A Protein Kinase That Phosphorylates Light-Harvesting Complex Is Autophosphorylated and Is Associated with Photosystem II†

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ABSTRACT: Thylakoid membranes were phosphorylated with [γ-^{32}P]ATP and extracted with octyl glucoside and cholate. Among the radiolabeled phosphoproteins in the extract was a previously characterized protein kinase of 64-kDa apparent mass. The ability of this enzyme to undergo autophosphorylation in situ was used to monitor its distribution in the membrane. Fractionation studies showed that the kinase is confined to granal regions of the thylakoid, where it appears to be associated with the light-harvesting chlorophyll–protein complex of photosystem II. The kinetics of kinase autophosphorylation were investigated both in situ and in extracted, purified enzyme. In the membrane, autophosphorylation saturated within 20–30 min and was reversed with a half-time of 7–8 min upon removal of ATP or oxidative inactivation of the kinase; the accompanying dephosphorylation of light-harvesting complex was slower and kinetically complex. Fluoride (10 mM) inhibited these dephosphorylations. Autophosphorylation of the isolated kinase was independent of enzyme concentration, indicative of an intramolecular mechanism. A maximum of one serine residue per mole of kinase was esterified. Autophosphorylation was more rapid in the presence of histone IIIs, an exogenous substrate. Dephosphorylation of the isolated enzyme was not observed.

Illumination of thylakoids promotes the phosphorylation of the LHC[1] complex on a threonine residue (Bennett, 1977) of an N-terminal peptide (Mullet, 1983). This phosphorylation is thought to be involved in regulating the distribution of absorbed excitation energy between photosystems II and I (Bennett et al., 1980) and is modulated by the redox state of the plastoquinone pool (Allen et al., 1981).

Lin et al. (1982) partially purified and characterized (Lucero et al., 1982) two protein kinases from spinach thylakoids, neither of which was able to phosphorylate isolated LHC, the presumed physiological substrate. Recently, a third protein kinase was described (Coughlan & Hind, 1986a), of apparent molecular mass 64 kDa, which phosphorylates purified LHC in vitro. Using a heterogeneous system consisting of this purified kinase and thylakoids deprived of endogenous kinase activity, it proved possible to reconstitute physiological rates of LHC phosphorylation (Coughlan & Hind, 1987a).

Protein kinases from mammalian cells commonly undergo autophosphorylation (Krebs & Beavo, 1979); this phenomenon has already been documented for the isolated, purified, 64-kDa protein kinase from thylakoids (Coughlan & Hind, 1986a). We show here that autophosphorylation and dephosphorylation of the kinase occur in native thylakoid membranes and present a kinetic description of these events. Self-radiolabeling, immunological detection, and activity assay of the membrane-resident kinase are used to assess the distribution of enzyme among membrane subfractions.

Materials and Methods

Preparation and Extraction of Thylakoid Membranes. Spinach thylakoid membranes were phosphorylated with [γ-^{32}P]ATP as previously described (Coughlan & Hind, 1986c). Protein kinase was isolated by extraction of phosphorylated membranes with OG–cholate (Coughlan & Hind, 1986a) and was followed by two cycles of affinity chromatography (Coughlan & Hind, 1987a).

Thylakoid membrane subfractions enriched in PS II were prepared from nonphosphorylated membranes by the method of Berthold et al. (1981) or from phosphorylated membranes by the modification of Widger et al. (1984). They were further fractionated into LHC and core proteins as described by Ghanotakis and Yocum (1986). Membrane vesicles enriched in PS I were prepared according to Peters et al. (1983).

Assay of Protein Phosphorylation. Washed thylakoids (500 μg of Chl) were resuspended to 900 μL in reaction medium [0.1 M sorbitol, 10 mM $MgCl_2$, and 10 mM Tricine–NaOH (pH 8.0), with 10 mM NaF where shown], sparged with argon, and preequilibrated for 1 min in the presence of either 5 mM dithionite or 100 W/m^2 light (tungsten filament) at 20 °C. Protein phosphorylation was initiated by the anaerobic addition of 100 μL of a solution of 2.5 mM ATP containing 100 μCi of [γ-^{32}P]ATP in reaction medium. At fixed time intervals, 50-μL aliquots were anaerobically withdrawn and kinase activities quenched by the addition of 50 μL of 0.2 M ethylenediaminetetraacetic acid on ice. The membranes were sedimented (12000*g*, 15 s) in an Eppendorf Minifuge; the pellet was rinsed twice with 200 μL of ice-cold reaction medium with the omission of $MgCl_2$ and was then solubilized in 30 μL of a solution containing 4% (w/v) sodium dodecyl sulfate, 4% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, 0.05% (w/v) bromphenol blue, and 25 mM Tris-HCl (pH 8.8). The samples were heated (1 min, 70 °C) to dissociate chlorophyll–protein complexes and electrophoresed; the gel was stained, destained, dried, and autoradiographed as described elsewhere (Coughlan & Hind 1986a,b). Radioactivity was quantified either by scintillation counting of excised radioactive areas of

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[1] Abbreviations: ATP, adenosine 5′-triphosphate sodium salt (Sigma Chemical Co.); Chl, chlorophyll; LHC, light-harvesting pigment–protein complex of photosystem II; OG, *n*-octyl β-D-glucopyranoside (Behring Diagnostics); PS, photosystem; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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the dried gel (LHC) or by scanning reflectance densitometry of the developed X-ray film (kinase).

