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Chloroplast Thylakoid Protein Phosphatase Is a Membrane Surface-Associated Activity

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ABSTRACT

Chloroplast thylakoid protein phosphatase activity was measured using 32P-labeled histone as an exogenous substrate and an assay of the 32P released involving formation of a phosphohistone complex and organic extraction. The activity was liberated from wheat (Triticum aestivum) thylakoids by washing the membranes in NaCl-containing solutions followed by centrifugation. The liberated phosphatase activity had a pH optimum of approximately 6.75, was inhibited by addition of 10 millimolar EDTA or EGTA, and was stimulated by addition of millimolar amounts of dithiothreitol, magnesium, manganese, or calcium ions. The rate of thylakoid protein dephosphorylation was decreased following liberation of a portion of the protein phosphatase activity and was increased by addition of salt-liberated phosphatase fraction. These results suggest that at least a portion of wheat thylakoid protein phosphatase is a peripheral, rather than an integral, membrane protein.

Although a number of protein phosphorylation systems have been observed in plants (cf. Ref. 20), the majority of this research has focused on chloroplast thylakoid phosphorylation. Phosphorylation of chloroplast thylakoid membrane proteins was first described by Bennett (5) and has been explored by a number of laboratories in the past decade (1, 4, 7, 10, 12, 14, 16, 19). Most of this work has focused on the phosphorylation of the light-harvesting complex apoproteins and the coincident transition from state I to state II within the photosynthetic apparatus. However, thylakoid protein phosphorylation is far from simple, with multiple protein kinases present (9) and as many as two dozen thylakoid proteins acting as phosphoryl acceptors (15). The phosphate in these phosphoproteins resides on surface-exposed regions which can be removed by treatment with proteases such as trypsin (6, 17). There is also protein kinase activity in the chloroplast stromal phase (8, 13) which is distinct from the thylakoid protein kinases.

In contrast to the situation with thylakoid protein kinases, we know relatively little about chloroplast protein phosphatases. Phosphorylation of many thylakoid phosphoproteins in mature tissues is stimulated by light and, when phosphorylated material is placed in the dark, it is dephosphorylated (5, 6, 17, 23). The dephosphorylation appears to involve phosphatase activity that is inhibited by NaF and not affected by inhibitors of photosynthetic electron transport (6). The phosphatase activity is also inhibited by molybdate ions (19). The half-time for dephosphorylation of the light-harvesting complex apoproteins is approximately 5 to 7 min, whereas dephosphorylation of other thylakoid proteins is slower (11, 17, 23). Addition of surfactants such as Triton X-100 results in a loss of dephosphorylation activity (6, 24). There also appears to be a NaF-sensitive protein phosphatase activity in the stromal fraction (13), but it is less well characterized and its relationship, if any, to thylakoid protein phosphatase activity is unknown.

The data in this report will demonstrate a thylakoid activity that will dephosphorylate [32P]phosphohistone. This activity is liberated from the thylakoid membranes by washing in solutions with elevated salt concentration, indicating that it is not an intrinsic membrane activity. This report will deal with initial characterization of the partially fractionated enzyme activity to establish that it can be accurately measured and to optimize its activity.

MATERIALS AND METHODS

Thylakoid Isolation

Wheat (Triticum aestivum L. cv Brule) was grown for 10 to 20 d in a soil-vermiculite mixture at 23°C, 60% RH, and 250 μmol m⁻² s⁻¹ (PAR) of light. Leaves were excised and homogenized twice with a Waring blender for 5 s in ice-cold 0.4 M sorbitol, 1 mM NaCl, 25 mM Na tricine (pH 7.6). All further steps were carried out at 0 to 5°C. The homogenate was filtered through four layers of Miracloth (Calbiochem, Inc.) and centrifuged at 3,000 g for 1 min. The chloroplasts in the pellet were disrupted in 25 mM tricine (pH 7.6), 1 mM KCl, and 5 mM MgSO₄, with the aid of a glass homogenizer. The solution was centrifuged at 27,000 g for 5 min to pellet the membranes. The pelleted thylakoid membranes were again washed by resuspension in the same buffer, homogenization, and recentrifugation. Following the second wash, the pelleted membranes were resuspended in 25 mM Na tricine (pH 7.6), 1 mM KCl, 5 mM MgSO₄. Chl (2) and protein (22) concentrations were determined as described in their respective references.

