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Articles

Characterization of membrane ATPase activities of spinach chloroplasts

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Mg^{2+} works as a key factor on photosynthesis in plant chloroplasts. We have a final purpose to make the Mg^{2+} transport proteins clear in chloroplasts. The existence of Mg^{2+}-dependent ATPase has been suggested in 1973, but has not been elucidated yet. ATPases of spinach chloroplast membranes were solubilized by detergents, and their activities were characterized. Octylglucoside was the most valuable detergent for solubilization of thylakoid ATPase; however, taurocholate was more suitable for solubilization of envelope ATPase. Both thylakoid and envelope ATPases had the optimum pH of around pH 7.5. Both ATPases required Mg^{2+} for activity, but our results indicated that the ATPases used Mg･ATP chelates as a substrate, and free Mg^{2+} was inhibitory. Thus, we could not identify "Mg^{2+}-dependent" ATPase in thylakoid and envelope membranes, and other approaches are necessary for purifying Mg^{2+} transport proteins and making them clear.

Key words: ATPase; Mg^{2+}; Detergent; Thylakoid; Envelope; Chloroplast; Spinach

The plasma membrane of plant cells and plant organellar membranes are involved in volume regulation, transepithelial transport, and regulation of the membrane potential. One of the factors that have decisive effects on the energy production and CO_2 fixation in the plant chloroplast is ion concentrations. In the chloroplast stroma, concentrations of the physiological important ions are on the order of 150 mM K^+, 50 mM Cl^-, and 5 mM Mg^{2+} [1].

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide.

During photosynthesis of the plant chloroplast, light-driven H^+ gradients are generated between the cytosolic site (~pH 7), the stroma (~pH 8), and the thylakoid lumen (<pH 6). Ion transport proteins present in the chloroplast inner envelope membrane and in the thylakoid membrane appear to be involved in the development and regulation of H^+ gradients and membrane potentials across these membranes[2]. Due to the pH optimum (~pH 8) of the key enzymes of the photosynthetic carbon reduction cycle, the rate of light-dependent CO_2 fixation in chloroplasts depends on the maintenance of a high stromal pH[3]. The uphill H^+ flux across the chloroplast envelope is probably driven by a H^+-ATPase and indirectly coupled to the uptake of potassium (K^+ channel) from the cytosol [1),4)]. Also, the concentration of Mg^{2+} increases due to the light-dependent release of Mg^{2+} from thylakoid membranes[5].

As ion transport systems in the chloroplast, the existence of Ca^{2+}/H^+ antiporter[6] and K^+-channel[7] is known. However, the existence of Mg^{2+} transport proteins is not yet clear, though the existence of Mg^{2+}-dependent ATPase has been suggested in 1973[8]. Mg^{2+} works as a key factor on the photosynthesis. We have a final purpose to make the Mg^{2+} transport proteins clear in chloroplasts. We here characterize ATPase enzymes in spinach chloroplasts, which act as important adjustment of the ion concentrations.

Materials and methods

Preparation of chloroplast membranes[4),9)]

Spinach leaves were obtained from local markets. Leaves were ground with grind medium (50 mM Hepes-KOH (pH 7.5), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbate) twice for 10 s in a Waring blender. The slurry was passed through four layers of Miracloth before centrifugation at 5,000 × g for 15 s. Pellets were resuspended in the grind medium and layered onto 30% Percoll solution in 50 mM Hepes-KOH (pH 8.0) and 0.33 M sorbitol. After centrifugation at 700×g for 15 min, the intact chloroplasts in the pellets were resuspended in the same solution and centrifuged at 1,000×g for 10 min.