Assay of Protein Dephosphorylation. Membranes were phosphorylated for a fixed time in the medium described above, whereupon the reaction was terminated by either (i) removal of ATP, where membranes were pelleted (15 s, Eppendorf Minifuge), washed twice in reaction medium, and finally resuspended to 0.5 mg of Chl/mL in reaction medium (total manipulation time 1–2 min), or (ii) addition of 2 mM potassium ferricyanide (final concentration), which inactivates kinase by oxidizing the plastoquinone pool. Dephosphorylation was monitored by incubation at 20 °C in the dark; at fixed time intervals, 50-μL aliquots were withdrawn and processed as described above. Identical kinetics were obtained with either quenching procedure.

Assay of Kinase Autophosphorylation. Autophosphorylation of the purified kinase was achieved by incubating 20 ng in 50 μL of 10 mM Tricine–NaOH (pH 8.0), 10 mM $MgCl_2$, 10 mM NaF, and 0.2 mM ATP containing 5 μCi of [γ-^{32}P]ATP, for a given time at 25 °C. The reaction was stopped by addition of 25 μL of 50% (w/v) trichloroacetic acid on ice after addition of 10 μL of a 5 mg/mL solution of cytochrome *c* as a carrier protein. The suspension was left on ice for 20 min and centrifuged (10 s, Eppendorf Minifuge). The pellet was rinsed twice with 250 μL of 5% (w/v) trichloroacetic acid and once with 250 μL of ether–ethanol (1:1 v/v) and finally redissolved in 50 μL of 4% (w/v) sodium dodecyl sulfate solution. Excess acidity was neutralized by the addition of 5 μL of 1 M Tris-HCl (pH 8.0). Electrophoresis, staining and destaining of the gel, and quantification of radioactivity were as described in Coughlan and Hind (1986a).

Phospho Amino Acid Analysis and Peptide Mapping. Purified kinase (5 μg) in 100 μL of 10 mM Tricine–NaOH (pH 8.0) and 10 mM $MgCl_2$ was incubated in the presence of 50 μM ATP containing 100 μCi of [γ-^{32}P]ATP for 30 min at 25 °C. The sample was then desalted over a 2-mL Sephadex G-25 column (6.5 × 2 cm) to remove unreacted ATP and divided into two 200-μL aliquots.

For phospho amino acid analysis, an aliquot was freeze-dried, 1 mL of 6 N HCl was added, and partial acid hydrolysis was conducted for 1–6 h at 110 °C. The hydrolysate was freeze-dried, redissolved in 10 μL of 50 mM NH_3 containing 2 μg each of phosphoserine, phosphothreonine, and phosphotyrosine as markers, and subjected to high-voltage thin-layer electrophoresis on cellulose thin-layer plates (20 × 20 cm, Eastman Chromogram 13255) at pH 3.5 and 6.5 in the buffer systems described in Walker et al. (1985). Radioactive areas of the electrophoretogram were detected by autoradiography and compared to the position of the marker phospho amino acids by ninhydrin staining (Perham, 1978).

To the second sample aliquot (2.5 μg of protein) was added 2.5 μL of a freshly prepared 10 μg/mL trypsin solution (Sigma type XIII, sp act. 12000 units/mg of protein) in 1 mM HCl (1:100, enzyme:substrate, w/w), and the solution was incubated for 24 h at 37 °C; supplemental 2.5-μL aliquots of trypsin were added at 9 and 18 h. The sample was freeze-dried, redissolved in 10 μL of 50 mM NH_3, and spotted onto a cellulose thin layer [see above and Perham (1978)] for electrophoresis in the first dimension (30 min at 1000 V). The plate was air-dried and then subjected to ascending chromatography at 90° (second dimension) in 1-butanol–acetic acid–water–pyridine (15:3:12:10 by volume). Radioactive peptides were detected by autoradiography with Du Pont Cronex X-ray film.

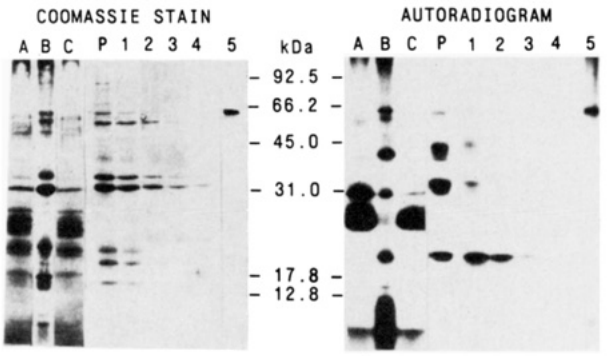

FIGURE 1: Purification of a protein kinase from phosphorylated thylakoid membranes. Thylakoids were phosphorylated with [γ-^{32}P]ATP, the membranes were treated with OG–cholate, and the protein kinase was partially purified to the Bio-Rad P-300 desalting step (Coughlan & Hind, 1986c). The P-300 eluate (400 μL containing 0.8 mg of protein) was loaded on a 1-ml histone IIIs–Sepharose affinity column and washed and the peak of histone kinase activity eluted (Coughlan & Hind, 1987). Fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (Left panel) Polypeptide composition; (right panel) distribution of ^{32}P-labeled phosphopeptides revealed by autoradiography. Lane markings are as follows: (A) thylakoid membranes; (B) supernatant; (C) pellet after OG–cholate treatment and ultracentrifugation; (P) eluate from P-300 column. Lane 1 shows the initial throughput from the affinity column, and lanes 2–4 show the sequential washes with 50 mM NaCl. Lane 5 is the fraction eluted by buffered 0.5 M NaCl (Coughlan & Hind, 1987). Molecular weight markers are (kDa) phosphorylase *b* (92.5), bovine serum albumin (66.2), ovalbumin (45), carbonic anhydrase (31.0), myoglobin (17.8), and cytochrome *c* (12.8).

