Plant Physiol. (1992) 99, 1029–1036
0032-0889/92/99/1029/08/$01.00/0

Received for publication August 26, 1991
Accepted January 31, 1992

Isolation and Characterization of an Alkaline Phosphatase from Pea Thylakoids[1]

Jan Kieleczawa, Sean J. Coughlan, and Geoffrey Hind*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT

Endogenous dephosphorylation of the light-harvesting chlorophyll-protein complex of photosystem II in pea (*Pisum sativum*, L. cv Progress 9) thylakoids drives the state 2 to state 1 transition; the responsible enzyme is a thylakoid-bound, fluoride-sensitive phosphatase with a pH optimum of 8.0 (Bennett J [1980] Eur J Biochem 104: 85–89). An enzyme with these characteristics was isolated from well-washed thylakoids. Its molecular mass was estimated at 51.5 kD, and this monomer was catalytically active, although the activity was labile. The active site could be labeled with orthophosphate at pH 5.0. High levels of alkaline phosphatase activity were obtained with the assay substrate, 4-methylumbelliferyl phosphate (350 micromoles per minute per milligram purified enzyme). The isolated enzyme functioned as a phosphoprotein phosphatase toward phosphorylated histone III-S and phosphorylated, photosystem II-enriched particles from pea, with typical activities in the range of 200 to 600 picomoles per minute per milligram enzyme. These activities all had a pH optimum of 8.0 and were fluoride sensitive. The enzyme required magnesium ion for maximal activity but was not dependent on this ion. Evidence supporting a putative function for this phosphatase in dephosphorylation of thylakoid proteins came from the inhibition of this process by a polyclonal antibody preparation raised against the partially purified enzyme.

The efficient utilization of absorbed light quanta in steady-state photosynthesis involves a mechanism for sensing the relative excitation rates of the two photosystems and optimally adjusting their electron throughputs. First recognized by Myers (25) as the phenomenon of state transition, this mechanism is now being examined at the molecular level (see ref. 5 for review) and is known to involve the reversible phosphorylation of a small fraction of the LHC-II[2] pool and of several other proteins associated with PSII. The responsible kinase is bound to the thylakoid and phosphorylates stromally exposed portions of its substrate polypeptides. Some properties of the protein kinase and phosphatase involved in this regulatory mechanism have been studied in thylakoid preparations, but a better understanding of the system has awaited the resolution and *in vitro* characterization of its component enzymes. The protein kinase from spinach thylakoids has been purified and characterized (11). Although several acid phosphatases have been isolated from thylakoid preparations (28, 30, 33), their activity is predominantly toward soluble phosphate esters rather than phosphoproteins. Furthermore, the stroma ranges from about pH 7.0 to 8.0 in the dark and light, respectively (32), and LHC-II dephosphorylation *in situ* has an optimum pH of 8.0 (4, 23). Thus, catalysis of dephosphorylation by a phosphatase with an alkaline pH optimum may be presumed.

An enrichment strategy based upon maximizing the ratio of alkaline to acid phosphatase activity in thylakoid subfractions led to the successful purification of an alkaline phosphatase whose isolation and preliminary enzymological properties are described below.

MATERIALS AND METHODS

Materials

Pea plants (*Pisum sativum* L. cv Progress 9) were grown under fluorescent (5160 W) and incandescent (3000 W) lights on a 12-h (27°C) light/12-h (24°C) dark cycle. Freshly harvested 2- to 3-week-old leaves were used for the isolation of the phosphatases. 4-MUP, 4-methylumbelliferone, sodium cholate, PMSF, and purified III-S histone were obtained from Sigma. Carrier-free [γ-^{32}P]ATP and [^{32}P]Pi were from New England Nuclear. MEGA-9 was purchased from Fluka and DEAE-cellulose (DE52) was from Whatman. Sephadex G-75, phenyl-Sepharose, Q-Sepharose, and heparin-agarose were from Pharmacia. Regular and prestained molecular mass standards were from Bio-Rad. All other chemicals were of the highest available purity.

Electrophoresis

SDS-PAGE was done according to the procedure of Laemmli (20), using 15% polyacrylamide. Apparent molecular masses were determined with reference to the migration of standard proteins, as described by Weber and Osborn (31). Semidenaturing electrophoresis was carried out on 15% gels (20), with an SDS to protein ratio of 5.0 in the samples, which were not heated, and only 0.05% SDS in the gel as poured. Fixed gels were stained with Coomassie brilliant blue or silver (7). Where indicated, radiolabeled proteins were

[1] Supported by grant 89–372612–4544 from the Competitive Research Grants Office (Photosynthesis Program) of the U.S. Department of Agriculture. This work was performed at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy, with additional support from its Division of Energy Biosciences, Department of Basic Energy Sciences.

[2] Abbreviations: LHC-II, light-harvesting Chl *a*/*b* protein complex of PSII; AS, ammonium sulfate; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonic acid; MEGA-9, *N*-nonanoyl-*N*-methylglucamine; 4-MUP, 4-methylumbelliferyl phosphate; pNPP, *p*-nitrophenyl phosphate; TN, Tricine-NaOH, pH 8.0.

detected by drying the gel and marking the positions of prestained protein standards with radioactive ink; the gel was then autoradiographed for 5 d at −70°C using DuPont Cronex x-ray film and an intensifying screen.

Protein and Chl Assay

Membrane protein concentrations were determined by the method of Fanger (15). Chl concentrations were determined according to the method of Arnon (2).

