curvi-linear in the absence of activating anions [17].

The rice mitochondrial ATPase activity was inhibited competitively by ADP with a K_1 (ADP) of 404 μM. The affinity of the enzyme towards ADP is quite high, which is evident from such data. The temperature optimum of the enzyme was 25° (± 1°) with *ca* 15–20% decrease in activity for every 5° above or below this temperature level. Thus the dependence of the enzyme activity on assay temperature was quite pronounced in this case. The purified protein was cold-labile and it lost almost all the activity at 0° within 30 min. The purified enzyme could be protected from cold lability by the addition of methanol or glycerol. Addition of methanol (5% as final concentration) stabilized the enzyme up to a period of 36 hr in soluble condition at 4°.

Differential sensitivity to oligomycin of ATPase activity of different fractions from rice seedling mitochondria is shown in Table 2. It is rather peculiar that the whole sonicate from rice mitochondria showed an ATPase activity that was only partially inhibited by oligomycin. The Triton X-100 solubilized fraction had an ATPase activity which was inhibited more than 98% by oligomycin. The purified protein fraction was not inhibited by oligomycin like the purified F_1 ATPase preparations from other sources. At least some bands on the SDS gel electrophoresis profiles of the Triton X-100 solubilized fraction disappeared after heat activation. It is quite apparent that those polypeptides responsible for oligomycin sensitivity of the membrane-bound or Triton X-100 solubilized ATPase were eliminated during heat treatment, thus making it less sensitive to oligomycin.

The new method adopted here has a number of improvements over the previous method [3] for the purification of the F_1 ATPase system from rice seedling mitochondria. For instance, the use of 200 mM KH_2PO_4 with Tris and EDTA as the buffer system gives better resolution of the enzyme activity as well as improvement of the percentage yield (Table 1). The use of KH_2PO_4–EDTA stabilizes the protein in a soluble condition which otherwise can be easily inactivated in the presence of divalent cations such as Mg^{2+} even though inorganic phosphate is present in the medium. The addition of an intermediate step of high speed centrifugation between the low speed precipitation of mitochondria and Triton X-100 addition helps to separate the membrane and the soluble fraction of the whole sonicate, thus providing the means for a higher degree of purification. Washing with a low concentration of Triton X-100 without homogenization avoids major contaminating factors which usually dissolve out in a single-step Triton purification procedure.

The presence of ATP in the solution is obligatory because the lack of ATP decreases enzyme activity by *ca* 10% during storage for every 24 hr. However, the purified enzyme can be preserved for at least 6 months by

Table 2. Inhibition by oligomycin of the ATPase activity of different preparations of mitochondrial membrane

Fraction	Treatment	Activity as per cent of original
Whole sonicate	None	100
	Oligomycin (1 μg/ml)	60
	Oligomycin (5 μg/ml)	58
Washed membrane	None	50
	Oligomycin (1 μg/ml)	20
	Oligomycin (5 μg/ml)	15
Triton X-100 fraction	None	33
	Oligomycin (1 μg/ml)	2
	Oligomycin (5 μg/ml)	0
Heat-treated fraction	None	98
	Oligomycin (1 μg/ml)	98
	Oligomycin (5 μg/ml)	98

The ATPase activities of the different fractions were measured under standard assay conditions with Tris–HCl as buffer, ATP as Na^+ salt and Mg^{2+} as divalent cation.

lyophilization. The use of the new gradient system for the final purification reduces the requirement of the centrifugation speed to a level which can be provided by an ultracentrifuge commonly available in a biochemical laboratory. The associated increment in time requirement is quite nominal. The total time taken for purification of the enzyme from the crude material is only 6–7 hr, which can help the worker to characterize the basically unstable enzyme protein instantly, without intermediate steps for storage. Thus the new method avoids complicated and lengthy procedures which usually lead to a reduction in the final yield as well as in the activity of the pure protein.

The methodology described here is rapid and constantly reproducible for plant mitochondrial ATPases. In the animal system as well, purification of mice liver mitochondrial F_1 ATPases by this method gave an equally good yield and activity. This method can also be adopted for the isolation of Racker factor from yeast and CF_1 (chloroplast coupling factor) from the leaves of higher plants.