The intact chloroplasts were suspended in hyperosmotic medium (0.6 M sucrose, 10 mM Tricine-KOH (pH 7.5), 2 mM EDTA, 5 mM 2-mercaptoethanol) and ruptured by exposure to two freeze/thaw cycles (freezing at -20°C for

a minimum of 1.5 h followed by thawing at room temperature). The medium containing ruptured chloroplasts was then diluted by adding 2 volumes of medium containing no sucrose. After gentle shaking for 5 min at 4°C, the medium was centrifuged at 15,000×g for 20 min to pellet membranes. The pellets were suspended in medium that had 0.2 M sucrose.

For separation of thylakoid and envelope membranes, the membrane fraction suspended in 0.2 M sucrose was centrifuged at 6,000×g for 30 min to pellet thylakoid membranes. The supernatant was centrifuged again at 40,000×g for 1 h to pellet chloroplast envelope membranes. The yellow pellets were resuspended in the medium that had 0.2 M sucrose.

Solubilization of membranes by detergents

Membranes were incubated for 1 h at 4°C in solubilization medium containing detergent as indicated. After incubation, the mixture was centrifuged at 60,000×g for 90 min. The supernatant was again centrifuged at 60,000×g for 1 h. The supernatant was recovered and assayed for ATPase activity.

Measurement of ATPase activity

ATP hydrolysis was determined by the release of Pi. Standard reaction mixtures (25 µl total volume) contained 40 mM Tricine-KOH (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, and 2.5 µg protein. Release of phosphate from ATP was determined by a colorimetric procedure using ammonium molybdate[10].

Results

Solubilization of chloroplast membrane ATPases by detergents

A variety of ionic, nonionic and zwitterionic detergents were tested for their ability to solubilize chloroplast membrane ATPases. Membranes were incubated for 1 h at 4°C in medium containing a detergent with gently stirring. The suspensions obtained were centrifuged, and the ATPase activity in the supernatant was assayed.

The effect of detergents on the solubilization of thylakoid membrane ATPase activity is shown in Table 1. Highest ATPase activity was obtained with octylglucoside, a non-ionic detergent, and dodecylmaltoside. The ionic and zwitterionic detergents tested were less effective in comparison with the non-ionic detergents. These results indicated that octylglucoside was most useful to solubilize ATPase from

Table 1. Effect of detergents on solubilization of ATPase activity from thylakoid fraction. Thylakoid fractions (2.5 mg/ml protein) were incubated in presence of various detergents and centrifuged as described under Materials and methods. The final concentration of detergent was 1.0% (v/w) except for Nonidet P40 and Tween 20. The supernatants were assayed for ATPase activity.

Detergent	ATPase activity (nmol Pi/hr)
Brij 35	0
Digitonin	0
Dodecylmaltoside	1.01
Nonidet P40 (0.50%)	0.22
Octylglucoside	1.70
Triton X-100	0.03
Tween 20 (0.50%)	0.46
Cholate	0.08
Deoxycholate	0.12
Taurocholate	0.08
CHAPS	0.38

Table 2. Effect of detergents on solubilization of ATPase activity from envelop membrane fraction. Envelope membrane fractions (3.3 mg/ml protein) were incubated in presence of various detergents and centrifuged as described under Materials and methods. The final concentration of detergent was 1.3% (v/w). The supernatants were assayed for ATPase activity.

Detergent	ATPase activity (nmol Pi/hr)
Brij-35	0.07
Digitonin	0
Dodecylmaltoside	0.28
Octylglucoside	0.19
TritonX-100	0.25
Tween 20	0
Cholate	0.45
Deoxycholate	0.20
Taurocholate	0.48
CHAPS	0.19

thylakoid membranes.

Table 2 shows the effect of detergents on the solubilization of envelope membrane ATPase activity. In contrast to the solubilization of thylakoid membrane ATPase, the ionic detergents were effective. Highest ATPase activity was obtained with taurocholate and cholate. With treatment of taurocholate, 41% of the ATPase activity was recovered in the supernatant after centrifugation. The non-ionic and zwitterionic detergents such as dodecylmaltoside, Triton X-100, and CHAPS were less effective. These results indicated that taurocholate was most useful to solubilize ATPase from envelope membranes.