Enzyme Activity Assay

Alkaline phosphatase activity with 4-MUP as substrate was determined by a modification of the method of Brunette *et al.* (8). Aliquots of 0.4 mL were preincubated for 5 min at 25°C in 20 mM TN buffer supplemented with 10 mM $MgCl_2$ and 2 mM CHAPS; the reaction was started by adding 0.1 mL 20 mM 4-MUP (0.5 mL, final volume) and terminated after 30 min by adding 3.5 mL 10 mM NaF in 0.25 M Tris (pH 10.0). Samples were immediately assayed for 4-methylumbelliferone in an Aminco SPF-125 spectrofluorometer, with excitation and detection wavelengths of 360 and 445 nm, respectively. Concentrations of dephosphorylated substrate were deduced by comparison of fluorescence intensities with those of a dilution series from a standard solution of 4-methylumbelliferone. Acid phosphatase activity was determined by replacing TN buffer in the incubation medium with 20 mM sodium acetate, pH 5.0. Although the detergent concentrations used did not interfere with the assay, Chl quenched the fluorescence strongly (30% quenching at 5 μg Chl/mL); hence, its concentration was kept below 0.5 μg/mL in the reaction mixture and correction for its effect was made. For comparison of 4-MUP with other substrates, alkaline phosphatase activity was determined at a standard substrate concentration of 1 mM, using 0.25 μg enzyme/mL; liberated Pi was assayed by the malachite green procedure (3). Kinetic data were obtained from triplicate assays of three individual enzyme isolates and represent combined means where indicated. Dephosphorylation rates were linear up to 1.4 μg alkaline phosphatase/mL standard assay medium.

Active Site Labeling and Semidenaturing SDS-PAGE

This followed the procedure of Engström (14). Two aliquots of enzyme (an amount that can release 82 nmol Pi/min at 25°C, pH 8.0, with 4-MUP as substrate) were incubated in 0.1 M potassium Tricine buffer, pH 8.0, or 0.1 M sodium acetate buffer, pH 5.0. Both mixtures (0.4 mL total volume) contained 10 mM $MgCl_2$, 0.5 μM K_2HPO_4, and approximately 15 μCi [^{32}P]Pi. After the mixtures had been on ice for 30 min and occasionally stirred, 30 μg of Polybrene was added, and protein was precipitated with 1 mL cold acetone. The pellets were washed with 1 mL acidic acetone (8 volumes acetone/2 volumes 0.5 N HCl). Finally, pellets were washed with 1 mL cold ethanol:ethyl ether (1:1). Samples were dissolved in 50 μL Laemmli SDS-PAGE sample buffer, sonicated for 1 min, and subjected to semidenaturing electrophoresis.

Activity Staining in Gels

An aliquot of the purified enzyme that can dephosphorylate approximately 0.4 μmol 4-MUP/min at 25°C and pH 8.0 was electrophoresed at 10°C for 3 h under semidenaturing conditions. Enzyme activity was renaturated at 25°C in 0.1 M TN buffer, 10 mM $MgCl_2$, 2.5 mM $CoCl_2$ (three incubations of 30 min in 250 mL, each change). The gel was soaked in 2 mM 4-MUP in the above described buffer, and fluorescence was observed on a long-wave UV illuminator. Optimum fluorescence was reached within 15 min; diffusion led to band broadening at longer times.

Preparation of ^{32}P-Labeled Thylakoids and Histone

Pea thylakoids containing 1 mg Chl were suspended in 2 mL 20 mM TN supplemented with 0.1 M sorbitol, 10 mM $MgCl_2$, and 10 mM NaF. After 10 μL 0.1 M ATP containing 200 μCi [γ-^{32}P]ATP was added, the suspension was placed under white light (50 W/m^2) for 15 min. Phosphorylation was terminated by placing the sample on ice and adding 100 μL 0.2 M EDTA. The membranes were washed twice in suspending medium and finally resuspended in 1 mL 20 mM TN containing 0.1 M sorbitol and 10 mM $MgCl_2$. Phosphorylated, PSII-enriched particles were prepared as described in ref. 6, but the phosphorylation step was included before treatment with Triton X-100. Phosphorylated histone III-S for use as a phosphatase substrate was prepared using the intrinsic kinase activity of the thylakoid membranes. Pea thylakoids (100 μg Chl) were incubated at 0°C with 100 μg histone III-S in 100 μL 20 mM TN containing 10 mM NaF, 0.1 M sorbitol, 0.1 mM ATP, and 5 μCi [γ-^{32}P]ATP. After the mixture was exposed to white light (50 W/m^2) for 15 min at 22°C, phosphorylation was terminated by adding 1 N HCl to a final concentration of 0.2 N. The mixture was kept on ice for 5 min and then centrifuged for 1 min at 12,000*g* in an Eppendorf microfuge. The pellet was washed twice with 100 μL 0.25 N HCl. Supernatant fluids were combined and dialyzed overnight against 20 mM TN.

Kinetics of Dephosphorylation

The dephosphorylation time course and pH dependence were studied in PSII particles (6) from pea that lacked endogenous phosphatase or on histone III-S as follows: 50-μL aliquots (10–15 μg Chl or 10 μg histone III-S) were withdrawn from the reaction mixture and placed in vials containing 2.5 μL 0.2 M K_2HPO_4 and 25 μL 4% SDS (w/v), 25% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue in 20 mM Tris-Cl buffer (pH 8.0). The samples were heated (1 min at 90°C) before electrophoresis. Developed gels were dried and autoradiographed as described above. For quantitation, relevant radioactive bands were excised from the dried gel, placed into scintillation vial inserts, supplemented with 2 mL Aquasol scintillation fluid, and counted in a Beckman LS-7800 scintillation counter (open window setting).

Preparation of Washed Thylakoid Membranes

Dark-adapted pea leaflets (300 g fresh weight) were washed in cold distilled water and blotted dry. All subsequent

steps in membrane preparation and enzyme purification were conducted at 0 to 4°C in buffers containing 0.2 mM PMSF and 2 mM ϵ-aminocaproic acid. Leaflets were ground for 10 s at medium speed in a 1-gallon Waring blender with 1 L 0.33 M sorbitol, 5 mM $MgCl_2$, 20 mM KCl, and 2 mM sodium ascorbate in 20 mM TN. The homogenate was filtered through four and then eight layers of muslin and centrifuged for 10 min at 3000*g*. The crude chloroplast pellet was resuspended in grinding medium without ascorbate and centrifuged as before. Thylakoids were obtained by washing twice with 250 mL 10 mM NaCl in 10 mM TN followed by centrifugation (12,000*g*, 10 min). The membranes were resuspended in 1 mM EDTA in 10 mM TN at <0.5 mg Chl/mL, stirred gently on ice for 10 min, and recovered by centrifugation as before. They were resuspended in 500 mL 2 M NaBr in 10 mM TN and stirred on ice for 30 min to complete the removal of coupling factor complex and nonspecifically bound proteins. These thylakoids were diluted threefold with 10 mM TN and sedimented at 20,000*g* for 15 min. The pellets were washed in 10 mM NaCl, 10 mM TN and finally resuspended in the same buffer at 3 mg Chl/mL.

