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Purification and Characterization of a Chloroplast Outer-Envelope-Bound, ATP-Dependent Protein Kinase[1]

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ABSTRACT

An ATP-dependent protein kinase was partially purified from isolated outer envelope membranes of pea (*Pisum sativum* L., Progress No. 9) chloroplasts. The purified kinase had a molecular weight of 70 kilodaltons, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was of the cyclic nucleotide and Ca^{2+}, calmodulin-independent type. The purification involved the detergent solubilization of purified outer envelopes by 0.5% cholate and 1% octylglycoside, followed by centrifugation on a linear 6 to 25% sucrose gradient. Active enzyme fractions were further purified by affinity chromatography on histone III-S Sepharose 4B and ion exchange chromatography on diethylaminoethyl cellulose. The protein kinase eluted at 100 millimolar and 50 millimolar NaCl, respectively. The protein kinase was essentially pure as judged by Western blot analysis. The enzyme has a K_M of 450 micromolar for ATP and a V_{max} of 25 picomoles of ^{32}P incorporated into histone III-S per minute per microgram. Inhibition by ADP is competitive (K_i 150 micromolar).

Covalent modification of proteins is a major and potent possibility to regulate their biochemical properties. The phosphorylation or dephosphorylation of proteins is one way to accomplish changes in enzyme activities or to transmit certain signals in inter- and intracellular communication (10). In recent years, more knowledge has accumulated on the existence of protein kinases in different subcellular locations in plants (24 and references therein). Each chloroplast compartment—namely, the outer envelope (27), the inner envelope (26), the soluble protein (14) and the thylakoid membranes (1, 8, 21)—contains its own set of protein kinases. Relatively little is known about the function of these protein kinases with the exception of that involved in the redox-regulated light-harvesting Chl-protein complex II phosphorylation (1, 8).

The plastid envelope is a conservatively retained structure through all developmental stages in plastid differentiation. The biochemical function of these membranes involves the control of metabolite flux in and out of the organelle and biosynthesis of galactolipids, prenyllipids, and isoprenoids (9), functions which are essential for plastid differentiation and throughout the life cycle of the organelle. These processes have to be under stringent control to be adjusted to the physiological needs and could be a target for regulation by protein phosphorylation-dephosphorylation mechanisms. The complex system of organelle to cell or organelle to nucleus interaction, which is necessary for chloroplast development and organelle function, also requires regulatory control mechanisms at different levels (23). This signal transduction or regulation can be accomplished by protein phosphorylation and protein dephosphorylation. A possible localization of such a regulatory system would be the outer chloroplast envelope, which forms the boundary and contact between the cytoplasm and the organelle. Earlier studies have concentrated on the *in situ* characterization of the outer envelope membrane-bound, ATP-dependent protein kinase (26, 27). In addition, to date no proteins have been purified in an active state from this membrane. In this report, I describe the partial purification and initial characterization of an ATP-dependent protein kinase from isolated outer envelope membranes of pea chloroplasts.

MATERIALS AND METHODS

Pea plants (*Pisum sativum* L., Progress No. 9) were grown in a growth chamber under a 12-h light-dark regime and 20°C constant temperature. Calmodulin antagonist W-5[2] and protein kinase inhibitor H-9 were from Seikagaku America, Inc., St. Petersburg, FL. Proteinaceous protein kinase inhibitor from bovine heart was from Sigma, St. Louis, MO. Cyanogen bromide-activated Sepharose 4B was from Pharmacia. [γ-^{32}P]ATP (110 TBq/mmol) and [γ-^{32}P]GTP (370 GBq/mmol) were from Amersham. GTP was used as lithium salt and stored at −80°C. DEAE-cellulose preswollen powder was from Whatman. All other chemicals were of reagent grade and were used without further purification.

Isolation of Purified Outer Chloroplast Envelope. Eight hundred to 1000 g of leaves were harvested from 14-day-old pea plants. Chloroplasts were isolated in low ionic strength buffer as described previously (26). The crude chloroplast fraction was purified on a linear silica sol gradient (Percoll) (18). Purified chloroplasts were resuspended for 10 min in hypertonic sucrose solution and ruptured by a Dounce homogenizer (18). Most of the thylakoids were pelleted by a low speed centrifugation (5000*g*, 15 min), while envelope membranes remained in the supernatant. Inner and outer envelope membranes were separated on a sucrose step gradient (0.996 M, 0.8 M, 0.465 M sucrose, 10 mM Tricine-KOH, pH 7.9, 250,000*g*, 3 h) (18). Purified outer envelope membranes were recovered from the 0.465 M to 0.8 M sucrose interface, pelleted, and stored in aliquots at −80°C. Total yield of outer membrane was about 2 mg of protein.

