Purification and Characterization of a Membrane-bound Protein Kinase from Spinach Thylakoids*

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A protein kinase was isolated from spinach thylakoid membranes by solubilization with octyl glucoside and cholate. The enzyme was purified to apparent homogeneity by ammonium sulfate precipitation, gel filtration, and sucrose density centrifugation, followed by affinity chromatography on either Affi-Gel blue (yielding denatured enzyme) or on histone cross-linked to Sepharose (yielding active enzyme). Electrophoresis on denaturing polyacrylamide gels, followed by staining with silver, revealed the kinase as a single band corresponding to an apparent molecular mass of 64 kDa. The active enzyme underwent autophosphorylation and could be detected by autoradiography following incubation with \( \gamma -{\text{32P}} \)ATP and Mg\(^{2+} \) ion. The specific phosphotransferase activity of purified kinase was \( \approx 90 \text{ nmol of phosphate } \text{min}^{-1} \text{(mg protein)}^{-1} \) with lysine-rich histone (III-S or V-S) as substrate; casein was phosphorylated at \( \approx 30\% \) of this rate.

The physiological substrate for the kinase is presumed to be light-harvesting chlorophyll \( a/b \) protein complex. In solubilized form, this was phosphorylated at \( \approx 10\% \) of the rate observed with histone III-S as substrate, or 10-100 times slower than the estimated rate of phosphorylation of the light-harvesting complex in situ. Possible reasons for this shortfall are considered. The kinase is proposed as the principal effector of thylakoid protein phosphorylation and associated State transition phenomena.

In chloroplasts, efficient co-operation of the two photosystems in tandem requires control mechanisms that balance their electron throughput. One such mechanism, as yet poorly understood, senses the redox status of the plastoquinone pool that serves as an electron buffer between the photosystems and as a vehicle for dispersion of reductant throughout the membrane. As the plastoquinone pool becomes reduced, the activity of a membrane-bound protein kinase is elicited (1). This enzyme phosphorylates a threonine residue near the N terminus of the light-harvesting chlorophyll \( a/b \) protein complex (LHC)\(^2 \) (3, 4), leading to electrostatic repulsion of some LHC away from photosystem II and toward association with photosystem I (5). In consequence, a higher proportion of excitation energy becomes available to photosystem I, net oxidation of plastoquinone is favored, and the kinase tends toward deactivation (6). This mechanism for balancing photosystem turnovers seems to operate within the time frame of several minutes (7); together with redox-independent dephosphorylation of LHC catalyzed by a membrane-bound phosphoprotein phosphatase (8), it is believed to account for the phenomenon of State transition in green plant photosynthesis (8).

Both the LHC kinase and LHC itself are intrinsic to the thylakoid membrane (9). LHC is a major membrane protein that contains 50-60% of the total chlorophyll and can be isolated without difficulty. LHC kinase, however, has remained elusive. Two protein kinases isolated from thylakoids by Lin et al. (10) and Lucero et al. (11) could not utilize LHC as a substrate. Clark et al. (12) showed that a procedure developed for isolating the cytochrome \( b/f \) complex from thylakoids led to solubilization of some protein kinase activity. The discovery that lysine-rich histones could serve as efficient substrates for this enzyme (12) facilitated its partial purification. Fractions enriched in histone kinase activity could phosphorylate solubilized LHC at the site phosphorylated in situ (3, 4). However, the reaction rates were low, and these fractions were also enriched in ferredoxin:NADP\(^{+} \) oxidoreductase and minor polypeptides among which the kinase could not be identified.

We now report the purification of this protein kinase to apparent homogeneity and document some of its important characteristics.

EXPERIMENTAL PROCEDURES

Materials—Spinach (Spinacea oleracea cultivar Hybrid 424, Park Seed Company) was cultivated in a growth chamber under conditions previously described (13). Leaves were washed and de-veined and then stored overnight at 4°C before use. Carrier-free \( \gamma -{\text{32P}} \)ATP was obtained from New England Nuclear. Octyl glucoside was supplied by Behring Diagnostics. Plastocyanine-1 was a gift of G. Hauska, University of Regensburg. Sodium cholate, casein, and purified histone fractions were from Sigma. Total histone was from Worthington. Cibacron blue F3GA was obtained as the free dye (Reactive Blue 2) from Sigma and dissolved in 100-200 mesh agarose (Affi-Gel blue), from Bio-Rad. The identity of this dye is still unconfirmed. Other chemicals were of highest commercial purity.