Isolation of Alkaline Phosphatase

This enzyme was solubilized from washed pea thylakoids by diluting the above described suspension with an equal volume of 30 mM MEGA-9, 25 mM sodium cholate, 20% (saturation) AS, 10 mM TN and gently stirring for 1 h. The 10% AS suspension was centrifuged for 1 h at 105,000*g*. Solid AS was added to bring the decanted supernatant fraction to 50% saturation. After 20 min, the resulting AS precipitate was collected by centrifugation for 10 min at 12,000*g*. The precipitate from 95% saturation with AS was obtained in a similar fashion. Both precipitates were separately redissolved in a minimum volume of 10 mM CHAPS, 10 mM TN and dialyzed overnight against this same medium.

Almost 70% of the alkaline phosphatase activity was in the 10 to 50% (saturation) AS-precipitated fraction. This was mixed with DE52 cellulose preequilibrated in 10 mM TN at a ratio of 1 mL wet bed volume/5 mL dialyzed 10 to 50% AS fraction. The mixture was gently stirred on ice for 15 min and then poured into a column (3 × 10 cm) overlying a thin layer of Sephadex G-25 (coarse) on a glass frit. After the green flow-through material (approximately 10% of the applied Chl remains bound and is not subsequently eluted) was collected, the column was washed with 10 mM and then 100 mM NaCl in 10 mM TN until no protein was detected by the Fanger method (15). Alkaline phosphatase activity was eluted batchwise with 300 mM NaCl in 10 mM TN (approximately 100 mL), at a flow rate of 1 mL/min. If a concentration step was needed, this eluate was adsorbed onto a 1- × 3-cm column of Q-Sepharose, which was preequilibrated with 300 mM NaCl in 10 mM TN. The enzyme was eluted in approximately 10 mL 0.7 M NaCl in 10 mM TN and concentrated in a Centriprep-30 (Amicon) to 1 mL with simultaneous exchange to 20 mM TN, 20 mM NaCl, 2 mM CHAPS. Further purification was performed on a Sephadex G-75 (fine) molecular sieve column (2 × 30 cm) equilibrated with this same buffer. Fractions of 1 mL were collected at a flow rate of 0.5 mL/min; 50- and 5-μL aliquots were withdrawn for electrophoresis and enzyme assays, respectively. To improve resolution, active fractions were concentrated and rechromatographed on a second Sephadex G-75 column (2 × 45 cm) as before. Active fractions were made 200 mM in NaCl and loaded on a phenyl-Sepharose column (1 × 4 cm) equilibrated with 200 mM NaCl in 10 mM TN. This column was washed with 5 volumes of equilibration buffer and developed in a simultaneously decreasing (200–0 mM) NaCl gradient and increasing (0–80%, v/v) ethylene glycol gradient. Fractions of 0.8 mL were collected every 2.5 min. Those containing the peak of enzyme activity (approximately 50 mM NaCl, 55% [v/v] ethylene glycol) were combined and after dialysis were loaded on a heparin-agarose column (1 × 3 cm) equilibrated with 10 mM TN. This column was washed with 10 volumes 25 mM NaCl in the equilibration buffer and developed with a linear NaCl gradient (25–200 mM) in the same buffer. Fractions (1 mL) were collected every 2 min. Active fractions were concentrated using a Centricon-30. For storage, glycerol was added up to 20% (v/v), and then samples were frozen in liquid N_2 and kept at −70°C.

Estimation of Native Molecular Weight

The first Sephadex G-75 column (2 × 30 cm, see above) was calibrated at a flow rate of 0.5 mL/min using electrophoresis standards in 20 mM TN, 20 mM NaCl, 2 mM CHAPS; protein distribution in the elution profile was analyzed by denaturing SDS-PAGE performed on a 50-μL aliquot of each 1.0-mL sample. The void volume, V_o, was measured with blue dextran. The native M_r was determined by mixing enzyme purified on heparin-agarose with standards and cochromatographing on the calibrated column; standard proteins were assayed as before, and alkaline phosphatase activity was assayed fluorimetrically using 4-MUP as the substrate. M_r was obtained from a plot of log M *versus* $(V_e - V_o)$.

Preparation of Antibody

Antibody was raised in a New Zealand white rabbit. After preimmune blood was withdrawn, partially purified antigen (see "Results") mixed with Freund's complete adjuvant was injected subcutaneously, followed in 4 weeks by a booster of antigen mixed with incomplete Freund's adjuvant. Blood was withdrawn 2 weeks later. Immune and preimmune sera were partially purified by precipitation with 40% (saturation) AS.

RESULTS

Association of Alkaline Phosphatase with Thylakoid Membranes

Endogenous dephosphorylation in pea thylakoids was unaffected by washing in 0.2 M NaCl. Even a wash in 2 M NaBr only slightly decreased the endogenous rate of dephosphorylation of the four major thylakoid phosphoproteins and did not modify the pH dependence of this process (not shown). In contrast, after a 2 M NaBr wash, only about 15% of the original activity toward 4-MUP at pH 5.0 or 8.0 remained associated with the membranes (Table I). This bound activity was persistent, however, and 70 to 80% survived four additional washes with buffered 10 mM NaCl (not shown). Be-