Radioactive areas of the plate were scraped off and the peptides eluted by incubating the silica gel with 200 μL of 50 mM NH_3 for 30 min. The silica was centrifuged (1 min, 12000*g*) in an Eppendorf Minifuge, the supernatant decanted, and the pellet extracted with 50 mM NH_3 a further 2 times. The pooled supernatants were freeze-dried, redissolved in 400 μL of 6 N HCl, and processed for phospho amino acid analysis as described above.

Other Analytical Techniques. Polyclonal antisera to the purified kinase were raised in mice, and the IgG fraction was purified as previously described (Coughlan & Hind, 1987a). Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose and immune blotting were carried out as in Coughlan et al. (1985). Protein concentration was determined either by the dye-binding method of Bradford (1976) or by the method described by Bensadoun and Weinstein (1976). Chlorophyll concentration was determined by the method of Arnon (1949).

RESULTS

The self-labeling of 64-kDa protein kinase upon incubation of a detergent extract of thylakoids with [γ-^{32}P]ATP was previously exploited (Coughlan & Hind, 1986a, 1987a) to monitor the purification of this enzyme. The membrane-bound kinase also labels itself during concomitant catalysis of LHC phosphorylation by [γ-^{32}P]ATP. Figure 1 shows that although a 64-kDa phosphoprotein is barely detectable on an autoradiogram of ^{32}P-labeled thylakoids, the labeled kinase is evident following extraction with OG–cholate and purification by affinity chromatography as previously described (Coughlan & Hind, 1986a, 1987a). No other phosphoprotein of apparent mass of ~64 kDa is seen in the extract or extracted membranes. Autophosphorylation thus provides a means of probing the distribution of kinase in the thylakoid.

Phosphorylated membranes were fractionated, with Triton X-100, to yield membrane preparations enriched in either PS I or PS II (Figure 2). The virtual absence from the PS II

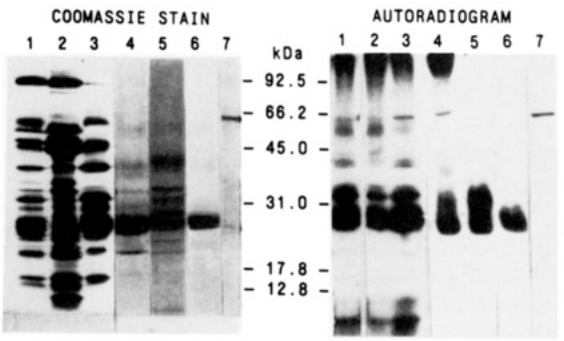

FIGURE 2: Fractionation of thylakoid membranes into PS I and PS II and subfractionation of PS II into LHC and core complex. Membranes (5 mg of Chl) were phosphorylated as in Coughlan and Hind (1986c), extracted with Triton X-100, and fractionated into supernatant (containing PS I and undissociated membrane fragments) and pellet (enriched in PS II). The pellet was resuspended (2 mg of Chl/mL) in 0.4 M sucrose, 0.5 M NaCl, 10 mM $CaCl_2$, 20 mM sodium 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 10 mM NaF, and 0.1 mM phenylmethanesulfonyl fluoride and either used immediately or frozen in liquid nitrogen and stored at −70 °C. The PS II enriched pellet was further fractionated by a modification of the method of Ghanotakis and Yocum (1986). Sufficient stock solution of 140 mM OG in water was added to a 100-μL aliquot (200 μg of Chl) of PS II particles to give a final concentration of 35 mM. The suspension was incubated for 30 min on ice and then centrifuged for 30 s at 100000*g* in a Beckman airfuge to sediment LHC. Detergent was removed from the supernatant by passage through a 1-mL column of Extractigel (Pierce) equilibrated in the PS II storage medium, with the omission of NaCl. The supernatant was sedimented (100000*g*, 2 min), the supernatant decanted, and the pellet redissolved in 100 μL of equilibration buffer. Aliquots of the various fractions were electrophoresed and further processed as in Figure 1. (Left panel) Polypeptide composition of the fractions; (right panel) distribution of ^{32}P-labeled phosphoproteins. Lanes 1–3 show respectively the starting material (thylakoids), supernatant, and pellet obtained by treatment with Triton X-100. Lanes 4 and 5 are the LHC and core pellets from subsequent treatment of the PS II fraction with OG. Lanes 6 and 7 show LHC and kinase isolated from phosphorylated membranes.

enriched fraction of polypeptides having mass of 105, 37, or 34 kDa (tentatively identified as PS I reaction center, ferredoxin:$NADP^+$ oxidoreductase and cytochrome *f*, respectively) is evident from the Coomassie-stained gel. This fraction was further resolved by OG into its constituent LHC and PS II core complexes. For purposes of comparison, LHC was also extracted directly from phosphorylated thylakoids by the method of Mullett (1983). The corresponding autoradiogram shows that autophosphorylated kinase is concentrated in the PS II enriched fraction and accompanies LHC when this is extracted therefrom with OG. The LHC preparation of Mullett is evidently more highly resolved, lacking not only kinase but also minor polypeptides in the 20–40-kDa range and a 32-kDa phosphoprotein.