Phosphatase Liberation

In order to salt-wash thylakoid membranes, the membrane-containing solution was diluted to a final concentration of 0.2 mg Chl mL⁻¹ in resuspension buffer. Sufficient solid NaCl was slowly added to the solution to produce a final concentration of 0.2 M. The solution was stirred for 10 min and then
centrifuged at 40,000 g for 30 min. The supernatant fraction was removed and adjusted to 30% saturation with solid ammonium sulfate in an ice bath. The solution was stirred for 15 min and then centrifuged at 40,000 g for 15 min. The supernatant fraction was removed and adjusted to 60% saturation with solid ammonium sulfate. This solution was stirred for 15 min and then subjected to centrifugation at 40,000 g for 15 min. The supernatant fraction was discarded and the pellet was resuspended in a small volume of 10 mM Na Mes (pH 6.75). The 30 to 60% ammonium sulfate fraction was dialyzed overnight against 500 volumes of 10 mM Mes (pH 6.75). This fraction was used for the majority of the assays reported here.

For isolation of intact chloroplasts, young leaves were cut into small pieces and placed into an ice-cold slurry of 0.4 m sorbitol, 1 mM NaCl, 25 mM Na Tricine (pH 7.6). Leaves were homogenized for about 3 s with a Waring Blender. The homogenate was squeezed through four layers of Miracloth with a thin layer of cotton wool between the first and second layer. The filtrate was centrifuged for 60 s at 2,000 g. The chloroplast pellet was resuspended and further purified by centrifugation in a gradient of Percoll as described by Moutioux and Douce (18). The intact chloroplasts were collected and lysed in 25 mM Na Tricine (pH 7.6), 1 mM KCl, 5 mM MgSO4, and centrifuged for 4 min at 12,000 g to separate the stromal (supernatant) fraction from the membrane (pellet) fraction. This thylakoid-containing membrane fraction was further salt-washed as described above.

Histone Phosphorylation

Histones were labeled in a 1 mL reaction containing 4 mg Histone III-S (Sigma), 25 mM Na Tricine (pH 7.5), 5 mM MgSO4, 10 mM NaF, 0.1 mM unlabeled ATP, and 0.4 mCi [γ-32P]ATP (>4000 Ci mmol−1, ICN Radiochemicals). The reaction was initiated with 250 units of bovine heart protein kinase catalytic subunit (Sigma) and incubated for 2 h at 30°C. Following incubation, 1 mL of 4 mM ammonium acetate was added to the labeling reaction and the solution loaded onto a Sephadex G-25 column (1 x 30 cm) prequillibrated with 4 mM ammonium acetate. Fractions (1.5 mL) were collected and a small aliquot of each was assayed by Cerenkov radiation. This revealed a rapidly eluting peak of radioactivity representing labeled histones and a much larger peak of small mol wt material, probably unincorporated nucleotides and inorganic hydrolysis products. The labeled fractions from the first peak were pooled and concentrated by freeze-drying. The dried, labeled histone was dissolved in a small volume of water and frozen in aliquots for use in the protein phosphatase assay.

The protein phosphatase assay contained 50 mM Na Mes (pH 6.75) and 13 µg histone (4 x 10 to 1 x 10^6 dpm) in a total volume of 100 µL unless otherwise indicated. Assays were normally carried out in triplicate in polypropylene 0.5 mL microcentrifuge tubes. After addition of enzyme, the tubes were incubated at 30°C for 30 min. Fifty microliters of the reaction mixture was assayed for 32Pi by formation of a phosphomolybdate complex and its extraction into an iso-butanol-toluene mixture (21).

Thylakoid Protein Dephosphorylation

The upper half of 14-day-old wheat leaves were used to measure thylakoid protein dephosphorylation. It has been demonstrated previously (10) that immature tissues from the base of wheat plants contain significant levels of a light-independent thylakoid protein kinase activity not inhibited by DCMU. To assess the effect of salt treatment on endogenous thylakoid protein dephosphorylation, two tubes containing 8 mL each of freshly prepared thylakoid membranes (0.44 mg Chl mL⁻¹ buffer) were prepared. Two mL of water was added to the control tube and 2 mL of 1 M NaCl was added to the other. The tubes were incubated for 10 min at 25°C and then centrifuged for 15 min at 40,000 g. The pellet in each tube was resuspended in 5 mL of 25 mM Na Tricine (pH 7.6), 5 mM MgSO4, and the Chl concentration redetermined. Two identical incubations were prepared containing 25 mM Na Tricine (pH 7.6), 5 mM MgSO4, 0.2 mM unlabeled ATP, 85.2 µCi [γ-32P]ATP, and 0.14 mg mL⁻¹ of Chl in 0.5 mL. The phosphorylation was initiated by the addition of thylakoid membranes and the solutions were incubated at 30°C in an illuminated (500 µmol; m⁻² s⁻¹) water bath. After 10 min of labeling, the thylakoid protein kinase was inhibited by addition of DCMU to a final concentration of 10 µM and the water bath was darkened. Samples (25 µL) were removed at the indicated intervals and the amount of label incorporated into protein was measured by a filter paper assay (16).