Table I. *Purification Procedure*

Fraction	Volume	Protein	Total[a] Activity	Specific[a'] Activity	Total[b] Activity	Specific[b'] Activity	Yield[a]	Purification[a',c]	pH 8[d]/pH 5
	mL	*mg*					%	*fold*	
Starting chloroplasts	404	2228	9091	4.08	16844	7.56			0.54
Washed thylakoids	175	1367	1463	1.07	2529	1.85			0.58
Detergent extract	375	270	1387	5.14	2316	8.58			0.60
10–50% AS	94.0	163	914	5.61	570	3.49			1.61
Column eluates									
DE-52	88.0	5.2	116	22.3	26.7	5.13	100	1	4.35
Sephadex G-75									
First	5.0	4.4	148	33.7	23.2	5.23	128	1.5	6.44
Second	19.0	1.6	106	66.2	12.4	7.75	91	3	8.54
Phenyl-Sepharose	21.5	0.43	67.8	157.7	3.0	7.00	58	7	22.5
Heparin-agarose	0.5	0.037	13.2	357.8	0.15	4.05	11	16	88.3

[a, b] Measured at pH 8.0 and 5.0, respectively, in μmol of Pi released from 4-MUP per min. [a', b'] Measured at pH 8.0 and 5.0, respectively, in μmol of Pi released from 4-MUP min^{-1} mg^{-1} protein. [c] In early stages of fractionation, acid phosphatases contribute substantially to the rate at pH 8.0; therefore, yield and purification are estimated only after DE52 chromatography. [d] Ratio of total activities.

cause washing in 2 M NaBr seemed to remove adventitious phosphatases and left the membrane-associated activity essentially unchanged, this treatment and additional washes in TN were done before solubilization with detergent (see "Materials and Methods"). Intact chloroplasts obtained from Percoll or sucrose gradients yielded comparable starting material after washing; furthermore, the chloroplast envelope fraction was found to be devoid of phosphatase activity (not shown). Detergent was required (Table I) to remove essentially all phosphatase activity from the thylakoid membrane.

Thermal Stability and Effect of Proteinase Inhibitors

After the alkaline phosphatase activity was removed from the membrane, it was unstable; therefore, we searched for conditions that would favor retention of activity during the purification protocol. The precipitate from 10 to 50% saturation AS (see "Materials and Methods") was redissolved in 10 mM CHAPS, 10 mM TN and dialyzed against 2 mM CHAPS, 10 mM TN. After 8 h at 22 or 4°C, the corresponding losses of alkaline phosphatase activity at pH 8.0 were 30 and 14%. After 24 h under the same conditions, these values became 77 and 67%, respectively. Similar studies were performed with the 300 mM NaCl eluate from the DE52 column: after 8 h of storage at 22 or 4°C, activity losses of 17 or 44% were found, respectively (apparent cold lability). After the preparation was purified on Sephadex G-75 (Table I, two passes), 8 h of storage resulted in about a 10% loss in activity at 22 or 4°C. A comparable loss occurred during a freeze-thaw cycle, regardless of the presence of ethylene glycol (up to 50%) as a cryoprotectant. Highly purified preparations were even more sensitive to freezing and thawing. Proteinase inhibitors were evaluated for their ability to preserve activity in the 10 to 50% AS fraction. No protection was observed during storage for 8 h at 4°C with PMSF (1 mM), ε-aminocaproic acid (5 mM), *N*-[*N*-(L-3 *trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (750 μg/mL), or a mixture of these. At room temperature, PMSF preserved the activity for 8 h without loss; there was 10 to 20% loss of activity in the presence of *N*-[*N*-(L-3 *trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine or ε-aminocaproic acid under the same conditions.

Enzyme Extraction and Purification

MEGA-9/sodium cholate/AS treatment solubilizes almost all the acid and alkaline phosphatases as judged from the almost unchanged activity ratio and >90% overall recovery (Table I). Partial separation of acid and alkaline phosphatase activities was achieved by AS fractionation. About 65% of the alkaline and about 25% of the acid activities are recovered in the precipitate from 50% saturated AS. Most of the remaining acid phosphatase activity is in the >50% AS fraction. The 10 to 50% AS precipitate was redissolved, dialyzed, and further purified by adsorption on DE52 cellulose followed by selective elution, yielding about a fourfold increase in specific activity at pH 8.0. Size-exclusion chromatography on Sephadex G-75 further separated the alkaline and acid phosphatase activities. Most of the acid phosphatase eluted close to the void volume (Fig. 1), along with some alkaline phosphatase activity. The retarded fraction carried alkaline phosphatase activity (slightly exceeding that applied to the column) and was used for further purification. Complete separation of the acid from alkaline phosphatase activities can be achieved by using a longer (2 × 100 cm) column or a second pass through a 2- × 45-cm column (Table I) and similar separation conditions. Subsequent fractionation on phenyl-Sepharose yielded a product displaying three to four major bands on a silver-stained SDS gel (Fig. 2B) and having more than twice the specific alkaline phosphatase activity of the final Sephadex G-75 product. There was no significant acid phosphatase activity at any point in the elution profile from phenyl-Sepharose (Fig. 2A). Final purification was achieved by chromatography on heparin-agarose. After the most active fractions were concentrated, a single band at 51.5 kD was seen by electrophoresis and silver staining (Fig. 3A). Positive identification of this band as an alkaline phosphatase came from its *in situ* activity staining with 4-MUP (Fig. 3B) and from active site-specific labeling with ^{32}Pi at pH 5.0 but not at pH 8.0 (Fig. 3C). (The use here of pH 5.0 and 8.0 to,

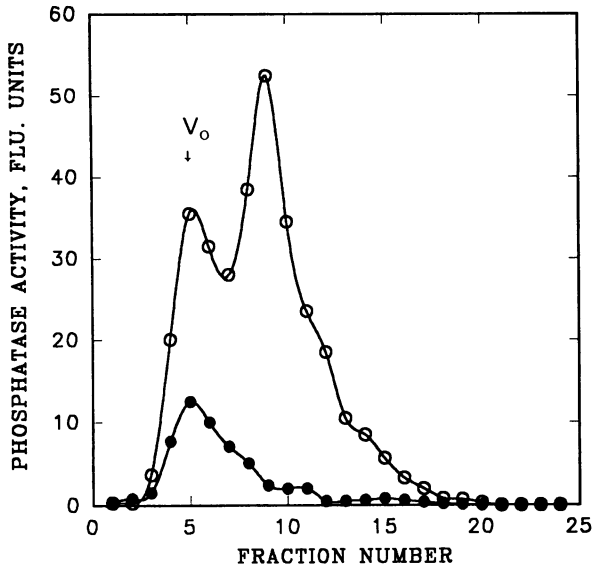

Figure 1. Alkaline (O, pH 8.0) and acid (●, pH 5.0) phosphatase activity profiles of fractions from chromatography on the first Sephadex G-75 column (2 × 30 cm). A total of 2.35 mg protein was loaded in the experiment shown. Activity was determined in 5-μL aliquots of column fractions. One fluorescence (Flu.) unit corresponds to the dephosphorylation of 0.37 nmol 4-MUP/min at 25°C.