Table I presents an analysis of the kinase activities of these various fractions toward LHC or histone as substrate. Only the untreated thylakoids catalyzed both LHC and histone phosphorylation following activation of kinase with reductant. Catalysis of histone phosphorylation survived exposure to detergent and was independent of reductive activation; indeed, 1 mM dithionite (Table I) or 1 mM dithiothreitol (not shown) was somewhat inhibitory. Most of the histone kinase activity was associated with the LHC pellet, though 27% remained associated with the PS II core. No activity was present in the remaining supernatant.

Verification of the above findings was obtained by immunoblot analysis of the membrane subfractions (Figure 3), using a polyclonal antiserum to the purified kinase. In thylakoids and in PS II particles, the immunoglobulin cross-reacted with

Table I: Distribution of Protein Kinase Activity among Thylakoid Subfractions

fraction	protein (μg)	specific activity[a] LHC	specific activity[a] histone	distribution (%)[b]
thylakoid		(0.60)	(1.0)[c]	
PS I		nd[d]	nd	
PS II	300	nd	1.5 (0.7)	100
LHC	180	nd	1.7	69
PS II core	100	nd	1.2	27
LHC supernatant	30	nd	1.0	7

[a] Nanomoles of phosphate incorporated per milligram of membrane protein in fraction, during 10 min at 20 °C in the presence of 250 μM [^{32}P]ATP. Endogenous LHC, or histone IIIs (1 mg/mL), was used as substrate. [b] Normalized to the total activity in the PS II fraction. [c] Reductant (1 mM dithionite) was added where indicated by parentheses. [d] nd, not detectable.

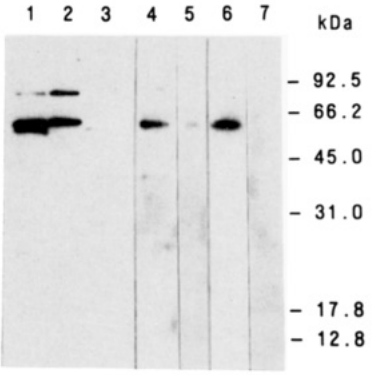

FIGURE 3: Distribution of kinase in thylakoid membrane subfractions by immunoblot analysis. Experimental conditions were as in Figure 2 except that the membranes were not phosphorylated. Electrophoretic transfer of proteins from the acrylamide gel to nitrocellulose was carried out in a Hoefer Transphor apparatus (100 V, 2 h). The blot was incubated with immunoglobulin raised against purified kinase, followed by ^{125}I-labeled protein A, and was then autoradiographed (Coughlan et al., 1985). Lanes 1–3 show thylakoids, PS II, and PS I particles, respectively. Lanes 4 and 5 are LHC and PS II core. Lane 6 shows purified kinase and lane 7, LHC purified as in Mullet (1983).

the 64-kDa protein kinase and with a higher molecular weight polypeptide (~82 kDa). This latter has not been identified and is under investigation; it was not apparent in the kinase preparation used to raise antibodies or that probed in lane 6 of Figure 3. Some cross-reactivity occurred with a polypeptide of ~60 kDa; however, this component did not appear if a protease inhibitor was included in media used for thylakoid isolation (not shown). Kinase was absent from PS I particles (lane 3). Probing LHC and core fractions from PS II with the antibody showed that kinase was predominantly associated with the LHC pellet fraction, only minor amounts being present in the photosystem core. Thus the results from immunology confirmed those obtained by the autophosphorylation patterns and by enzymatic assays. It is also noteworthy that a negative result was obtained upon probing LHC isolated by conventional means with the kinase antiserum (Figure 3, lane 7), signifying that the postulated interaction between kinase and LHC is disrupted during the LHC purification procedure of Mullet (1983).

The kinetics of autophosphorylation and subsequent dephosphorylation were determined for both membrane-resident and extracted kinase. Kinase in the membrane phosphorylated itself and endogenous LHC with closely similar kinetics (Figure 4a,b). The phosphorylation rate was initially linear but began to decline after 2–5 min, the maximal extent of phosphorylation being attained after 20–30 min. Membrane-bound kinase was rapidly dephosphorylated with a half-time of 7–8 min. In complete contrast, and as previously