To determine the effect of exogenously supplied salt-liberated protein phosphatase on dephosphorylation of endogenous thylakoid proteins, the following experiment was conducted. Thylakoid membranes were resuspended in a 1.0 mL incubation containing 25 mM Na Tricine (pH 7.6), 5 mM MgSO4, 0.2 mM unlabeled ATP, and 200 µCi [γ-32P]ATP. The solution was incubated in an illuminated water bath as above for 10 min. Aliquots of 0.25 mL were transferred to two tubes containing equal volumes of 25 mM Na Tricine (pH 7.6), 5 mM MgSO4, and 20 µM DCMU; one of the tubes also contained 25 µg of protein from the ammonium sulfate precipitated, salt-liberated protein phosphatase activity. The samples were incubated in a darkened water bath at 30°C and samples were removed as above for assay of remaining label in thylakoid phosphoprotein.

RESULTS AND DISCUSSION

Our previous study on thylakoid protein phosphatase (24) utilized TCA precipitation to determine label released from substrate phosphoprotein. We have since employed a different assay method, organic extraction of a phosphomolybdate complex, for the following reasons: we were unable to completely precipitate histone with TCA; background activity in organic extraction assays lacking added enzyme is negligible; the assay is specific for Pi released during the incubation and shows no interference by protease activity; and the assay is convenient to use with a large number of samples. Using labeled histone and the phase partition assay, we were able to reproducibly detect protein phosphatase activity in thylakoid membrane preparations. The results presented in Figure 1 demonstrate the linearity of the dephosphorylation rate versus time.

During studies of the protein phosphatase activity, it be-
came apparent that the activity was somewhat loosely associated with the thylakoid membranes. Centrifugation to establish that the activity was associated with the membranes usually resulted in approximately 10 to 15% of the activity remaining in the supernatant fraction. Washing the membrane with increasing concentrations of NaCl followed by centrifugation resulted in greater amounts of protein phosphatase activity remaining in the supernatant fraction. However, it was also observed that supernatant fractions containing greater than 0.2 to 0.25 M NaCl had apparently less activity than those with lower concentrations. We also found that addition to the assay of increasing amounts of these enzyme fractions resulted in an apparent decrease in enzyme activity. This decrease could be mimicked by keeping the enzyme concentration constant and adding increasing final concentrations of NaCl to the assay. We have concluded that washing with solutions of increased ionic strength is able to liberate at least a portion of the protein phosphatase activity from the thylakoid membranes. The amount of phosphatase liberated from the membranes as a function of the salt concentration could be measured following dialysis and is shown in Figure 2. The amount of activity in the supernatant fraction is not increased by NaCl concentrations higher than approximately 0.3 M.

To demonstrate that the observed phosphatase activity was of chloroplast origin, intact plastids were fractionated from wheat leaves by centrifugation through a Percoll gradient. This chloroplast fraction was hypotonically lysed and the membrane fraction recovered. This thylakoid-containing fraction was then washed with NaCl and the membranes removed by centrifugation. All fractions were assayed for histone phosphatase activity. The data from one such experiment are shown in Table I. The amount of thylakoid associated protein phosphatase which was liberated by the salt wash was routinely 50 to 60% of the activity originally pelleted with the thylakoid fraction. These data corroborate that the histone phosphatase activity liberated from the thylakoids by salt solutions is probably a chloroplast enzyme. However, it should be noted that similar experiments with intact chloroplasts isolated from spinach leaves indicated that, at most, 15% of the thylakoid protein phosphatase activity was released by similar salt washes and results from wheat may not be applicable to all species of plants.

We arbitrarily chose to conduct further studies on the protein phosphatase activity liberated by washing the wheat thylakoid membranes with 0.2 M NaCl. It was empirically determined that this activity could be conveniently concentrated by precipitation with ammonium sulfate as described in "Materials and Methods." Extensive dialysis of the concentrated fraction was necessary to remove residual ammonium sulfate, which, like NaCl, was inhibitory when added to the assay. The standard assay used for the majority of experiments reported herein contained 16 μg of protein from this fraction. The release of 32Pi from labeled histone was linear for at least 30 min using the standard assay. The measured activity was also proportional to the amount of added protein in the phosphatase fraction (Fig. 3) over the range tested (up to 65 μg). The response of the standard assay to changes in labeled histone concentration are shown in Figure 4. It is evident that
the activity is not saturated by the histone concentrations used.