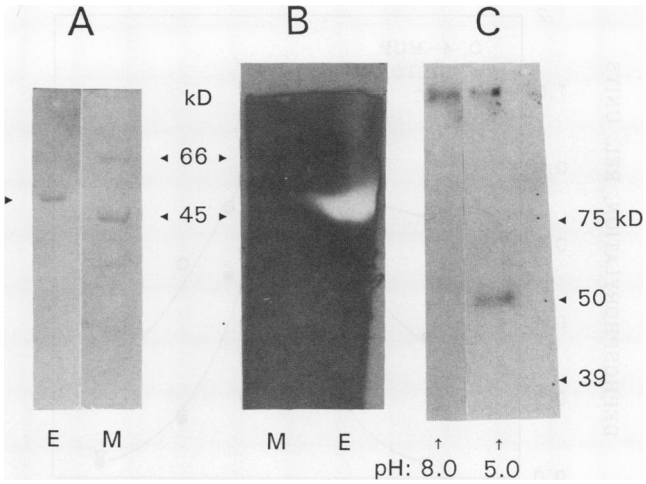

Figure 3. Identification of purified pea thylakoid phosphatase following SDS-PAGE. A, Silver stain; lane E, enzyme (concentrated fractions after heparin-agarose chromatography, 70 ng protein loaded); lane M, molecular mass markers as in Figure 2B. B, Activity staining (4-MUP fluorescence) in the gel; lanes E and M, as in A but 0.3 μg protein loaded in E. C, Self-labeling of the active site with ^{32}Pi at pH 5.0 and 8.0 (0.8 μg protein loaded). Dots are radioactive ink on Bio-Rad prestained molecular mass markers (BSA, 75 kD), hen ovalbumin (50 kD), carbonic anhydrase (39 kD). For other details see "Materials and Methods."

respectively, stabilize and destabilize the characteristic enzyme-phosphate adduct [10] of an alkaline phosphatase should not be confused with use of these pH values to differentiate between catalysis by an acid or alkaline phosphatase.)

Kinetic Properties of Alkaline Phosphatase

The dephosphorylation of 4-MUP, histone III-S, and *in membrano* LHC-II by thylakoid alkaline phosphatase had a pH optimum of 8.0 (Fig. 4). The pH profiles with artificial substrates were reproducibly sharp, possibly indicative of substrate ionizations. Magnesium ion is required for maximal enzyme activity, with 10 mM Mg^{2+} increasing activity two- to threefold, whereas 10 mM Ca^{2+} or 20 mM NaCl increase activity by 1.5-fold. Almost a 20-fold enhancement of alkaline phosphatase activity was observed in the presence of 5 mM $CoCl_2$. Corresponding enhancement effects of these ions on the *in situ* phosphatase activity of isolated pea thylakoids were sixfold (1 mM $MgCl_2$) and threefold (5 mM $CoCl_2$). At concentrations of 0.5 to 2.5 mM and in the absence of Mg^{2+},

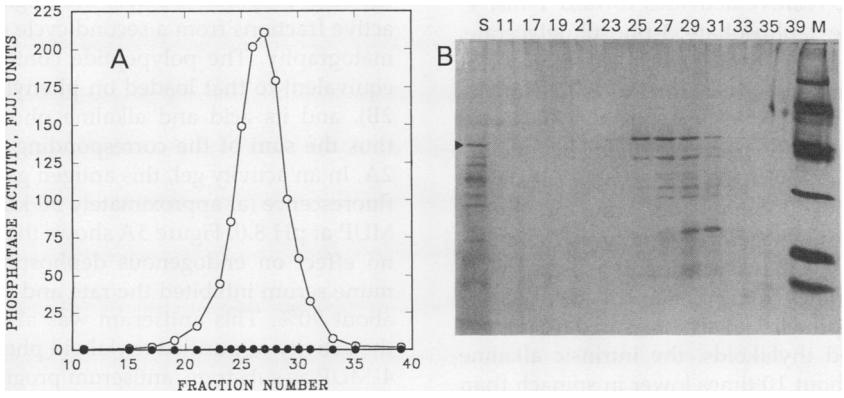

Figure 2. A, Phosphatase activity profiles at pH 8.0 (O) and pH 5.0 (●) in fractions from phenyl-Sepharose chromatography. The column was loaded with 1.05 mg protein (concentrated active fractions from the second G-75 column [2 × 45 cm]); enzyme assays were performed on 20-μL fractions. B, Analysis by SDS-PAGE of aliquots (50 μL) from phenyl-Sepharose chromatographic fractions corresponding to A. Lane S, material loaded; lanes 11–39, selected fractions. The molecular mass markers (lane M) were: rabbit muscle phosphorylase b (97.4 kD), BSA (66.2 kD), hen egg-white albumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and hen egg-white lysozyme (14.4 kD). The gel was stained with silver (7).