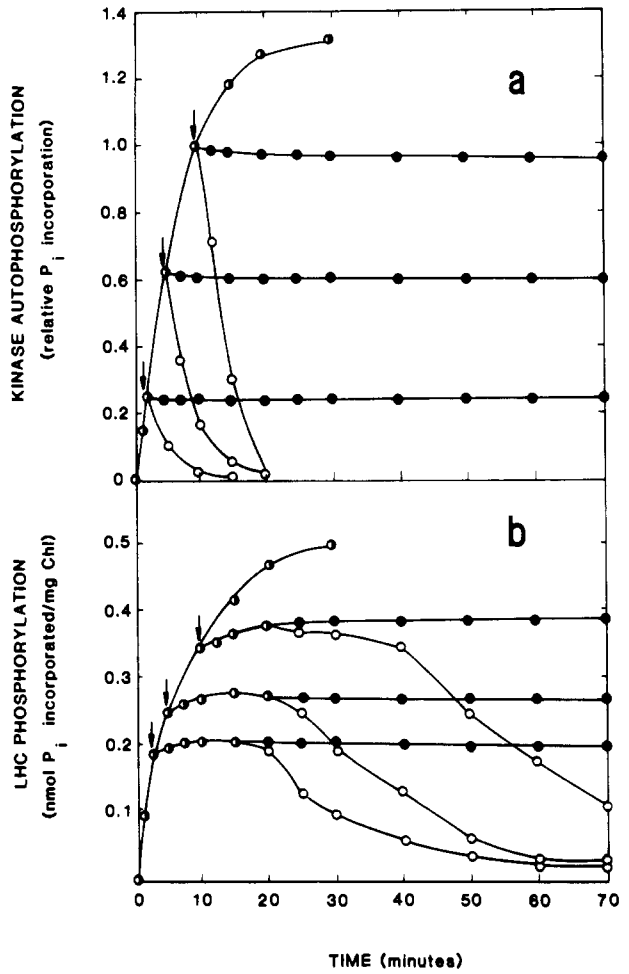

FIGURE 4: Kinetics of phosphorylation and dephosphorylation of (a) kinase and (b) LHC in situ. Experimental details are given under Materials and Methods. (○) Minus NaF; (●) plus 10 mM NaF.

reported by Horton and Foyer (1983), LHC was not dephosphorylated for a period of between 10 and 20 min after the cessation of phosphorylation, following which dephosphorylation set in and was half complete in 30–60 min, depending on the initial extent of phosphorylation.

Dephosphorylation of both proteins was inhibited by the presence of 10 mM NaF, an inhibitor of thylakoid phosphatases (Bennett, 1981). Fluoride inhibition did not increase the net rate of LHC phosphorylation, consistent with the low phosphatase activity of spinach thylakoids and contrasting with rate data from pea thylakoids (Steinback et al., 1982; Horton & Foyer, 1982), which have high intrinsic phosphatase activity.

The kinase autophosphorylates much more slowly following isolation from the membrane, a 1–2-h incubation period being necessary for complete phosphorylation to a maximum of 1 residue/mol of enzyme (Figure 5a). The presence of exogenous substrate (histone IIIs) doubled the initial rate but had no effect on the final extent of autophosphorylation. Dephosphorylation of the kinase was not observed under the conditions described in Figure 5a, regardless of the presence of NaF (not shown).

Analysis of the kinetics of autophosphorylation revealed a K_m (Mg^{2+}-ATP) of 25 μM, a K_e (Mg^{2+}) of 1 mM, and a pH optimum of 8.0 (Figure 5b,c). The initial rate of autophosphorylation was a linear function of kinase concentration over the range 5–120 ng/50 μL (Figure 5d). This result suggests that the mechanism of autophosphorylation is intramolecular, as reported for mammalian kinases (King et al., 1983; Todhunter & Purich, 1977). The K_m and pH optimum are consistent with self-phosphorylation by the substrate catalytic site (Coughlan & Hind, 1986a). The calculated specific activity of autophosphorylation was ~1 nmol of phosphate incorporated·min^{-1}·(mg of enzyme)$^{-1}$, which compares with a catalytic rate of 50–100 nmol of phosphate transferred·min^{-1}·(mg of enzyme)$^{-1}$ with histone IIIs as a substrate.

The presence of a single autophosphorylation site on the enzyme was supported by peptide mapping and phospho amino acid analysis of a total tryptic digest of the self-labeled kinase. A single, neutral phosphopeptide was detected by two-dimensional mapping; the phosphorylated amino acid was identified as serine (Figure 6).

Discussion

The control of light-energy distribution between PS I and PS II is thought to be exercised through reversible phosphorylation of a pool of LHC. In the dark, this pool is dephosphorylated and associated with PS II; activation of kinase in the light leads to LHC phosphorylation and subsequent migration to stromal lamellar regions, where PS I complexes are predominant (Staehelin & Arntzen, 1983).

LHC kinase presumably occurs in contact with its substrate, in the granal regions of the membrane. Whitelegge et al. (1987) showed that granal fractions prepared by Yeda press or aqueous-phase partition fractionation are comparable to thylakoids in ability to phosphorylate membrane proteins, suggesting that the protein substrates and the kinase reside in the grana. We have now verified this supposition by fractionating the membrane with detergents and assaying the kinase distribution among subfractions by use of immune blotting, by use of self-radiolabeling, and on the basis of enzymatic activity.

The cofractionation of LHC and kinase upon further extraction of a PS II enriched fraction with OG is believed to indicate a functional association between enzyme and substrate in the membrane. This presumably involves hydrophobic forces for, if the pelleted material from OG is resuspended in Triton X-100 and the LHC is reaggregated by addition of Mg ions, the kinase remains in the supernatant (data not shown). Correspondingly, the preparation of LHC from phosphorylated membranes by direct extraction with Triton X-100 followed by precipitation with Mg ions (Mullet, 1983) yields a kinase-free LHC preparation (Figures 2 and 3). The presumed LHC–kinase association is also sensitive to the combination of OG and cholate or nonanoyl-*N*-methylglucamide and cholate (Coughlan & Hind, 1986a,b; 1987a), which solubilize the kinase but leave LHC in the membrane.