Assays and Analyses—Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as in Ref. 15. Gels were fixed and stained with Coomassie Brilliant Blue (15) or by silver nitrate (16). Protein was determined by the Bradford method (17) except where high concentrations of detergent were present and then the method of Bensadoun and Weinstein (18) was used. Cytochrome \( b \) and \( f \) contents and plastocyanin-plastocyanin oxidoreductase activity were determined as in Ref. 13; ferredoxin:NADP\(^{+} \) oxidoreductase activity was determined according to Vallejos et al. (19) and chlorophyll content according to Arnon (20). The density of the gradient fractions was estimated with an Abbé refractometer.

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The filter paper method of Lin et al. (10) was used for protein kinase assays except that 5 mM dithiothreitol and 250 µg ml−1 histone were present in the incubation mixture. LHC was isolated (21) under subdued light from spinach leaves which had been stored in the dark for 24 h at 4 °C. Phosphorylation of LHC was assayed as in Clark et al. (12) with omission of ascorbate and addition of 5 mM dithiothreitol. The radioactive gels were dried down and allowed to expose x-ray film through an intensifier screen for 2–4 days at −70 °C and were developed as before (12). Radioactive ink was used to visualize molecular weight markers. For quantitation of radioactivity, relevant areas of the dried gel were excised, placed in scintillation vial inserts, supplemented with 3 ml of Aquasol scintillation fluid, and counted in a Beckman LS-33 scintillation counter (open window setting).

Isolation and Treatment of Thylakoid Membranes—Thylakoids devoid of coupling factor and residual stomatal contamination were prepared by a modification of the method of Hurt and Hauska (22): 300 g of leaves were ground for 10 s at full speed in a 1-gallon Waring blender with 1 liter of 0.4 mM sucrose, 3 mM MgCl₂, 2 mM sodium ascorbate, and 25 mM sodium Tricine (pH 8.0). The homogenate was filtered through two, then eight layers of muslin, then centrifuged for 20 min at 12,000 × g in a Sorvall GSA rotor (6 × 100 ml). The pellet was resuspended in 400 ml of 1.0 M NaR₃, 10 mM sodium chloride, 10 mM Tricine (pH 8.0), and 30% ethanol, before centrifugation at 30,000 × g for 1 h. The supernatant was carefully decanted and the pellet resuspended in 450 µl of 1.0 M NaBr, 10 mM sodium Tricine (pH 8.0), and stirred gently on ice for 30 min to dissociate the coupling factor complex. The membranes were then centrifuged for 5 min at 10,000 × g (Sorvall GS-33 rotor, 6 × 100 ml), rinsed once in grinding medium without ascorbate, rinsed twice with 300 ml of 1.0 M NaCl, 10 mM sodium Tricine (pH 8.0), and desalted by dialyzing for 12 h against 1 liter of 0.1% Triton, 10 mM Tricine, and 10 mM Mes (pH 6.5), 0.1 mM PMSF, and 0.1% sodium deoxycholate, 4% 2-mercaptoethanol, 25% glycerol, 25 mM Tris chloride (pH 8.0). Residual acidity was removed by addition of 5 µl of 1 M Tris chloride (pH 8.0). The samples were electrophoresed and the gel stained and autoradiographed as described under “Experimental Procedures”. Gradient fractions are described from bottom (left) to top (right); fraction 0 = pellet.

![Fig. 1](image1.jpg)