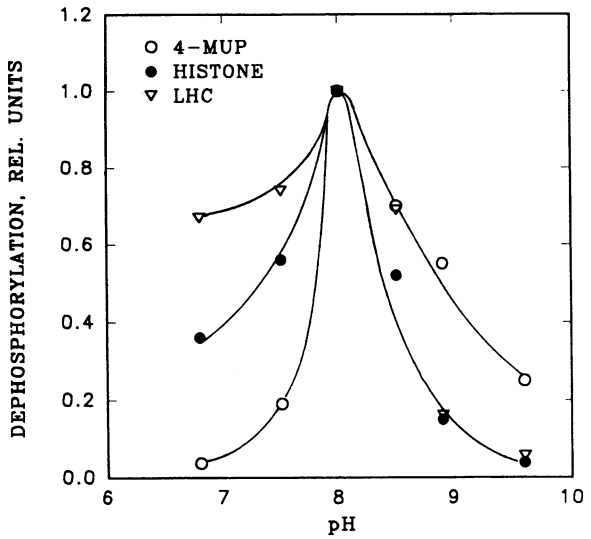

Figure 4. pH profiles for dephosphorylation of 4-MUP, histone III-S, and LHC-II by purified alkaline phosphatase. For comparison, activities were normalized to 1.0 at the optimum (pH 8.0). The specific activities at this pH were 354 μmol Pi·min^{-1}·mg protein^{-1} (4-MUP), 568 pmol Pi·min^{-1}·mg protein^{-1} (histone), and 210 pmol Pi·min^{-1}·mg protein^{-1} (LHC-II). LHC-II dephosphorylation was measured using PSII-enriched particles (6) from pea as substrate, as described in "Materials and Methods."

zinc ion increased activity of the isolated enzyme by about 72%, although higher concentrations were inhibitory (80% inhibition at 10 mM Zn^{2+}). In the presence of 10 mM $MgCl_2$, zinc was inhibitory at all concentrations (up to 92% at 10 mM).

Purified pea thylakoid phosphatase utilizes 4-MUP as a substrate, with measured specific activity up to about 350 μmol Pi·min^{-1}·mg protein^{-1} and with an apparent K_m value of 1.3 mM. This is well within the range of activities documented for other alkaline phosphatases hydrolyzing pNPP under similar conditions (10). Other nonphosphoprotein substrates were utilized; the relative activities toward 1 mM 4-MUP, pNPP, PPi, glucose-1-phosphate, 3-phosphoglycerate, and β-glycerophosphate were, respectively: 1.0, 0.55, 0.49, 0.37, 0.32, and 0.16 (the value given for PPi is half of the actual Pi released).

Relative to 4-MUP and pNPP, phosphorylated histone III-S and PSII-enriched particles (6) from pea are poor substrates (specific activities of 200–600 pmol·min^{-1}·mg protein^{-1}). When partially purified enzyme (corresponding to the material shown in lane S of Fig. 2B) was used to dephosphorylate stacked thylakoid membranes from spinach, an activity of 25 to 50 pmol·min^{-1}·mg protein^{-1} was measured (data not shown; in unfractionated thylakoids, the intrinsic alkaline phosphatase activity is about 10 times lower in spinach than in pea and was almost completely inhibited for this study by heating the thylakoids for 5 min at 55°C). With 4-MUP, pNPP, or histone III-S as a substrate, the isolated alkaline phosphatase did not observe simple Michaelis-Menten kinetics (not shown). This is probably not due to aggregation of the enzyme because a similar sigmoidal substrate saturation profile was obtained in the presence of 5 mM CHAPS (for which we find a critical micellar concentration of 4.5 mM in 20 mM TN containing 10 mM $MgCl_2$). Similar data are obtained if Mg^{2+} is omitted from the assay medium or if the assay is carried out in 0.5 M TN (not shown). This kinetic behavior cannot be attributed to a multisubunit structure of the enzyme because its apparent molecular mass estimated both by semidenaturing (Fig. 3, B and C) and denaturating gel electrophoresis (Fig. 3A) was 51.5 kD. Furthermore, the molecular mass determined by gel filtration on Sephadex G-75 (see "Materials and Methods") was 52.5 kD, with an indicated Stokes radius of 30 (this estimate may include one to two bound molecules of CHAPS [mol wt, 615]). Analysis of the substrate saturation profile for 4-MUP by the method of Hill (13) suggests that some cooperativity may occur (Hill coefficient = 1.7).

The enzyme was weakly sensitive to sulfhydryl chemical modifiers, the hydrolysis of 4-MUP being 30 to 40% inhibited by 1 mM *p*-chloromercuribenzoate, iodoacetate, or *N*-ethylmaleimide (not shown). AMP, ADP, and PPi at 1 mM were stronger inhibitors (giving 86, 85, and 69% inhibition, respectively) most likely through competition for the active site. Pi and NaF were competitive inhibitors with K_i values of 0.82 ± 0.06 and 3.66 ± 0.26 mM, respectively, as determined from Dixon and Lineweaver-Burk double-reciprocal (13) plots (not shown). Although the *in membrano* dephosphorylation of thylakoid phosphoproteins was reported to be insensitive to 5 mM Pi (23), we found that Pi was inhibitory if the thylakoids were unstacked (data not shown). The fluoride sensitivity of 4-MUP dephosphorylation by isolated enzyme is compared with that of protein and 4-MUP dephosphorylations by pea thylakoids in Table II. In common with its effect on isolated enzyme, 10 mM NaF gave >90% inhibition of the *in membrano* dephosphorylation of LHC and the 45-kD phosphoprotein. The activities of endogenous phosphatase toward added 4-MUP and the endogenous 32-kD phosphoprotein were relatively less sensitive to fluoride.

Antibody raised to partially purified thylakoid phosphatase was tested for its effect on dephosphorylation *in membrano*. Owing to inadequate recovery of alkaline phosphatase from the final preparative steps, the original antigen consisted of active fractions from a second cycle of Sephadex G-75 chromatography. The polypeptide content of this material was equivalent to that loaded on phenyl-Sepharose (lane S, Fig. 2B), and its acid and alkaline phosphatase activities were thus the sum of the corresponding values shown in Figure 2A. In an activity gel, this antigen gave only a single band of fluorescence (at approximately 50 kD) upon reaction with 4-MUP at pH 8.0. Figure 5A shows that preimmune serum had no effect on endogenous dephosphorylation, whereas immune serum inhibited the rate and extent of the reaction by about 90%. This antiserum was also effective in inhibiting the activity of purified thylakoid phosphatase (Fig. 5B). With 4-MUP as substrate, antiserum progressively inhibited the 4-MUP dephosphorylation rate by >80%, compared to an inhibition of about 15% given by preimmune serum.