Sucrose Density Gradient Centrifugation. About 500 μg of outer envelope protein were solubilized with 0.5% cholate, 1% octylglycoside, 25 mM Tricine-KOH, pH 7.2, in a final volume of 200 μl for 30 min at 4°C (21). The mixture was then centrifuged for 30 min at 50,000*g*. The supernatant was layered on top of a 5-ml, 6 to 25% sucrose gradient (10 mM Tricine-KOH

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[2] Abbreviatons: oATP, adenosine 2′,3′-dialdehyde 5′-triphosphate; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; W-5, *N*-(6-aminohexyl)-1-naphtalene sulfonamide hydrochloride; H-9, *N*-(2-aminoethyl)-5-isoquinoline sulfonamide dihydrochloride.

[pH 7.2], 0.1% Triton X-100, 0.1% asolectin). The gradient was centrifuged at 270,000*g* for 12 to 15 h. Gradients were fractionated manually from the top into 200-μl fractions. Fractions containing the highest protein kinase activity were pooled and used for further purification.

Histone III-S Sepharose Column. Histone III-S was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's recommendation. Fresh column material was used for each purification. Histone-Sepharose in the amount of 0.5 ml equilibrated with 10 mM Tricine, pH 7.9, 0.1% Triton X-100 (buffer A) was incubated on ice for 10 min with the pooled fractions of the sucrose density gradient. The material was then transferred into a Pasteur pipette, and proteins were eluted by an NaCl step gradient in buffer A. The column was first washed twice with 1 ml of buffer A and then with 1 ml of 25 mM NaCl, 0.5 ml of 50 mM NaCl, 1.5 ml of 100 mM NaCl, and finally 0.5 ml of 250 mM NaCl in buffer A. All fractions were desalted by Sephadex G-25 filtration prior to further use.

DEAE Column. The 100 mM NaCl eluate of the histone-Sepharose column was bound to 0.5 ml of DEAE-cellulose previously equilibrated with buffer A, for 10 min at 4°C. The column material was treated as above and was washed twice with 1 ml of buffer A, 1 ml of 25 mM NaCl, 1.5 ml of 50 mM NaCl, 0.5 ml of 100 mM NaCl, and finally with 0.5 ml of 250 mM NaCl in buffer A. All fractions were immediately desalted by Sephadex G-25 filtration. Active enzyme preparations were stored at −20°C in 50% glycerol.

Protein Kinase Assay. Protein kinase activity was measured in the presence of histone III-S (1 mg/ml) and, if not otherwise defined, 4 mM $MgCl_2$, 25 mM Tricine-KOH, (pH 7.9), 8 μM ATP, and 15 μl of active kinase fraction in 50% glycerol at 20°C for 30 min in a final volume of 25 μl. The reaction was stopped by the addition of 25 μl of 2-fold concentrated solubilization buffer (20). Products were analyzed by SDS-PAGE (20) using a 10% separating gel. Gels were stained by Coomassie brilliant blue and destained in the presence of 20 mM KH_2PO_4, and the dried gel was autoradiographed (Kodak X-AR5) overnight at −80°C using an intensifying screen. ^{32}P incorporation was quantified by excising the gel slices off the gel, rehydrating with 0.5 ml of H_2O, and subsequent liquid scintillation counting.

Miscellaneous. Protein was estimated by bicinchoninic-protein reagent from Pierce Chemicals (Rockford, IL) using BSA as standard. Silver staining was done as described previously (2). Western blot analysis was carried out as described previously (3) using goat anti-rabbit alkaline phosphatase conjugate. Phosphorylated amino acids were determined as described by Hunter and Senfton (17).

RESULTS

Each chloroplast subfraction has its own protein kinase activities (24); however, the outer chloroplast envelope represents less than 0.5% of the total chloroplast proteins (9). From this, it was obvious to start with the kinase purification from isolated outer membranes to avoid cross-contaminations from other chloroplast subfractions. The outer membrane fraction used was devoid of thylakoids as determined by the absence of Chl (not shown). The cross-contamination of the outer membrane by inner envelope membrane is generally around <5% (9, 18). SDS-PAGE analysis of the isolated envelope fractions showed that the purity of the preparations used was within this range (see below). Different membrane solubilization protocols were examined; finally the solubilization of chloroplast outer envelope was done at 0.5% sodium cholate (w/v) and 1% octylglycoside (w/v) at a protein concentration of 2.5 mg/ml (Fig. 1). After solubilization of 500 μg of outer envelope membrane protein, the second purification step was centrifugation on a linear sucrose density gradient. The main protein kinase activity was well separated