**Fig. 1.** Autoradiograph of ³²P-labeled P300 eluate, before and after fractionation on a sucrose density gradient, directed toward endogenous substrates or added histone. Lanes A and B show the results of incubating P300 material with [γ⁻³²P]ATP for 20 min in absence (A) and presence (B) of histone V-S, as described under “Experimental Procedures”. Fractions 0–17 show sucrose gradient fractions prepared from P300 eluate and individually assayed for kinase activity by incubation with histone and [γ⁻³²P]ATP, followed by electrophoresis and autoradiography. Gradient fractions (25 µl) were added to 25 µl of a substrate mixture to give 0.1% Triton X-100, 10 mM MgCl₂, 10 mM NaF, 0.5 mM ATP, 250 µg/ml histone V-S, 10 mM sodium Tricine (pH 8.0), and 1 µCi of [γ⁻³²P]ATP and were incubated for 15 min at 25 °C. The reaction was quenched on ice by addition of 50 µl of 25% (w/v) trichloroacetic acid. After 30 min, the samples were centrifuged (1 min, 12,000 × g). The pellets were rinsed with 400 µl of 5% (w/v) trichloroacetic acid, then 400 µl of etherethanol (1:1, v/v), and dissolved in 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 25% glycerol, 25 mM Tris chloride (pH 8.0). The samples were electrophoresed and the gel stained and autoradiographed as described under “Experimental Procedures”. Gradient fractions are described from bottom (left) to top (right); fraction 0 = pellet.

![Fig. 2](image2.jpg)

**Fig. 2.** Autoradiograph showing protein kinase activity of the P300 material, before and after fractionation on a sucrose density gradient, directed toward endogenous substrates or added histone. Lanes A and B show the results of incubating P300 material with [γ⁻³²P]ATP for 20 min in absence (A) and presence (B) of histone V-S, as described under “Experimental Procedures”. Fractions 0–17 show sucrose gradient fractions prepared from P300 eluate and individually assayed for kinase activity by incubation with histone and [γ⁻³²P]ATP, followed by electrophoresis and autoradiography. Gradient fractions (25 µl) were added to 25 µl of a substrate mixture to give 0.1% Triton X-100, 10 mM MgCl₂, 10 mM NaF, 0.5 mM ATP, 250 µg/ml histone V-S, 10 mM sodium Tricine (pH 8.0), and 1 µCi of [γ⁻³²P]ATP and were incubated for 15 min at 25 °C. The reaction was quenched on ice by addition of 50 µl of 25% (w/v) trichloroacetic acid. After 30 min, the samples were centrifuged (1 min, 12,000 × g). The pellets were rinsed with 400 µl of 5% (w/v) trichloroacetic acid, then 400 µl of etherethanol (1:1, v/v), and dissolved in 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 25% glycerol, 25 mM Tris chloride (pH 8.0). The samples were electrophoresed and the gel stained and autoradiographed as described under “Experimental Procedures”. Gradient fractions are described from bottom (left) to top (right); fraction 0 = pellet.

with 200 ml of 10 mM NaCl, 10 mM sodium Tricine (pH 8.0), and resuspended in this same buffer to a final concentration of 2.3 mg of chlorophyll/ml. Isolation and treatment of thylakoid membranes was performed at 0 °C. All solutions contained 0.1 mM PMSF as a proteolysis inhibitor.

**Extraction of Protein Kinase—**Protein kinases were extracted from treated thylakoids by a modification of the method of Hurt and Hauska (22) for isolation of the cytochrome b₅ complex. The final NaBr-washed thylakoid suspension was slowly diluted 1:1 with 60 mM octyl glucoside, 25 mM sodium cholate, 0.1 mM PMSF, 20 mM sodium Tricine (pH 8.0), and stirred gently on ice for 1 h. The suspension was then centrifuged (Beckman Ti-60, 8 × 30 ml) for 1 h at 300,000 × g. The supernatant was carefully decanted and the pellet resuspended in 0.5% Triton X-100, 10 mM Tricine (pH 8.0) to a final concentration of 2 mg of chlorophyll/ml. Solid (NH₄)₂SO₄ was added to the supernatant to give 40% saturation and then the suspension was left for 20 min on ice and centrifuged (Sorvall GSA rotor, 6 × 100 ml) for 15 min at 12,000 × g. Solid (NH₄)₂SO₄ was again added to the supernatant to a final saturation of 60%. After 20 min at 0 °C, the suspension was centrifuged as before, the supernatant decanted, and solid (NH₄)₂SO₄ added to 90% saturation. The suspension was again left for 20 min, then centrifuged, and the supernatant decanted. The pellets from 0 to 40% and 60 to 90% of saturating (NH₄)₂SO₄ were separately redissolved in minimum volumes of 0.1% Triton, 10 mM sodium Tricine (pH 8.0), and desalted by dialyzing for 12 h against 1 liter of each of the same buffer. The 40–60% pellet was redissolved in 30 mM octyl glucoside, 12.5 mM sodium cholate, 0.1 mM PMSF, 10 mM sodium Tricine (pH 8.0), and desalted by passage through a Bio-Gel P-300 column (20 × 2 cm) pre-equilibrated with 10 mM CHAPS, 0.1 mM PMSF, 10 mM sodium Tricine (pH 8.0). This product is referred to as “P300 eluate.” It was stored in liquid N₂ after the addition of glycerol to 5% (v/v) and a few grams of PMSF.