DISCUSSION

Two previous attempts (30, 33) were made to relate the activity of a partially purified phosphatase to the endogenous

Table II. *Comparison between the Fluoride Sensitivities of Isolated and Endogenous Alkaline Phosphatases*

The substrate for thylakoid-bound alkaline phosphatase was added 4-MUP (4 mM) or the indicated endogenous thylakoid phosphoprotein previously radiolabeled as described in "Materials and Methods."

Enzyme Source: Substrate:	Isolated	Thylakoid Membranes			
	4-MUP	4-MUP	LHC	32 kD	45 kD
		% activity remaining			
NaF, 1 mM	78	86	53	78	86
NaF, 10 mM	8	40	0	22	8
Control rate[a]	72.0	4.7	36.6	7.3	7.6

[a] Control rates are expressed as μmol min^{-1} mg^{-1} protein (purified, isolated enzyme), μmol min^{-1} mg^{-1} Chl (endogenous activity with 4-MUP), or μmol min^{-1} mg^{-1} Chl (endogenous dephosphorylation of LHC, 32- or 45-kD phosphoproteins). The initial radiolabel content of the LHC, 32-kD, 45-kD, and 9-kD membrane components was in the ratio 1.0:0.4:0.4:0.25, respectively. Reliable kinetic data for the 9-kD phosphoprotein could not be obtained in this study, owing to high background radioactivity at the bottom of the gel.

dephosphorylation of thylakoid proteins. A phosphatase in wheat thylakoids, with an optimum at pH 6.75 for histone dephosphorylation, was loosely associated with the membrane and could be released by treatment with 0.2 M NaCl. About 50% of the endogenous dephosphorylation rate persisted after this salt treatment. Restoration of the salt extract increased the rate of dephosphorylation in washed membranes by about 20% (30). In contrast, we conclude that the principal protein phosphatase of pea thylakoids is an integral membrane protein or is tightly bound. The claim (30) that in wheat thylakoids protein phosphatase activity is surface associated could indicate a difference between species, although substantial activity was tightly bound in wheat also, as discussed above.

The low recovery of protein and activity in our purification procedure is mainly due to a significant loss at the DE52 chromatography step. Studies of thermal stability, cryoprotection and different proteinase inhibitors did not suggest how to improve the yield. Comparably low yields of phosphoprotein phosphatases have been documented (26).

Phosphatases are noted for their breadth of substrate specificity, and the division between alkaline and phosphoprotein phosphatases is not distinctly drawn; *e.g.* in addition to using a wide range of phosphoesters as substrates, *Escherichia coli* alkaline phosphatase also dephosphorylates PSII and other phosphoproteins (17, 22). The enzyme isolated here resembles a broad class of Zn-containing alkaline phosphatases isolated from a variety of sources (10, 19), although the presence of zinc in our preparation remains to be established; it has an alkaline pH optimum, it can be radiolabeled with Pi at pH 5.0 (Fig. 3C), and it can utilize sugar phosphates, pNPP, 4-MUP, or PPi as a substrate. Although an alkaline inorganic pyrophosphatase activity is associated with spinach thylakoids (16), this differs from thylakoid alkaline phospha-

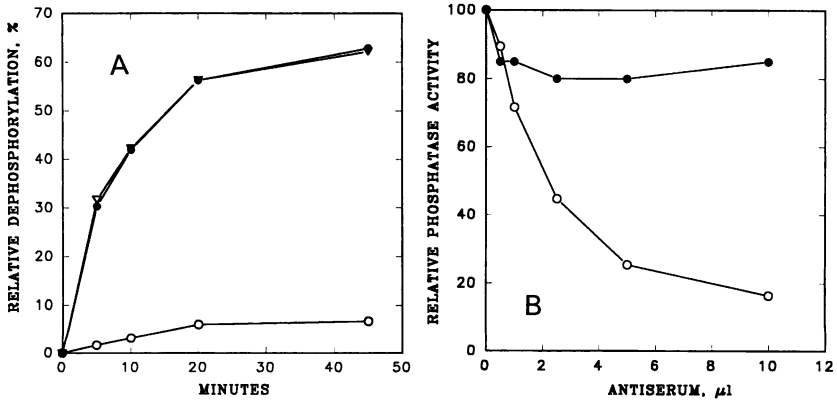

Figure 5. A, Effect of antiserum to partially purified phosphatase (see lane S, Fig. 2B) on the time course of LHC-II dephosphorylation in PSII-enriched particles from pea (6). Partially purified sera were used at 1 μL/μg Chl. Aliquots of 20 μg Chl in 50 μL were withdrawn at the indicated times and analyzed by SDS-PAGE and autoradiography as described in "Materials and Methods." O, with antiserum; ●, with preimmune serum; ∇, with 10 mM TN buffer. Comparable data were obtained for the effects of antiserum on the dephosphorylation of 45- and 32-kD phosphoproteins (not shown). B, Inhibition of 4-MUP dephosphorylation by purified thylakoid phosphatase following preincubation with antiserum. Enzyme (0.7 μg/assay) was preincubated with antiserum (O) for 30 min on ice in 50 μL buffer; activity was measured as described in "Materials and Methods." Samples with preimmune serum (●) and with TN buffer (not shown) were treated identically.

tase in being completely removed by washing the membranes in hypotonic buffer or buffered EDTA. Good utilization of PPi is not unprecedented among phosphatases (24) and is noteworthy here in view of the reported ability of PPi to serve as an alternative phosphate donor in the phosphorylation of thylakoid proteins by the endogenous kinase (27). Phosphoprotein phosphatases also use artificial substrates such as pNPP with differing, but often quite high, efficiencies (1, 12).