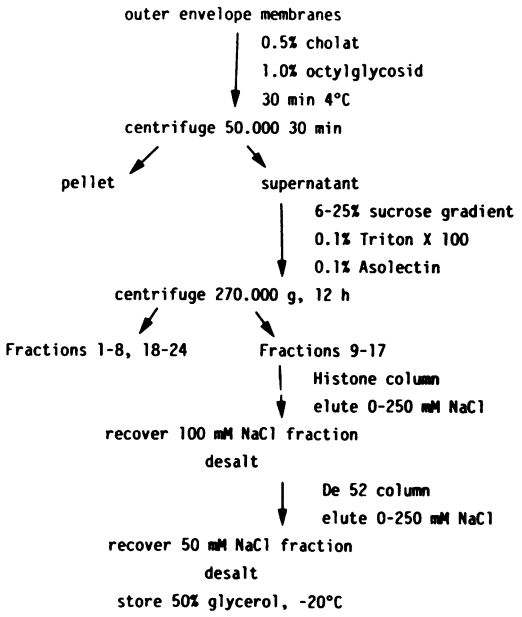

FIG. 1. Purification scheme of outer chloroplast envelope-bound, ATP-dependent protein kinase.

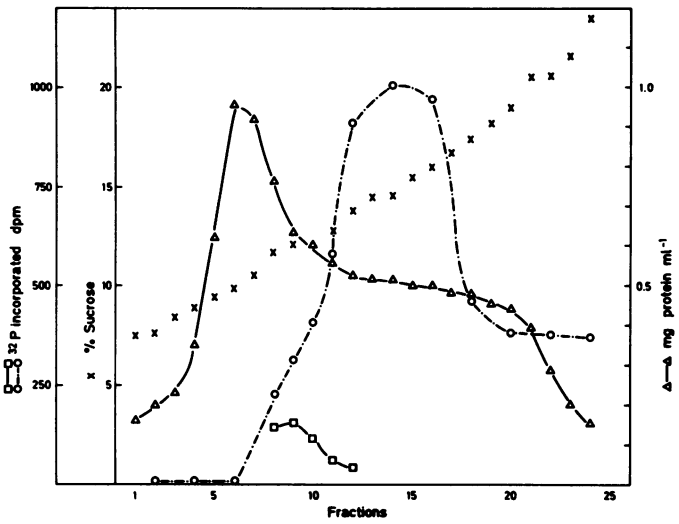

FIG. 2. Purification of the protein kinase on a linear sucrose gradient. Solubilized outer envelopes were centrifuged for 12 h on a linear 6 to 25% sucrose gradient (×). Protein kinase activity was measured in the presence of histone III-S (○-·-○); □ represents the ^{32}P incorporation into a 64 kD endogenous phosphoprotein; △, protein content. Fifteen microliters of each fraction were used in the standard phosphorylation assay.

from the main protein peak (Fig. 2). Only one endogenous protein with an apparent molecular mass of 64 kD was labeled in gradient fractions of lower density, while the histone III-S phosphorylating activity peaked at a higher density (Fig. 2). The nature of this endogenous phosphoprotein was not further investigated.

As a third purification step, I employed affinity chromatography using lysine-rich histone III-S coupled to a Sepharose 4B matrix. The kinase activity bound completely to the matrix. The column was developed by an NaCl step gradient. The kinase activity was eluted by prolonged washing (1.5 ml) at 100 mM NaCl (Fig. 3). The activity was not completely eluted by this treatment: about one-fourth of the activity that eluted at 100 mM salt could still be found in the 250 mM NaCl eluate (0.5 ml) (Fig. 3). Assays for protein kinase activity were done only after desalting of the fractions by molecular sieving. Of each fraction, 20 μl