**Purification of Protein Kinase—**The 40–60% ammonium sulfate fraction
Purification of Thylakoid Protein Kinase

Fig. 3. Autoradiograph showing protein kinase activity of P300 eluate and its density gradient fractions, with isolated LHC as substrate. Experimental conditions were as described for Fig. 2, except that LHC (250 ng of protein/ml) was present in the P300 material without (A) and with (B) added substrate (LHC). Fractions 6-13 show the only gradient fractions containing 32P-phosphoproteins.

Fig. 4. Analysis of proteins in P300 eluate by density gradient fractionation, denaturing gel electrophoresis, and silver staining. Experimental conditions were as described in Fig. 1, but the P300 eluate was applied to the gradient directly, without exposure to [32P]ATP. Fraction numbering corresponds to the system of Figs. 1 and 2. Unfractionated P300 eluate is also shown.

Fig. 5. Distribution of minor polypeptide components in the range 40-70 kDa in the density gradient fractions of P300 eluate. Experimental procedures were as described for Fig. 1, except that silver staining was prolonged to emphasize minor constituents. Arrow, 32P-labeled band.

contains most of the protein kinase activity as well as the cytochrome b6 complex. P300 eluate (45 nmol of cytochrome f) was applied to a 38-ml, 10-30% (w/v) sucrose gradient containing 10 mM CHAPS, 0.1% asolectin, 15 mM sodium Mes (pH 6.5), and centrifuged for 8 h at 250,000 × g (VT50 Beckman rotor, A × 40 ml). Gradient fractions (1.2 ml) were frozen in liquid N2 and stored at −70°C. Fractions containing kinase activity were pooled and purified by affinity column chromatography, using either Affi-Gel blue or lysine-rich histone linked to Sepharose.

For the former procedure, kinase-enriched fractions (5 ml total) from the sucrose density gradient were made to 0.5% (v/v) with 10% (v/v) Triton X-100 and loaded on a 1-ml (0.5 × 6.5 cm) Affi-Gel blue column, pre-equilibrated with 0.1% (v/v) Triton X-100, 10 mM sodium Tricine (pH 8.0), 0.1 mM PMSF at 4°C. After 10 min, the throughput was collected (fraction 1). The column was washed sequentially with 2 ml of equilibration buffer (fraction 2) and then 0.5 ml each of equilibration buffer supplemented with 50 mM NaCl (fraction 3); 50 mM NaCl, 5 mM NADP* (fraction 4); 50 mM NaCl (fraction 5); 50 mM NaCl, 5 mM ATP (fraction 6); 50 mM NaCl (fraction 7); 0.25 M NaCl (fraction 8); 0.5 M NaCl (fraction 9); 1 M NaCl (fraction 10); 2 M NaCl (fraction 11); 2 ml of buffer alone (fraction 12); 1 ml of 10 mM CHAPS in buffer (fraction 13), and 0.5 ml of 1% (w/v) lithium dodecyl sulfate (fraction 14).

For purification on a histone affinity column, CNBr-activated Sepharose 4B was coupled to histone III-S essentially as described by Cuatrecasas (23, 24). Swollen, activated Sepharose (10 ml) was reacted with 50 mg of histone III-S in 0.1 M NaHCO3 buffer (pH 8.5); under our experimental conditions, the coupling efficiency was 100%, and the final yield was 5 mg of histone/ml of packed Sepharose. The pooled sucrose gradient fractions were loaded onto a 1-ml histone III-S Sepharose column (6 × 0.5 cm) previously equilibrated with 10 mM CHAPS, 0.1 mM PMSF, 10 mM sodium Tricine (pH 8.0). The column was washed with 5 ml of equilibration buffer and then with a further 2 ml containing 50 mM NaCl. Bound kinase activity was eluted with 0.25 M NaCl in equilibration buffer, dialyzed for 12 h at 0°C against 1 liter of buffer, and then stored at −70°C.