The activity we describe here is not inhibited by 10 μM okadaic acid (not shown) in contrast to the cytosolic protein phosphatase that dephosphorylates sucrose-phosphate synthase in spinach leaves (18). This okadaic acid insensitivity, the sensitivity to fluoride, activity in the absence of magnesium ion, and ability to dephosphorylate histone do not allow assignation of thylakoid alkaline phosphatase to an established class of eukaryotic phosphoprotein phosphatase (9, 29). It is noteworthy, however, that mitochondrial and prokaryotic protein phosphatases are recognized as falling outside this classification (21, 29).

Although our results have identified an enzyme that is appropriately sited and is catalytically able to accomplish the dephosphorylation of thylakoid phosphoproteins, it would be incautious to claim this as the *in vivo* phosphatase involved in state transitions without more detailed study.

LITERATURE CITED

1. **Anthony FA, Merat DL, Cheung WY** (1986) A spectrofluorimetric assay of calmodulin-dependent protein phosphatase using 4-methylumbelliferyl phosphate. Anal Biochem **155:** 103–107
2. **Arnon DI** (1949) Copper enzymes in chloroplasts. Polyphenoloxidases in *Beta vulgaris*. Plant Physiol **24:** 1–15
3. **Baykov AA, Evtushenko OA, Avaeva SM** (1988) A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal Biochem **171:** 266–270
4. **Bennett J** (1980) Chloroplast phosphoproteins. Evidence for a thylakoid-bound phosphoprotein phosphatase. Eur J Biochem **104:** 85–89
5. **Bennett J** (1991) Protein phosphorylation in green plant chloroplasts. Annu Rev Plant Physiol **42:** 281–311
6. **Berthold DA, Babcock GT, Yocum CF** (1981) A highly resolved, oxygen- evolving photosystem II preparation from spinach thylakoid membranes. FEBS Lett **134:** 231–234
7. **Blum H, Beier H, Gross HJ** (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99
8. **Brunette MG, Chan M, Lebrun M** (1981) A microfluorometric method for alkaline phosphatase: application to the various segments of the nephron. Anal Biochem **115:** 236–242
9. **Cohen P** (1989) The structure and regulation of protein phosphatases. Annu Rev Biochem **58:** 453–508
10. **Coleman JE, Gettins P** (1983) Alkaline phosphatase, solution structure, and mechanism. Adv Enzymol **55:** 381–452
11. **Coughlan S, Hind G** (1986) Purification and characterization of a membrane-bound protein kinase from spinach thylakoids. J Biol Chem **261:** 11378–11383
12. **Deguzman A, Lee EYC** (1988) Preparation of low-molecular-weight forms of rabbit muscle protein phosphatase. Methods Enzymol **159:** 356–368
13. **Dixon M, Webb EC** (1979) Enzymes, Ed 3. Longman, London, United Kingdom, pp 332–467
14. **Engström L** (1964) The amino acid sequence around the reactive serine in calf-intestinal alkaline phosphatase. Biochim Biophys Acta **92:** 79–84
15. **Fanger BO** (1987) Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents. Anal Biochem **162:** 11–17
16. **Gould JM, Winget GD** (1973) A membrane-bound alkaline inorganic pyrophosphatase in isolated chloroplasts. Arch Biochem Biophys **154:** 606–613
17. **Harrison MA, Allen JF** (1991) Light-dependent phosphorylation of photosystem II polypeptides maintains electron transport at high light intensity: separation from effects of phosphorylation of LHC-II. Biochim Biophys Acta **1058:** 289–296
18. **Huber SC, Huber JL** (1990) Activation of sucrose-phosphate synthase from darkened spinach leaves by an endogenous protein phosphatase. Arch Biochem Biophys **282:** 421–426
19. **Kim EE, Wyckoff HW** (1991) Reaction mechanisms of alkaline phosphatase based on crystal structures. J Mol Biol **218:** 449–464
20. **Laemmli UK** (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:** 680–685
21. **MacKintosh C, Coggins J, Cohen P** (1991) Plant protein phosphatases. Subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major quinate dehydrogenase phosphatase. Biochem J **273:** 733–738
22. **Mellgren RL, Slaughter GR, Thomas JA** (1977) Dephosphorylation of phosphoproteins by *Escherichia coli* alkaline phosphatase. J Biol Chem **252:** 6082–6089
23. **Michel HP, Shaw EK, Bennett J** (1987) Protein kinase and phosphatase activities in thylakoid membranes. *In* CJ Leaver, H Sze, eds, Plant Membranes: Structure, Function, Biogenesis. Alan R. Liss, New York, pp 85–102
24. **Moss DW, Eaton RH, Smith JK, Whitby LG** (1967) Association of inorganic pyrophosphatase activity with human alkaline-phosphatase preparations. Biochem J **102:** 53–57
25. **Myers J** (1971) Enhancement studies in photosynthesis. Annu Rev Plant Physiol **22:** 289–312
26. **Polya GM, Haritou M** (1988) Purification and characterization of two wheat-embryo protein phosphatases. Biochem J **251:** 357–363
27. **Pramanik AM, Bingsmark S, Lindahl M, Baltscheffsky H, Baltscheffsky M, Andersson B** (1991) Inorganic-pyrophosphate-dependent phosphorylation of spinach thylakoid membranes. Eur J Biochem **198:** 183–186
28. **Rengasamy A, Selvam R, Gnanam A** (1981) Isolation and properties of an acid phosphatase from thylakoid membranes of *Sorghum vulgare*. Arch Biochem Biophys **209:** 230–236
29. **Shenolikar S, Nairn A** (1991) Protein phosphatases: recent progress. Adv Second Messenger Phosphoprotein Res **23:** 1–121
30. **Sun G, Bailey D, Jones MW, Markwell J** (1989) Chloroplast thylakoid protein phosphatase is a membrane surface-associated activity. Plant Physiol **89:** 238–243
31. **Weber K, Osborn M** (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem **244:** 4406–4412
32. **Werdan K, Heldt HW, Milovancev M** (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochim Biophys Acta **396:** 276–292
33. **Yang CM, Danko SJ, Markwell JP** (1987) Thylakoid acid phosphatase and protein phosphatase activities in wheat (*Triticum aestivum* L). Plant Sci **78:** 17–22