Comparison of thylakoid and envelope ATPase activities

The effects of pH on the thylakoid

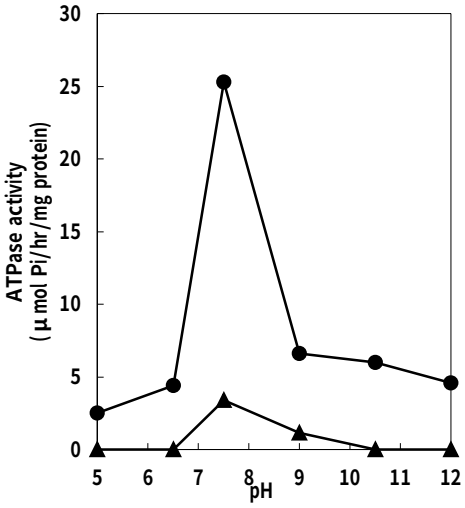

Fig. 1. pH dependence of the chloroplast ATPase activity. The ATPase activity of envelope membrane (▲) and thylakoid (●) fraction was measured at various pH.

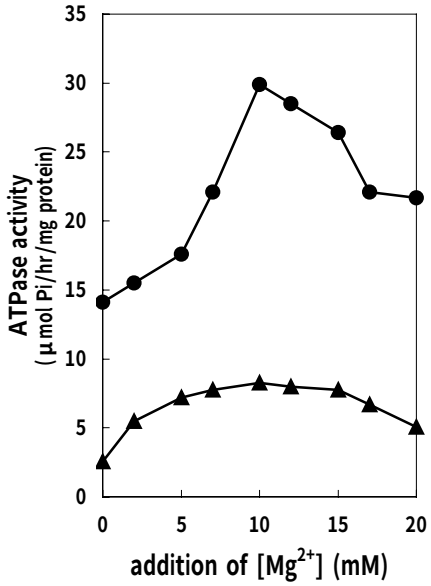

Fig. 2. Effect of Mg^{2+} on the chloroplast ATPase activity. The ATPase activity of envelope membrane (▲) and thylakoid (●) fraction was measured except that the concentration of $MgCl_2$ was varied.

and envelope ATPase activities were examined (Fig. 1). The optimum pH of both activities was estimated to be around pH 7.5.

The ATPase associated with the chloroplast envelope is typically referred to as a "Mg^{2+}-dependent" enzyme[8]. Previous studies[11),12)] have demonstrated that the Mg^{2+} dependency of various ATPases is not cue to the presence of a catalytic site on the enzyme for Mg^{2+} but, rather, because the Mg·ATP complex is the actual substrate for the enzyme. The characterization of the ATPase activity associated with the chloroplast thylakoid and envelope, therefore, focused on this Mg^{2+} dependency.

The effects of Mg^{2+} added were examined in the presence of the fixed concentration of ATP (10 mM). Highest activity of the thylakoid and envelope ATPase was obtained at equimolar (10 mM) concentration of Mg^{2+} and ATP (Fig. 2). This is consistent with the substrate being Mg・ATP. In the presence of higher concentration of Mg^{2+} (allowing for the presence of free Mg^{2+} in the reaction solution), inhibition of the enzyme activity was noted. Thus, free Mg^{2+} actually inhibited the thylakoid and envelope ATPases.

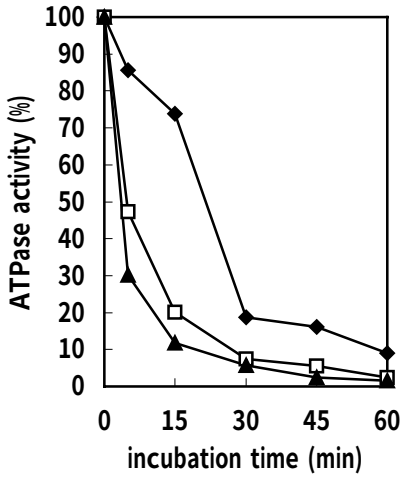

Fig. 3. Effect of DCCD on the ATPase activity of thylakoid fraction. After incubation with DCCD at 37°C, the ATPase activity of thylakoid fraction was assayed. The concentration of DCCD was 0 (♦), 10 (□), 50 (▲) μM.