Independent support for the association of kinase with LHC comes from work on nuclear mutations of *Chlamydomonas reinhardtii* (de Vitry et al., 1987), barley (Haworth et al., 1982), and clover (Markwell et al., 1985), showing that, in mutants lacking a functional LHC, phosphorylation of the other thylakoid proteins is diminished, implying a reduction in total protein kinase activity. In a range of *Chlamydomonas* mutants lacking either PS I or PS II, the cytochrome *b/f* complex, or LHC, only the LHC-deficient mutant lacked protein kinase activity. One interpretation of this finding is that kinase requires complexation with LHC for its stable incorporation into the membrane.

Useful comparisons can be made with the large body of available information on mammalian protein kinases. The oligomeric phosphorylase kinase of muscle possesses multiple sites whose autophosphorylation is associated with autoactivation of the isolated enzyme. Substrate and peptide analogues thereof accelerate these processes (Carlson & Graves, 1976;

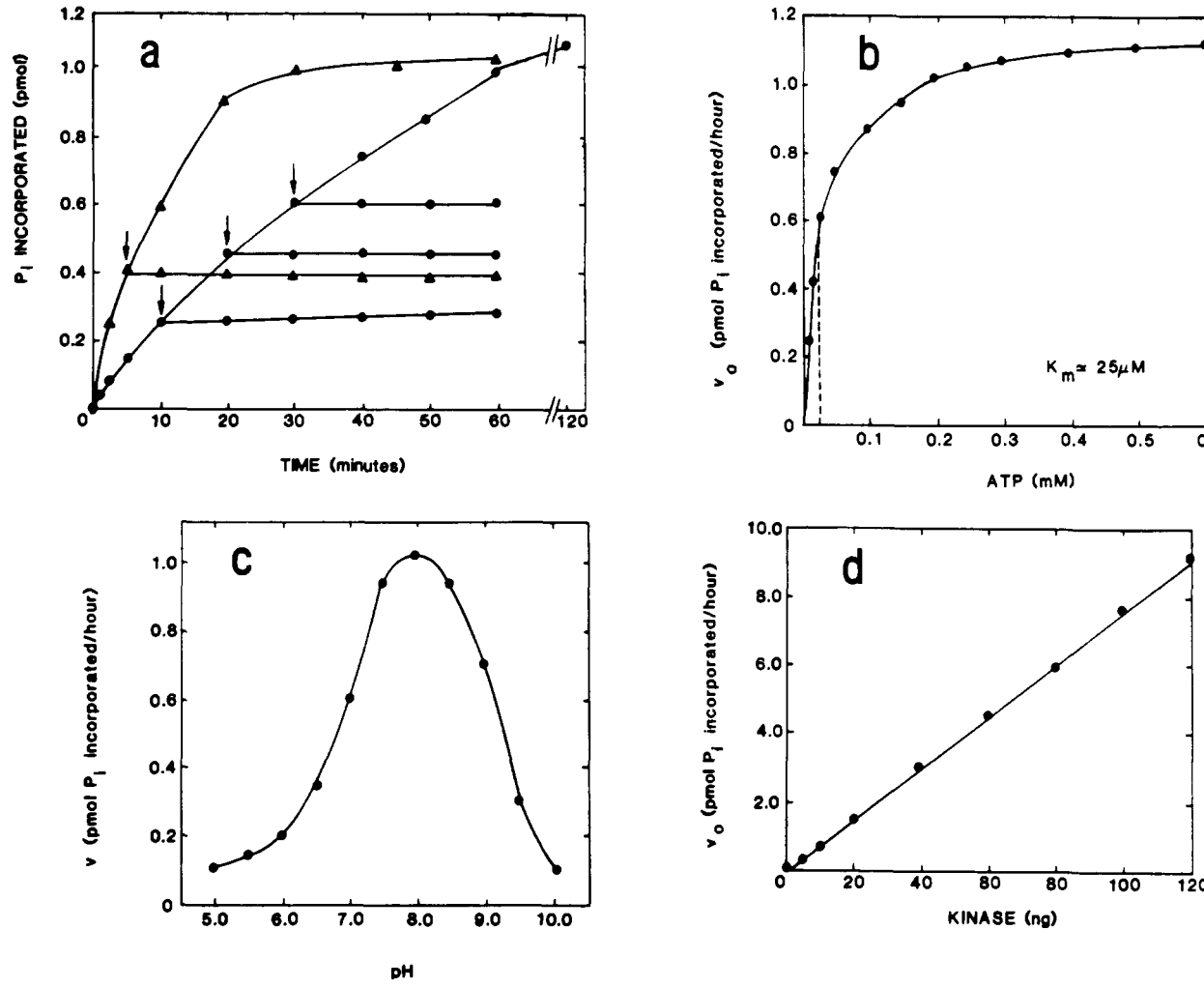

FIGURE 5: Kinetics of autophosphorylation of purified kinase. (a) Time course: (●) minus histone; (▲) plus 50 μg of histone IIIs. At the arrows, the sample was desalted over a Sephadex G-25 column to remove substrates, and the incubation was continued. (b) Dependence on ATP concentration: the ATP concentration was varied as shown. (c) Effect of pH: the buffer contained 5 mM each of 2-(*N*-morpholino)ethanesulfonic acid, Tricine, and 3-(*N*-morpholino)propanesulfonic acid adjusted with NaOH to the desired pH. (d) Relation of initial autophosphorylation velocity to kinase concentration: the kinase concentration was systematically varied over the range shown and a complete time course (0–60 min) obtained for each value. Except for the variations noted above, experimental details were as given under Materials and Methods.