Concerning the activity of the enzyme purified by this method, it could compare favourably with that obtained by the standard method described for animal sources. For instance, rat liver enzyme had an activity up to 70 μmol of Pi/min/mg protein when coupled with an ATP recovery system. The rice enzyme, on the other hand, had an activity up to 60 μmol of Pi/min/mg protein even without the recovery system, the addition of which might enhance the rate considerably.

EXPERIMENTAL

Isolation and purification of enzyme. Rice (*Oryza sativa* L.) seeds were surface-sterilized with 0.1% $HgCl_2$ soln and germinated at 30–33° in a dark and humid atmosphere. After 72 hr, the etiolated seedlings were harvested and both light and heavy mitochondria were isolated by a differential centrifugation procedure described in ref. [3]. The mitochondria were suspended in a washing medium composed of 0.35 M mannitol, 0.25 M sucrose and bovine serum albumin (1 mg/ml in 200 mM Tris–HCl buffer, pH 7.8—buffer A) and precipitated by centrifugation at 14 000 *g* for 10 min. The washed mitochondrial pellet was suspended in 200 mM KH_2PO_4–Tris buffer (pH 7.4) containing 5 mM EDTA (buffer B) and sonicated for 1 min at full power on an MSE sonicator, keeping the temp. below 4°. After sonication, the temp. of the suspension was raised to 8–10° and the unbroken mitochondria, along with other cell debris, were removed by precipitation at 10 000 *g* for 15 min. The translucent supernatant, diluted to 10 ml by the addition of the same buffer, was next washed by high speed centrifugation at 110 000 *g* for 30 min at 4° in a 50 L angle rotor of a Beckman L3-50 ultracentrifuge. The pellet was washed by repeated homogenization and precipitation and the ppt. was resuspended in buffer and treated with 10% Triton X-100 to make the final concn as 0.1% Triton without homogenization at 4°. The suspension was gently shaken without any frothing and immediately centrifuged at 110 000 *g* for 15 min. Finally, the pellet was carefully washed with buffer and homogenized with 10% Triton X-100, which was carefully added to make the final concn as 0.25% Triton in the same buffer. Triton was added at this step to solubilize the membrane-bound proteins, and the excess insoluble proteins were removed by centrifugation again at 110 000 *g* for 15 min at 4°. To the yellow transparent supernatant, ATP soln was added to make the final concn at least 25 mM ATP per ml. It was then heated on a water bath at 50° for 2 min and MeOH was added to a final concn of 5%. The addition of MeOH was an obligatory step to retain the activity of the protein during prolonged exposure to cold. The clear suspension was centrifuged at 110 000 *g* for 15 min at 4° and the ppt. discarded. The supernatant was ultimately layered on a 4–12% discontinuous glycerol gradient and centrifuged at 248 000 *g* (48 000 rpm) on a SW-65 rotor in a Sorvall OTD-50B ultracentrifuge for 3 hr at 4°. Twenty equal fractions of 10 drops each were collected from the gradient and assayed.

Analytical methods. The medium used for assaying the enzyme consisted of 5 mM $MgCl_2$, 15 μmol ATP and 25 mM Tris–HCl at pH 7.8 at 30°. Throughout the experiments, 25 μg enzyme protein was added. The reaction was started by adding the fraction to be assayed and was stopped after 10 min by adding 1 ml of ice-cold quench soln, after which the amount of Pi in the reaction mixture was measured [8]. One unit of ATPase activity was taken as the amount of enzyme required to liberate 1 μmol of Pi in 1 min. ATPase activity was expressed as the number of units per mg protein which was measured according to ref. [18]. Analytical PAGE of the protein samples as well as of the membranes was carried out principally following the method described in ref. [7]. The gel electrophoretic profile of whole submitochondrial particles and membranes was carried out according to ref. [19], which gave better resolution of bands on gels. Catalase activity was measured by the disappearance of peroxide spectrophotometrically at 240 nm, according to ref. [20].

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