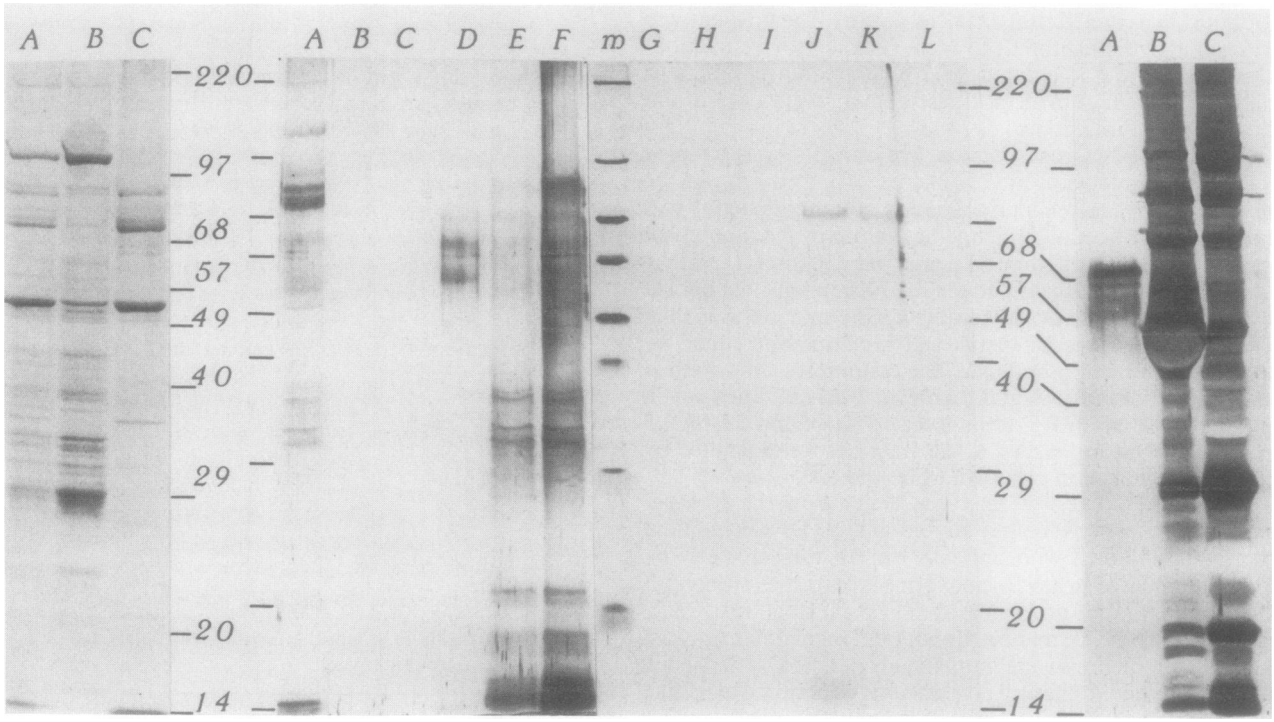

FIG. 3. Isolation of outer envelope-bound protein kinase from pea chloroplasts. The left panel shows a Coomassie brilliant blue stain of an SDS-PAGE; A, mixed envelope membranes; B, purified inner envelope membranes; C, purified outer envelope membranes. The central panel shows a silver-stained SDS-PAGE of fractions obtained from histone III-S Sepharose 4B (lanes A to F) and DEAE anion exchange chromatography (lanes G to L). Lanes A and G, void volume; B and H, wash; C and I, 25 mM NaCl; D and J, 50 mM NaCl; E and K, 100 mM NaCl; F and L, 250 mM NaCl. The 100 mM NaCl eluate of the histone III-S column (lane E) was desalted and applied to a DEAE column. All solutions were in buffer A. The right panel of Figure 3 shows a Western blot analysis, stained with alkaline phosphatase conjugated anti-rabbit immunoglobulin G. The antibody was raised in a rabbit using mixed (outer and inner) envelope membranes. Lane A of the Western blot shows the response of the antibody for the 50 mM NaCl eluate of the DEAE column (equivalent to 400 μl fraction, lane J). Lanes B and C show the antibody response to outer and inner envelope membranes, respectively. M_r standards (m) used were in kilodaltons: lactalbumin, 14; soybean trypsin inhibitor, 20; carbonic anhydrase, 29; aldolase, 40; fumarase, 49; pyruvate kinase, 58; bovine serum albumin, 68; phosphorylase *b*, 97.

were used to determine the enzyme activity. Incorporation rates of ^{32}P from [γ-^{32}P]ATP into histone III-S were (dpm $\times 10^2$): void volume, 9 (450); wash, 2 (100); 25 mM NaCl, 25 (1250); 50 mM NaCl, 25 (625); 100 mM NaCl, 100 (7500); and 250 mM NaCl, 90 (1800) (figures in parentheses indicate total activity present in each fraction). The 250 mM NaCl fraction was discarded for two reasons: (a) it contained a high amount of contamination by other proteins (Fig. 3), and (b) the 64 kD phosphoprotein described earlier eluted at this salt concentration. The protein kinase was very sensitive to higher salt concentrations (see below), so samples were immediately desalted by Sephadex G-25 filtration.