The pH optimum for the protein phosphatase activity was found to be approximately 6.75. The activity is inhibited by chelating agents such as EDTA and EGTA, with a 10 mM concentration of each inhibiting the activity by 64 and 70%, respectively. The addition of 10 and 25 mM NaF, previously reported to inhibit thylakoid dephosphorylation (6), inhibited the protein phosphatase activity by 80 and 91%, respectively. The activity was also influenced by the addition of sulfhydryl reducing agents and divalent cations to the assay. Both 2-mercaptoethanol and DTT stimulated protein phosphatase activity. The results of DTT addition are demonstrated in Figure 5. Concentrations of DTT as low as 0.1 mM markedly stimulate the activity. Bennett (6) previously established that Mg$^{2+}$ greatly increased the rate of thylakoid protein dephosphorylation, so we examined the effect of a number of divalent cations on the liberated phosphatase activity (Fig. 5). Magnesium and manganese ions stimulate activity in the concentration range 0.1 to 1 mM. Calcium ions caused a slight stimulation, whereas copper, zinc, and cadmium were all strongly inhibitory in this concentration range. NaCl and Na$_2$SO$_4$ (5 mM) were added to the assay to assess the effect of their respective anions on the phosphatase activity. Sodium chloride had a negligible effect at this concentration, whereas Na$_2$SO$_4$ caused an approximately 15% inhibition. Thus, the majority of the ion effects observed in Figure 5 were therefore probably due to the cations. It should be emphasized that we consider the above data of value in optimizing the assay of the salt-liberated protein phosphatase fraction, and not necessarily as relevant to modulation of thylakoid protein dephosphorylation *in vivo*.

The ionic nature of the liberated phosphatase was assessed by examining its behavior on ion-exchange columns. The activity was not retarded by passage through DEAE-cellulose or DEAE-Sephadex columns at pH 8. When applied to a CM-Sephadex column at pH 6.0, the activity was retained by the column. These experiments suggest that the protein phosphatase may have a net positive charge. It is not surprising that an activity resident on the thylakoid surface should be posi-
The location of this activity on the thylakoid surface presents a challenge in comparing the properties of the liberated activity with that of the activity in situ. The thylakoid has a negative surface charge density, resulting in a negative potential which makes the ionic composition in the proximity of the membrane different from that of the bulk solution (3, 4). For instance, Bennett (6) reports that magnesium ions at bulk concentrations as low as 10 μM markedly stimulate thylakoid protein dephosphorylation. Because of the membrane’s negative surface charge, the magnesium ion concentration in the vicinity of the membrane would be much greater than that of the bulk phase and not comparable to results on liberated enzyme. This phenomenon could also affect the apparent affinity for a positively charged protein substrate such as histone. Similarly, Michel et al. (17) report that the pH optimum for thylakoid protein dephosphorylation is 9.0, whereas we find an optimum of 6.75 for the liberated phosphatase activity. As discussed by Barber (3), the thylakoid negative surface charge can lower the pH at the membrane surface by 1 to 2 pH units below that measured in the bulk phase and the addition of a divalent cation, such as the magnesium usually added to enhance thylakoid dephosphorylation, can selectively lower the surface concentration of monovalent cations such as H⁺, further decreasing the local pH. Thus, the properties of the membrane-associated thylakoid protein phosphatase may markedly differ from that of the liberated form of the enzyme.

An electrostatically induced surface association between the thylakoid membrane and protein phosphatase activity is not at odds with our current understanding of the system. The majority of thylakoid protein phosphorylation takes place on residues exposed to the stromal phase; as a result, the labeled residues are readily removed by treatment of the thylakoids with trypsin (6). Phosphorylation of thylakoid proteins may cause a reorganization of the membrane constituents, with the phosphorylated proteins migrating to regions of greater negative surface charge density (4) and has been shown to regulate the distribution of a Mᵣ = 12,000 protein between the thylakoid surface and the stromal phase (8). Formation of such regions could tend to attract positively charged proteins such as the protein phosphatases, thereby modulating the rate at which the protein phosphorylation is reversed. Examination of such a phenomenon will have to await a better characterization of the thylakoid protein phosphatase(s).

**LITERATURE CITED**

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