RESULTS

Phosphoproteins in P300 Eluate—As a preliminary control, we examined the reactivity of kinase(s) in P300 eluate toward accompanying protein substrates. P300 eluate was incubated with [γ-32P]ATP and then subjected to denaturing gel electrophoresis. Autoradiography revealed that polypeptides of 9- and 25-kDa molecular mass had become strongly labeled (Fig. 1). A third component of mass ~64 kDa also incorporated 32P. Density gradient centrifugation of the phosphorylated P300 material substantially resolved the radioactive phosphoproteins, with the 64-kDa phosphoprotein migrating furthest down the gradient, as shown in Fig. 1.

Protein Kinase Activity of P300 Eluate—P300 eluate was incubated with [γ-32P]ATP and with lysine-rich histone, whereupon extensive incorporation of radioactivity into histone occurred. Although concomitant labeling of the endogenous 9-kDa component was suppressed, labeling of the 64-kDa species was relatively unaffected, remaining at about 5-10% of the histone level (Fig. 2, lane B). Fractionation of

2 Relative to the 64- and 9-kDa phosphoproteins, the 25-kDa component is less prominent in the minus-histone control (Fig. 2, lane A) than was observed in Fig. 1. This variability may result from different phosphorylation kinetics.
Enzymatic profile of density gradient fractions of P300 eluate. Fractions and assays are described under "Experimental Procedures". Fractions are labeled as in Fig. 4.

- P300 eluate. This narrow peak between fractions 9-11 more closely resembles that of the kinase activity profile, centering around fraction 5 (Figs. 2 and 3). Two possible candidates which are not resolved by the gradient. The most prominent, seen in fraction 5, is a component of M, ~37,000, previously identified as ferredoxin:NADP⁺ oxidoreductase (25). The second candidate, barely visible in Fig. 4 but clearly seen in Fig. 5 (which is the relevant part of the silver-stained gel from which the autoradiogram of Fig. 2 was derived), is a polypeptide of ~64 kDa. This is presumably the phosphoprotein detected by autoradiography in Figs. 1-3.

Fig. 6 is an analysis of enzyme activities in sucrose gradient fractions. It confirms that the 37-kDa species is the reductase, by reason of its diaphorase activity. In contrast to an earlier study (13) the broad peak of diaphorase activity was well separated from the cytochrome b/f complex. Although there was substantial overlap between histone kinase and diaphorase activities, the kinase migrated to slightly higher buoyant density and possessed a much narrower distribution profile. This narrow peak between fractions 9-11 more closely resembl-
Purification of Thylakoid Protein Kinase

Affinity Chromatography Histone-Sepharose

**Fig. 8.** Affinity chromatography of the kinase-enriched density gradient fractions on histone Sepharose: profile of enzyme activities.

An aliquot (1 ml) of the pooled, kinase-enriched fractions from sucrose density gradient centrifugation was mixed with 0.3 ml of histone III-S cross-linked to Sepharose (pre-equilibrated with 10 mM CHAPS, 10 mM sodium Tricine (pH 8.0), 0.1 mM PMSF) and slowly shaken on ice for 15 min. The slurry was poured into a 2 × 0.5-cm column and the throughput (fraction 1) collected. The packed column was washed with 2 ml of equilibration buffer (fraction 2), 1 ml of buffer containing 25 mM NaCl (fraction 3), then with successive 0.5-ml aliquots of buffer containing 50, 100, 250, and 500 mM, 1 M, and 2 M NaCl (fractions 4–9). The fractions were desalted by dialysis against 1 liter of 10 mM sodium Tricine (pH 8.0), 0.2 mM EDTA, 0.1 mM PMSF.

Enzyme activities and protein content were assayed as described under "Experimental Procedures". protein kinase activity; a total activity of 400 pmol of phosphate incorporated into histone III-S/min was loaded, 112% of which was recovered in fractions 6–7. protein content (μg/fraction); 250 μg of protein was loaded on the column and 255 μg recovered. NaCl concentration (mM).