The final step in the purification scheme (Fig. 1) involved a DEAE-anion exchange column. The pooled 100 mM NaCl eluate of the histone III-S Sepharose column was applied to the DEAE column. The main kinase activity was eluted by a prolonged 1.5 ml wash with 50 mM NaCl (Fig. 3). Residual enzyme still eluted at 100 and 250 mM NaCl (0.5 ml). All samples were desalted as above and used for further analysis. Of each fraction, 20 μl were used to locate the kinase activity. Incorporation rates of ^{32}P from [γ-^{32}P]ATP into histone III-S were (dpm $\times 10^2$): void volume, 5 (250); wash, 3 (150); 25 mM NaCl, 6 (300); 50 mM NaCl, 26 (1950); 100 mM NaCl, 13 (325); and 250 mM NaCl, 12 (300) (figures in parentheses indicate total activity present in each fraction). The whole purification procedure was completed within 16 h to minimize loss of enzyme activity. The purified kinase was fairly stable at −20°C in 50% glycerol for 8 to 10 weeks.

All fractions were analyzed by SDS-PAGE followed by silver staining of the gel. The only protein that visibly copurified with the kinase activity on the histone and DEAE column had a molecular weight of 70 kD (Fig. 3). This polypeptide was always detected in all SDS-PAGE analyses run of the column purification steps, while the other bands, probably due to contamination and degradation, changed from purification to purification (not shown). In isolated outer envelope membranes, the 70 kD protein is barely detectable by Coomassie brilliant blue stain of an SDS-PAGE gel or by Western blot analysis (Fig. 3). However, when the 50 mM NaCl eluate of the DEAE column was further analyzed by Western blot using an antibody raised against a mixture of outer and inner envelope membrane (gift of K. Keegstra, University of Wisconsin), the staining resulted also in only one major band, with several in molecular weight smaller but minor contaminations. These bands are probably degradation products of the 70 kD proteins, because 400 μl of active kinase preparation had to be concentrated 20-fold under low vacuum and 60°C for about 2 h prior to electrophoresis to yield enough protein to run a gel. The lower molecular weight bands in the Western blot analysis do not correspond to bands stained in the outer membrane either by Coomassie brilliant blue or by immunological methods (Fig. 3, left and right panels). Neither do they correspond to the low molecular contaminations seen occasionally in the silver stained SDS-PAGE of active kinase preparations (Fig. 3). It was possible to use Western blot analysis to establish the purity of the enriched kinase fraction, because the outer envelope membrane fraction used for the protein kinase purification as

well as the envelope membrane fraction used to raise antibodies were devoid of other chloroplast membranes, *e.g.* thylakoids. Contaminations in the partially purified protein kinase fraction by other envelope membrane proteins should therefore be detectable by this technique. To run SDS-PAGE analyses which were later silver stained, again 400 μl of each fraction had to be concentrated as above prior to electrophoresis (Fig. 3, middle panel). Further attempts to identify the protein kinase by direct photoaffinity labeling using [α-^{32}P]ATP (7) or autophosphorylation using carrier-free [γ-^{32}P]ATP failed.

It was not possible to obtain reliable data for the calculation of the yield or purification factor, since the enzyme was rapidly deactivated by the detergents used and by NaCl (8, see below) used for the elution of the proteins off the column matrix. Kinase activity was only partly restored by desalting the enzyme fractions on G-25 columns. The best possible estimate of the purification factor is given from the SDS-gel and the Western blot analysis of outer envelope membranes, where the 70 kD protein is a very minor component, while in fractions J and K of the DEAE column, this polypeptide was the most abundant protein band (Fig. 3).