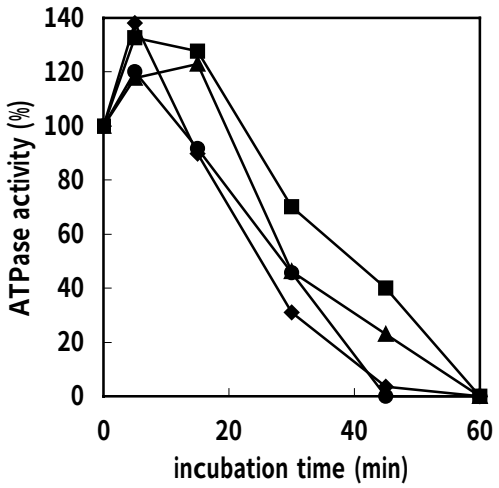

Fig. 4. Effect of DCCD on the ATPase activity of envelope fraction. After incubation with DCCD at 25°C, the ATPase activity of envelope fraction was assayed. The concentration of DCCD was 0 (♦), 50(▲), 100(●), and 500 (■) μM.

Effect of DCCD on the ATPase activity

DCCD is an inhibitor of thylakoid (and mitochondrial) H^+-ATPase[13]. The effects of DCCD on the thylakoid and envelope ATPase activity were examined. Both ATPase activities were thermolabile and lost rapidly during incubation, but Fig. 3 indicated that DCCD inhibited the thylakoid ATPase. Incubation with 10 and 50 μM DCCD for 15 min induced 73% and 84% inhibition, respectively. In contrast, no inhibition of the envelope ATPase activity was observed with 50-500 μM DCCD (Fig. 4).

Discussion

A better understanding of the enzymatic and transport functions of the chloroplast thylakoid and envelope membranes requires a detailed knowledge of their constituent proteins. However, purification of their proteins, especially envelope proteins, is hampered by several factors: (a) envelope membranes are only a minor membrane structure within the plant cell; they do not represent more than 1-2% of the total chloroplast proteins; (b) compared to most plant cell membranes, envelope membranes have a very high lipid to protein ratio (1.2-1.5

mg lipids/mg protein), and their complete solubilization requires large amounts of detergents that might be deleterious to enzymatic activities. Therefore, a compromise between solubilization and activity should be found, and the detergent carefully selected

The choice of a detergent for the solubilization of a membrane is rather empirical. The experiments described above have indicated that only a few of detergents are useful for studies of thylakoid and envelope ATPases. Two detergents were found to be superior to the others for the studies of the thylakoid ATPase, *i. e*. octylglucoside and dodecylmaltoside. In contrast, taurocholate and cholate were found to be superior for the envelope ATPase.

It is well known that illumination of chloroplasts causes stromal alkalinization and a release of Mg^{2+} from thylakoid membranes. In thylakoid, H^{+}-ATPase exists, which catalyzes the synthesis of ATP at the expense of a transmembrane proton gradient, and is sensitive to DCCD. In addition, an ATP-dependent pump could provide the necessary mechanism in envelope, and an ATPase is known to be a major component of the total intrinsic protein in envelope. The envelope ATPase was previously characterized as a "Mg^{2+}-dependent" enzyme in review articles. However, we could not identify the Mg^{2+}-dependent ATPase, but our results indicated that the enzyme uses Mg・ATP chelates as a substrate, and Mg^{2+}-dependent ATPase is a minor component, if any. Mg^{2+} transport should be the necessary mechanism in thylakoid and envelope. Other approaches are necessary for purifying the Mg^{2+} transport proteins and making them clear.

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