**Fig. 9.** Affinity chromatography of the kinase-enriched density gradient fractions on a histone-Sepharose column: gel electrophoretic analysis of column eluate. Aliquots (30 μl) of the column eluate fractions were processed as in Fig. 1 (legend). Gel lanes represent the fractions described in Fig. 8 (lane 0: sample as applied).

On the above evidence alone, definitive identification of the kinase polypeptide could not be made. When, however, the kinase-enriched gradient fractions were pooled and subjected to chromatography on Affi-Gel blue (Fig. 7), all protein kinase activity (capable of phosphorylating 400 pmol of histone V-S/min) was bound to the column. No measurable kinase activity was eluted by NADP+, ATP, 2 M NaCl, 1% (v/v) Triton X-100, or 10 mM CHAPS. In contrast, 96% of the diaphorase activity was recovered by eluting the column with NADP+ (data not shown). A final treatment of the column with denaturing detergent (1% (w/v) lithium dodecyl sulfate) released all the remaining protein of molecular mass 64 kDa (Fig. 7A, lane 14). A small amount of a 33-kDa protein, also present in the throughput and initial wash, could be removed from this final product by washing the column with 20–30 bed volumes of equilibration buffer prior to elution with NaCl (lane 14b).

Identity between the 64-kDa protein and the phosphoprotein of Figs. 1–3 was established by preincubating the concentrated, kinase-enriched, sucrose gradient fractions with [γ-32P]ATP before application to the Affi-Gel blue column. After developing the column as before, elution with 1% (w/v) lithium dodecyl sulfate removed most of the radioactivity in the form of a protein of molecular mass ~64 kDa (Fig. 7B). There was no kinase activity associated with any of the collected fractions.
Purification of Thylakoid Protein Kinase

Purification of a protein kinase from spinach thylakoids

| Fraction          | Volume | Protein | Chlorophyll | Cytochrome f | Reductase | Kinase total | Kinase specific activity | Kinase yield | Purification |
|-------------------|--------|---------|-------------|--------------|-----------|--------------|-------------------------|--------------|--------------|
| Thylakoids        | 268    | 715     | 183         | 300          | 1.1       | 36           | 50                      | 100          | 0            |
| NaBr-washed thylakoids | 80     | 512     | 180         | 300          | 1.0       | 30           | 58                      | 83           | 0            |
| Detergent suspension | 160   | 500     | 180         | 250          | 1.1       | 0.7          |                         |              |              |
| Supernatant       | 152    | 150     | 20          | 200          | 1.1       | 0.5          |                         |              |              |
| Pellet            | 380    | 180     | 50          |              |           | 0.1         | 0.05                    |              |              |
| (NH₄)₂SO₄ 0-40%   | 1.2    | 26      | 1.5         | 3            |           | 0.4         | 0.02                    |              |              |
| 40-60% supernatant| 2.6    | 40      | 0.6         | 190          | 0.4       | 2.5         | 62.5                    | 7            | 1.25         |
| P300 eluate       | 5.3    | 33      | 0.4         | 170          | 0.4       | 5.0         | 150                     | 14           | 3            |
| Sucrose gradient  | 12     | 2       | 0.2         |              |           | 4.0         | 2,500                   | 11           | 50           |
| Histone column    | 1.0    | 0.13    | 4.0         |              |           | 30,000      | 11 (0.02)               | 600          |              |

*Total ferredoxin:NADP⁺ oxidoreductase activity, millimoles of 2,6-dichlorophenolindophenol reduced min⁻¹.

*Total activity, nanomoles of phosphate incorporated into histone V-S min⁻¹.

Specific activity, picomoles of phosphate incorporated into histone V-S min⁻¹ (mg protein)⁻¹.

Relative to the total activity of thylakoids in phosphorylating histone V-S.

*The value for % yield in parentheses is for protein, relative to the starting material.

The ability to recover diaphorase but not kinase activity from Affi-Gel blue is evidence against reductase and in favor of the alternative candidate, the 64-kDa protein, as being the kinase. This view is reinforced by the more closely comparable distributions of the 64-kDa protein and kinase activity on sucrose density gradients, noted above. Positive proof of this identity was sought by fractionation of the combined, enriched sucrose gradient fractions on adsorbents which might permit elution of the 64-kDa protein under nondenaturating conditions.