Characterization of the Kinase Properties. All assays were done with the purified kinase fraction obtained from the DEAE column by 50 mM salt elution. ^{32}P was incorporated into histone III-S by this kinase-enriched fraction in a time- and temperature-dependent manner. Enzyme activity was linear during the time range tested (3 to 60 min). The enzyme had a temperature optimum between 30 and 37°C, but still had 25% and 10% of its activity at 0°C and −12°C, respectively. Different proteins were tested as acceptor substrates for the ^{32}P-transfer reaction (Table I). Lysine-rich histone III-S turned out to be the best external protein substrate. Peptides that were used as external substrates for a thylakoid-bound kinase (4) were not phosphorylated. Synthetic peptides, analog to the first and second halves of the N-terminal transit sequence of ribulose-bisphosphate carboxylase small subunit precursor protein, were also not accepted as external substrates (Table I). In general, all kinase assays were done at 1 mg/ml of histone III-S for 30 min at 20°C. The purified protein kinase had a K_M of 450 μM for ATP and a V_{max} of 25 pmol μg^{-1} min^{-1} (Fig. 4). The V_{max} deduced from the data in Figure 4 gives the lowest possible ^{32}P-incorporation rate, since an unknown percentage of the enzyme was irreversibly inactivated during the isolation procedure (see above). A pH optimum of 7.2 was determined, with a steep decrease in activity to the acidic pH range but a much slower decrease to more alkaline pH values (Fig. 4, insert). Acid hydrolysis of the phosphorylated histone III-S showed that serine was the only phosphorylated amino acid present. Dixon plot analysis of competitive ADP inhibition revealed a K_i of 155 μM for ADP (Fig. 5), while the effect of PPi was less pronounced, and 50% inhibition was obtained at 1 mM PPi (not shown). AMP (0.1 to 2 mM) was also without influence. Cyclic AMP or cyclic GMP (2 to 40 μM) did not alter the enzyme activity, neither did the cyclic AMP-dependent protein kinase inhibitor H-9 (2 to 20 μM) (16). High concentrations of the proteinaceous, cAMP-dependent protein kinase inhibitor from bovine heart (40 μg/ml) resulted in only a 30% decrease of ^{32}P incorporation (10) (not shown).

Protease treatment of protein kinases can result in an increased enzyme activity due to the removal of the regulatory subunit (10). A similar treatment of the purified kinase by trypsin (40 U/ml) up to 5 min at 4°C prior to the assay did not influence the enzyme activity. Increasing the trypsin concentrations up to 400 U/ml finally resulted only in a decrease of enzyme activity. Trypsin treatment was stopped by the addition of soybean trypsin inhibitor. Soybean trypsin inhibitor does not influence the kinase activity.

As already mentioned, the protein kinase was very sensitive to higher salt concentrations (Fig. 6). No differences in the extent of inhibition were observed between NaCl, LiCl, and KCl. A 40% inhibition was evoked by 50 mM salt. The protein kinase was still active in the presence of 0.5 to 10 mM EDTA. The addition of Mg^{2+} (15 mM final concentration) resulted in only a 50% increase in ^{32}P incorporation. Mn^{2+}, however, was a stronger activator and resulted in 1.5-, 3-, 4-, and 6-fold incorporation rates at 0.5, 1.5, 3.5, and 5.5 mM Mn^{2+}, respectively. Ca^{2+} (1 to 100 μM) in the presence or absence of calmodulin (0.1 to 0.3 μg) had no influence on the enzyme activity. These results were corroborated by experiments using the calmodulin antagonist W-5, which was without effect up to 2 mM. Reported concentrations for 50% inhibition of calmodulin-dependent protein kinases are in the range of 250 μM (6).

Heparin, which is a strong inhibitor for protein kinases of the casein kinase II type (15), was without influence on the purified kinase (up to 12 μg of heparin/ml). FSBA has been successfully used to affinity-label nucleotide binding sites in protein kinases (7, 11). FSBA reacts primarily with lysine, histidine, or cysteine residues, where the sulfonyl fluoride is the reactive functional group that can act as electrophilic agent (7). When the purified kinase was preincubated with FSBA (up to 2 mM) for 10 min the ^{32}P incorporation rates decreased about 40%. This inhibition was not reversed by 10 mM DTT, thus meaning that FSBA did not react with a cysteine residue (7). Preincubation with DTT alone (10 min, 10 mM DTT) had no effect on the kinase activity. Periodate oxidation of ATP results in the formation of oATP, which can also be used to covalently modify protein kinases under reducing conditions (7). Purified kinase activity, however, was not influenced by preincubation with o-ATP (0.08 to 0.8 mM) for 15 min in the presence or absence of Mg^{2+} (5 mM). The arginine binding reagent phenylglyoxal (5) also did not influence protein kinase activity, when preincubated for 10 min at 20°C and 2.5 mM phenylglyoxal. A recent paper (22) describes the Ca^{2+} and phospholipid activation of a solubilized envelope protein kinase after an initial acetone precipitation. I have compared the purified kinase activity in the presence and absence of phosphatidylcholine (2 mg/ml) or a mixture of authentic outer membrane lipids, mainly galactolipids and phosphatidylcholine extracted from purified outer membranes (2 mg/ml). Lipids were added in ethanol; control experiments received the same amount

Table I. *Substrate Specificity of the Partially Purified Kinase from Outer Chloroplast Envelopes*