King et al., 1983). Cohen et al. (1975) showed that an autophosphorylation site on one subunit of phosphorylase kinase is identical with that phosphorylated upon activation by a cAMP-dependent protein kinase. These processes in mammalian kinase systems are incompletely understood, but by analogy, a regulatory role for autophosphorylation of the thylakoid protein kinase can be postulated. Although there is no evidence for autoactivation or cascade activation of the thylakoid kinase, it may be premature to reject these possibilities until autophosphorylation has been studied in the presence of a native or reconstituted chloroplast stromal fraction.

Perhaps the strongest indication of some important role for autophosphorylation of the thylakoid kinase comes from the converse process: its rapid dephosphorylation. The sensitivity of this to fluoride, its absence from purified enzyme, and its occurrence in spinach thylakoids having low LHC phosphatase activity may argue for the existence of a distinct kinase phosphatase. Dynamic regulation of phosphatase could account for the anomalous progress curve of autophosphorylation, which seems indifferent to the presence of fluoride, suggesting that dephosphorylation is not concomitant and competitive. These observations, and the stimulation by histone noted in Figure 5a, hint at complex features of reversible autophosphorylation, which require further study.

It is noteworthy that the rate of LHC phosphorylation declines during a 20-min progress curve,[2] even when reductive activation of the kinase is maintained by an exogenous electron donor (Figure 4b of this work; Islam & Jennings, 1985). Feedback control of kinase activity through the redox-sensing mechanism cannot occur under these reductive conditions. Progressive inhibition of kinase by oxidation of the plastoquinone pool may also be impossible in light-activated systems lacking a catalyst of PS I turnover (Bennett et al., 1980; Steinback et al., 1982; Haworth et al., 1982). In these cases too, the progressive decline in LHC phosphorylation rate might indicate the functioning of a second regulatory mechanism.

Activation of kinase in response to its autophosphorylation, a possibility discussed above, would tend to give an initial lag, or concavity, in the LHC phosphorylation time course. The time courses of autophosphorylation and LHC phosphorylation are both nonlinear and convex (Figure 4); thus, we must entertain the contrasting notion that autophosphorylation of kinase inhibits its catalytic site and hence the phosphorylation of LHC. Such a self-inhibitory mechanism would have greater

[2] Data to be presented elsewhere show that <10% of the total LHC is phosphorylated after 20 min under comparable conditions.

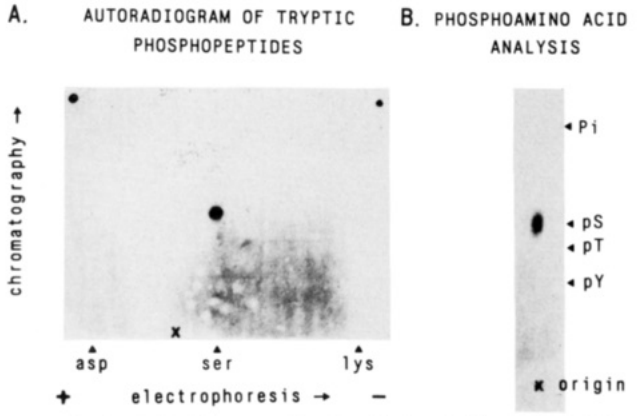

FIGURE 6: Analysis of the autophosphorylation site(s) on the 64-kDa protein kinase. (A) Autoradiogram of tryptic ^{32}P-labeled phosphopeptide(s) derived from autophosphorylated kinase by two-dimensional mapping. The origin and position of neutral (serine), acidic (aspartate), and basic (lysine) amino acids revealed by ninhydrin staining, after electrophoresis in the first dimension (1000 V, 30 min), are indicated. The thin-layer sheet and X-ray film were aligned by spotting the former with radioactive ink, which can be seen on both upper corners of the autoradiogram. The point of sample application is marked (×). (B) Phosphoamino acid analysis of the ^{32}P-labeled phosphopeptide. The positions of the origin and the phosphoamino acids revealed by staining the thin-layer sheet with ninhydrin are shown on the right side of the autoradiogram, which was run at pH 3.5 (1000 V, 20 min). Radioactivity equivalent to 1200 cpm was spotted on the fingerprinting thin layer, and 1050 cpm was recovered in the radioactive peptide shown. This was hydrolyzed (110 °C, 3.5 h), and radioactivity equivalent to 700 cpm was spotted on the phosphoamino acid resolving thin layer. The thin layers were developed with Cronex X-ray film and an intensifying screen (24 h, −70 °C).

significance where intrinsic protein phosphatase activity is low, as in spinach thylakoids. However, histone or LHC phosphorylation by isolated kinase not only accelerates autophosphorylation (Figure 5a) but also proceeds linearly [unpublished data; see also Alfonzo et al. (1980)], as does histone phosphorylation by thylakoids (data not shown). Thus in order to be tenable, any self-inhibition hypothesis must be restricted to catalysis of LHC phosphorylation by the in situ kinase.