Success was realized using specific adsorption of kinase to lysine-rich histone covalently linked to Sepharose. Fig. 8 shows a column elution profile obtained using this affinity procedure. All diaphorase activity and about 90% of the total protein loaded on the column were recovered in the throughput and initial washings; all histone kinase activity remained bound to the column. Development with a discontinuous NaCl gradient yielded the kinase between 0.25 and 0.5 M NaCl (112% recovery of activity in ~5% of the total applied protein). Analysis of these column fractions by denaturing gel electrophoresis followed by silver staining revealed the presence of a single polypeptide of apparent molecular mass ~64 kDa (Fig. 9). If the sucrose gradient fractions were incubated with [γ³²P]ATP as before (Fig. 7), but with the omission of Triton X-100, and the affinity chromatography procedure was repeated, a single radioactive polypeptide of apparent molecular mass 64 kDa was recovered in precise relation to the previously documented kinase activity data (not shown). It can thus be concluded that the same 64-kDa polypeptide which is phosphorylated by ATP in the absence of substrate is, in fact, a protein kinase. Table I shows recoveries and activities throughout the purification procedure. The ~20% loss in activity on the sucrose gradient is consistent with identification of the minor ~64-kDa component (Fig. 1, fraction 5) as aggregated, denatured kinase.

Enzymological Properties of the Kinase—The purified kinase is similar to the protein kinases isolated by Lin et al. (10) in that lysine-rich histones (III-S, V-S) are labeled preferentially while arginine-rich histones are inactive as substrates (Table II). Casein and LHC are also labeled but less extensively (~30 and 10%, respectively, in relation to histone III-S). With histone III-S as substrate, the Kₘ for ATP was ~35 μM, the Vₘₐₓ 30–50 nmol min⁻¹ (mg protein)⁻¹, and the pH optimum was 8.0 (Fig. 10). Mg²⁺ ion was required for activity. Dithiothreitol (5 mM) gave a 2–3-fold increase in activity, but reductants such as dithionite and plastoquinol-I were without effect (data not shown). Inclusion of asolectin in reaction mixtures did not improve activity and resulted in substantially higher radioactive background in the filter paper adsorption assay. Incubation of purified enzyme for 30 min with ATP led to phosphorylation of 1–10% of the total kinase present (not shown), essentially as noted for P300 material (Figs. 1–3). The rate of autophosphorylation was not inhibited by concomitant phosphorylation of histone or LHC nor did...
the resultant difference in level of enzyme phosphorylation measurably influence its catalytic activity toward these substrates.

**DISCUSSION**

The literature on protein kinases from animal tissues abounds with reports of autophosphorylation, for which a function is usually unclear. Most commonly, autophosphorylation is intramolecular, is enhanced by concomitant substrate phosphorylation, and occurs at a site distinct from the catalytic site (26-28). The significance of autophosphorylation is intramolecular, is enhanced by concomitant substrate phosphorylation, and occurs at a site distinct from the catalytic site (26-28). The significance of autophosphorylation is intramolecular, is enhanced by concomitant substrate phosphorylation, and occurs at a site distinct from the catalytic site (26-28).

The strong binding of the 64-kDa kinase to Affi-Gel blue is reminiscent of the findings of Holston et al. (26) for the P protein of the histidine permease of Salmonella typhimurium, which also required denaturing conditions for its elution. The affinity of bound dye for the nucleotide-binding site is, in such cases, considered less significant than the contribution from hydrophobic interactions. This is apparent here, upon comparison of the relative sensitivities of the reductase and kinase to inhibition by the free dye, Cibacron Blue, and the corresponding affinities of these enzymes for Affi-Gel blue. The Km for the reductase with respect to Cibacron Blue is ~3 μM (29), and the dye is a strong inhibitor of diaphorase activity (Km = 1.5 μM); hence, even lower Km values would be predicted for the more tightly adsorbed kinase (Fig. 7). Cibacron Blue was indeed found to inhibit histone phosphorylation,3 but the Km was ~75 μM. This is inconsistent with the observed behavior of kinase on Affi-Gel blue, and hydrophobic interactions considerably stronger than those invoked (29) for the reductase must exist. In turn, this suggests that the kinase is a hydrophobic protein which may be deeply buried in or may span the thylakoid membrane.