	Addition								
	None	Histone III-s	Histone V-s	Phosvitin	Casein	Peptide			
						1[a]	2[a]	3[b]	4[b]
Activity (%)	0	100	80	11	7.3	0	0	0	0

[a] Peptides 1 and 2 had the following sequences: 1: Ala Thr Glu Thr Val Glu Ser Ser Ser Arg Cys; 2: Met Arg Lys Ser Ala Thr Thr Lys Lys Ala Val Cys. [b] Peptides 3 and 4 had the following sequences: 3: Met Ala Ser Met Ile Ser Ser Ser Ala Val Thr Thr Val Ser Arg Ala Ser Arg Gly Gln; 4: Ser Ala Ala Val Ala Pro Phe Gly Gly Leu Lys Ser Met Thr Gly Phe Pro Val Lys Lys.

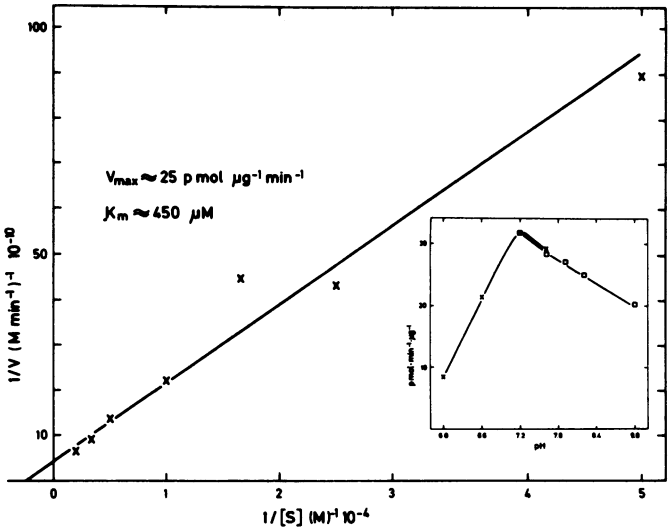

FIG. 4. Substrate and pH dependence of the purified protein kinase. A Lineweaver-Burk plot was used to determine K_M and V_{max}. The insert demonstrates the pH dependence of the kinase. Fifty millimolar [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane]-HCl (×) was used as buffer in the acidic range; 50 mM Tricine-KOH (□) was used in the alkaline range.

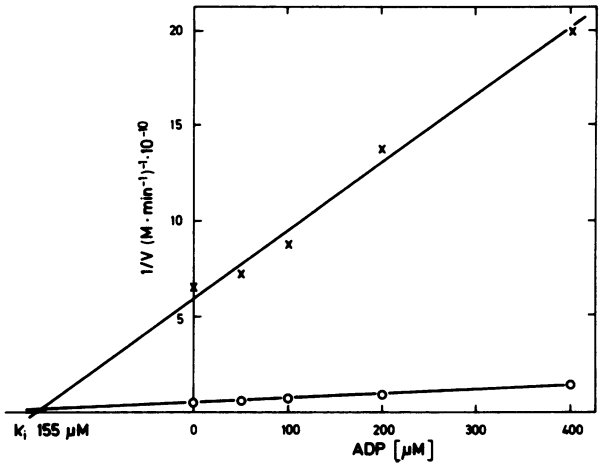

FIG. 5. Competitive inhibition of the protein kinase by ADP. The K_i was determined by a Dixon plot; ×, 0.04 μM ATP; o, 4 μM ATP.

of ethanol. I could not confirm the results reported by Muto and Shimogawara (22) and found no alteration in enzyme activity by the addition of lipids.

DISCUSSION

The present purification protocol results in the enrichment of an outer envelope membrane-bound, ATP-dependent protein kinase. This is the first protein purified in an active state from this membrane. The protein kinase is of the cyclic nucleotide and Ca^{2+}, calmodulin-independent type. The data obtained from the characterization of kinase activity still bound to the membrane or in the purified stage are comparable with each other (26, 27); although the *in situ* characterization of the outer envelope-bound protein kinase revealed an ATP concentration for half maximal activity of 37 μM (27), which is much lower than the K_M described in this paper for the partially purified enzyme. The K_M of 450 μM reported here for the partially purified enzyme is also higher than those generally found for protein kinases (10). These differences could be due to the change of environment