Other hypotheses to account for the gradual decline in LHC phosphorylation envisage limitation imposed by (i) the availability to kinase of only a small subfraction of LHC (Islam & Jennings, 1985) having phosphorylation sites defined by specific components of the LHC multigene family (Dunsmuir, 1985), (ii) a saturation of protein-free space in the stromal lamellae so that diffusion of phospho-LHC away from the kinase becomes strongly rate limiting (O'Leary, 1987), (iii) direct product inhibition by phospho-LHC (Clark et al., 1983), and (iv) a migration of the autophosphorylated kinase, in response to its increased negative charge, toward the stromal lamellae and away from its substrate. The observation (Whitelegge et al., 1987) that low concentrations of detergent selectively increase the rate of LHC phosphorylation could bear importantly on evaluation of these mechanisms.

More work is clearly needed toward a kinetic understanding of protein phosphorylation in thylakoids. Especially with regard to its autophosphorylation, the 64-kDa thylakoid kinase has much in common with mammalian kinases; we may expect to discover further points of resemblance as this subject unfolds.

Registry No. Light-harvesting protein II kinase, 102925-40-6.

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Purification and Partial Characterization of the Membrane-Bound Cytochrome *o*(561,564) from *Vitreoscilla*†

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ABSTRACT: Cytochrome *o*(561,564) terminal oxidase was solubilized from the membrane fraction of the bacterium *Vitreoscilla* sp., strain C1, and purified by differential pH dialysis, gel filtration chromatography, and ion-exchange chromatography. Subunit molecular weights, determined on sodium dodecyl sulfate–polyacrylamide gels by the Ferguson plot method, were 49 500 and 23 500. There were two protohemes IX, two coppers, and 45 mol of phosphorus per mole of protomer (73 000). The molecular weight of the cytochrome *o* complex estimated by chromatography on Sephacryl-400 in deoxycholate was 265 000, which is consistent with the enzyme complex under these conditions being a dimer (146 000) with the remaining molecular weight contribution arising from bound phospholipid, deoxycholate, and possibly other, smaller subunits. Difference spectra of the dithionite-reduced enzyme have split α absorption maxima at 561 and 564 nm at room temperature and 558 and 561 nm at 77 K. The CO difference spectrum at room temperature has absorption maxima at 570, 534, and 416 nm. Dissociation constants for CO and cyanide binding to the reduced and oxidized forms of the oxidase are 5.2 μM and 3.5 mM, respectively. The hemes in the cytochrome are one electron accepting centers, both with midpoint potentials around +165 mV at pH 7.0. The enzyme is highly autoxidizable, and its menadiol oxidizing activity is stimulated by phospholipids.

Cytochrome *o* was first identified in bacteria more than 30 years ago (Chance et al., 1953). There now appear to be two general types of *o*-type terminal oxidases (Wood, 1984): oxidases of the *c*–*o* type, like those purified from *Azotobacter vinelandii* (Jurtshuk et al., 1981) and *Methylophyilus methylotrophus* (Carver & Jones, 1983), and the terminal oxidases of the *b*–*o* type, e.g., the cytochrome *b*-562–*o* purified from *Escherichia coli* (Kita et al., 1984; Matsushita et al., 1984). The "soluble cytochrome *o*" from *Vitreoscilla* sp. was formerly believed to be a unique member of this latter group, but recent experiments have shown it to be a bacterial hemoglobin (Orii & Webster, 1986; Wakabayashi et al., 1986). Photolysis experiments on intact cells of *Vitreoscilla* had previously indicated the existence of a second CO-reactive *b*-type cytochrome (DeMaio et al., 1983). This second *o*-type cytochrome could be differentiated from the cytoplasmic hemoglobin because its CO compound underwent detectable photolysis at temperatures lower than −80 °C and its reaction with oxygen at −100 °C to form an oxygenated intermediate was similar spectrally and kinetically to that of the membrane-bound cytochrome *o* from *E. coli* (Poole et al., 1979). Additional evidence for the existence of a membrane-bound cytochrome *o* in *Vitreoscilla* came from studies on the supernatant and respiratory membrane fractions from cells disrupted by osmotic lysis after lysozyme treatment (DeMaio & Webster, 1983). CO difference spectra showed that this membrane fraction contained a CO-binding cytochrome with a Soret maximum at 416 nm, significantly different from the Soret band (420 nm) of the hemoglobin found in the supernatant fraction. Membranes free of soluble hemoglobin showed rapid respiratory rates with either NADH or ascorbate–DCPIP as substrate. Here we report on the purification and characterization of the membrane-bound cytochrome *o* from *Vitreoscilla*.

Materials and Methods

Purification of Cytochrome o(561,564). The organism used for these studies, *Vitreoscilla* sp., strain C1, was obtained originally from R. G. E. Murray, University of Western Ontario, and is his culture 389. The growth of *Vitreoscilla*, the preparation of respiratory membrane fragments, and their solubilization with sodium deoxycholate have been described

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