This is the first reported purification of a thylakoid protein kinase that supports significant rates of LHC phosphorylation, and we propose that the 64-kDa polypeptide is the principal protein kinase of the thylakoid membrane. Its Vmax (with histone) is 50-130 times greater than values reported previously for thylakoid kinases (10, 11) and compares favorably with values reported for soluble kinases from animal cells (30). The Km for ATP is close to the 25 μM determined (10, 11) for kinases solubilized from spinach thylakoids. It also approximates a value (40 μM) obtained (31) for in situ phosphorylation of pea LHC.

Our isolation protocol follows the strategy of Lin et al. (10) in employing octyl glucoside and cholate to solubilize kinase from the membrane, but whereas they used a modification of the method of Pick and Racker (32) for CF1-CF0 isolation, we adapted a version of that method used (22) for isolation of the cytochrome b6f complex. There is no a priori reason to expect major differences between the two sets of work; hence, our conclusion that a 64-kDa protein accounts for most of the kinase activity, whereas Lin et al. (10) did not detect this component (claiming instead to have identified 25- and 38-kDa proteins as kinases), is surprising. Their identification procedure rested primarily on the use of 8-azido-ATP as a photoaffinity label. In our experience, this reagent clearly cross-links to a number of thylakoid proteins throughout the kinase isolation procedure, but is ineffective as a probe for the kinase itself.3

Farchaus et al. (33) claim to have identified a 50-kDa component of the thylakoid membrane as a candidate for the LHC kinase, on the basis of studies with the nucleotide affinity inhibitor 5′-p-fluorosulfonylbenzoyladenosine. There is no evidence of such a protein in the present or previous studies (10-12), although it may well exist in the membrane and be resistant to extraction by octyl glucoside and cholate. Direct evidence for kinase activity associated with the 50-kDa component is required.

Physiological rates of LHC phosphorylation are between 0.1 and 1.0 nmol of phosphate incorporated min⁻¹ (mg membrane protein)⁻¹ (3, 6, 33). The specific activity of purified 64-kDa kinase, using isolated LHC as a substrate, was between 3 and 10 nmol min⁻¹ (mg enzyme)⁻¹. A simplistic calculation that assumes random distribution of the enzyme in the membrane and a concentration there of ~0.01% relative to total protein suggests that a specific activity of ~1000 nmol of phosphate incorporated min⁻¹ (mg enzyme)⁻¹ would be required to account for in vivo rates of LHC phosphorylation. Thus, there is a factor of 100-300 shortfall in the activity of purified LHC kinase toward micellar LHC. In contrast, the in vitro rates of histone phosphorylation (30-60 nmol min⁻¹ mg protein⁻¹) by purified and in situ LHC kinases are comparable.

The low activity of solubilized kinases toward solubilized LHC is unsurprising in the light of recent studies3 showing that detergent treatments too mild to solubilize kinase or LHC nonetheless inactivate in situ LHC phosphorylation. Detergent-induced modifications to the enzyme or substrate can be invoked in explanation; however, the relatively high rate of histone phosphorylation clearly indicates some inadequacy of isolated LHC as a substrate. LHC isolated in Triton X-100, for example, has undergone conformational change as judged by circular dichroism studies (34) and may be a less suitable substrate for phosphorylation than is the native protein. It is also difficult to saturate the enzyme with LHC.

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3 S. J. Coughlan and G. Hind, manuscript in preparation.
in detergent micelles because inhibition by detergent super-venes. In the thylakoid, by contrast, interaction between enzyme and substrate may be sterically favored, and the enzyme may be concentrated at the margins of the grana stacks.

The lack of redox control over the kinase activity of the solubilized enzyme signals additional complexity and may arise from the dissociation of an unidentified, plastoquinone-binding, regulatory subunit. The majority of known protein kinases are oligomers, often involving regulatory subunits; thus, the molecular mass of the native holoenzyme may be significantly higher than the 64 kDa indicated by denaturing gel electrophoresis.

We are attempting to reconstitute LHC and kinase into a lipid environment to evaluate the influence of steric factors on phosphotransferase activity. Antibodies are being raised to the isolated kinase to probe for its involvement in LHC phosphorylation in situ and in State transitions.

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