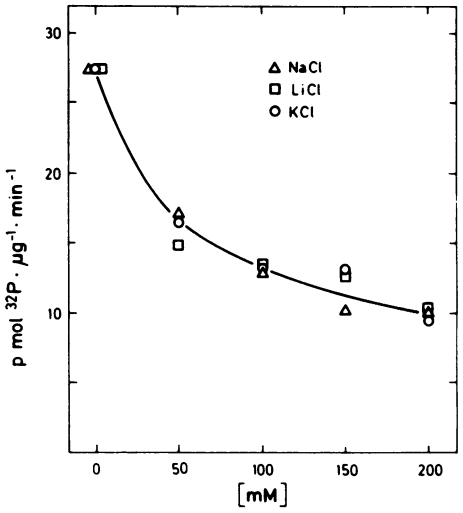

FIG. 6. Effect of various salts on the purified protein kinase activity.

that is experienced by the enzyme, *e.g.* from the hydrophobic, membrane-bound stage to a solubilized hydrophilic test system. The outer envelope-bound, ATP-dependent protein kinase (26) phosphorylates about 10 endogenous proteins in the outer envelope (27). The nature of these proteins was not investigated. *In vitro* histone III-S is the preferred protein substrate for the purified kinase; earlier studies (26, 27) have used histone II-S, casein, and phosvitin, which were poor substrates or were not accepted at all. The protein kinase has a molecular weight of 70 kD as determined by SDS-PAGE. It is of very low abundance in the outer envelope and not visible on SDS-PAGE by normal Coomassie brilliant blue stain. However, it is possible to detect the partially purified kinase in its purified state by Western blot techniques using an antibody raised against mixed envelope membranes. This analysis further demonstrates that an envelope protein was purified and that this protein fraction was near homogeneity.

Even though the attempts to obtain direct proof of the identity of the 70 kD protein as the protein kinase by direct photoaffinity labeling technics using [α-^{32}P]ATP or by autophosphorylation using carrier free [γ-^{32}P]ATP failed, indirect evidence supports the view that the 70 kD protein is indeed the protein kinase. The reasoning is the following: (a) The 70 kD protein is the only polypeptide that was enriched and copurified with the kinase activity through the affinity purification step and through the ion exchange purification. Four silver-stained SDS-PAGE analyses were run on individual protein purifications; while the small amounts of contaminations (also visible in Fig. 3) changed from experiment to experiment, the appearance of the 70 kD protein was always reproducible. (b) The purification protocol resulted in the enrichment of a very minor component of the outer chloroplast envelope and not, as is often the case, in membrane protein purification in the isolation of one of the major membrane polypeptides.

The protein kinase isolated from thylakoid membranes by Coughlan and Hind (8) shows some similar characteristics to the protein kinase isolated from the outer envelope membranes. Both can be solubilized using related solubilization protocols (8, 21). The reported molecular weight on one-dimensional SDS-PAGE differs only by about 6 kD. Both use histone III-S as preferred exogenous protein substrate and can be affinity-purified using histone III-S Sepharose. However, there are located in different chloroplast compartments, and I took very great care to start the protein kinase isolation from thylakoid-free outer chloroplast envelope membranes only. The isolated outer chlo-

roplast envelope fraction was devoid of Chl, which was used as a marker for thylakoid membranes.

To date, no exact function has been determined for this protein kinase. This is a very common phenomenon for the majority of enzymes described in the literature (19, 24). Many protein kinases were isolated and extensively studied before their exact regulatory function was finally found. The localization of this protein kinase at the interphase between the organelle and the cell anticipates interesting functions, which can now be addressed in the necessary diligence using the purified enzyme.

Our current working hypothesis predicts the involvement of a protein phosphorylation-dephosphorylation cycle in the ATP-dependent protein translocation into chloroplasts (25). Protein transport into chloroplasts requires the hydrolysis of ATP at the outer chloroplast envelope (13, 25). Phosphorylation of precursor proteins could make them import competent by evoking conformational changes (28). In my experiments, the protein kinase did not accept synthetic peptides, homologous to the first and second halves of the transit sequence of the small subunit of ribulose bisphosphate carboxylase. This might be due to conformational requirements for the protein substrate that are not met by the synthetic peptides. Second, a possible phosphorylation site (−Arg Gly Glu Ser−, 19) is situated right at the end of the first and the beginning of the second peptide (Table I). Another possibility could be the phosphorylation of a protein of the protein translocation apparatus. An antibody raised against the protein kinase should enable us to further study this question in greater detail.

The outer chloroplast envelope forms the boundary between the cytoplasm and the plastid. Although small molecules can freely pass through the membrane by porin (12), molecules over 10,000 D cannot pass the membrane. Signal transduction has been postulated to occur between the organelle and the nucleus in both directions to coordinate protein synthesis (23). The protein kinase might also be involved in a signal transduction chain.

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