Phosphorylation of Chloroplast Ribulose Bisphosphate Carboxylase/oxygenase Small Subunit by an Envelope-bound Protein Kinase in Situ

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A new protein kinase of the cAMP independent type was found to be bound to the outer envelope membrane of spinach chloroplasts. While stimulated by Mg** and inhibited by ADP, the enzyme showed no response to conventional protein substrates and was essentially independent of pH in the physiological (pH 7 to 8) range. The new protein kinase phosphorylated the mature form of the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (henceforth called Rbu-Pn) in the cytoplasm (1, 2), thus, a small polypeptide, designated the transit peptide, is attached to the protein during translation and serves as a substrate for a Mg-stimulated protein kinase. The synthesis of the small subunit is dependent on energy (ATP) at 30°C by rupturing the hypotonic buffer solution followed by centrifugation on a 3-phase discontinuous sucrose gradient (1.2, 0.93, 0.6 M sucrose) (7). When protease inhibitors were used, they were present throughout the entire isolation procedure.

Separation of Inner and Outer Envelope Membranes—Purified chloroplasts (equivalent to 50 mg of chlorophyll) were suspended in hypotonic sucrose solution, ruptured by a freeze/thaw cycle, and purified by two consecutive sucrose gradients essentially as described by Cline et al. (8), except that centrifugation times were shortened from 14 to 8 h and EDTA was omitted.

Thermolysin Treatment—Intact chloroplasts (equivalent to 20 mg of chlorophyll) were incubated with Bacillus thermoproteolyticus thermolysin in 0.33 M sorbitol, 25 mM Tricine-KOH buffer, pH 7.7, 5 mM CaCl₂ for 30 min at 4°C (135 µg of thermolysin/mg of chlorophyll). The reaction was stopped by adding EDTA (final concentration, 5 mM) and the chloroplasts were resuspended on a Percoll gradient. The envelope fraction from the treated chloroplasts was isolated as described above. Control experiments (untreated chloroplasts) were done in the same way except that thermolysin was omitted.

Assay of Protein Kinase Activity—Protein kinase activity was measured by following the incorporation of [γ-32P]ATP into the 18 kDa chloroplastic acid and ethanol insoluble protein fraction. Standard incubation mixtures (final volume 0.5 ml) contained 50 mM Tricine-KOH, pH 7.7, 4 mM MgCl₂, 100 µM ATP containing 3 pCi [γ-32P]ATP and the indicated amounts of envelope membranes. The reaction was incubated at 22°C for 10 min unless otherwise specified, and appropriate aliquots were removed and added to an Eppendorf microtube containing 0.5 ml of 12% (w/v) trichloroacetic acid to stop the reaction. Following centrifugation in an Eppendorf microfuge (4 min), the precipitated protein was subjected to three washings with 0.5 ml of 0.5% trichloroacetic acid and centrifuged at 12,000 x g for 10 min in a Beckman microfuge. The pellet was solubilized in 0.1 M NaOH and radioactivity was determined by liquid scintillation counting using a Beckman scintillation counter (LS100C) and a 2,5-diphenyloxazole-based scintillation fluid (Bioclear obtained from ICN, Irvine, CA). Alternatively, when proteins were analyzed, the ethanol-washed pellet was subjected to polyacrylamide gel electrophoresis as described below.

Electrophoretic Analysis of [32P]-labeled Envelope Membranes—SDS- and LDS-polyacrylamide gel electrophoresis were used to analyze unlabeled and [32P]-labeled chloroplast envelope membrane polypeptides. Following the ethanol wash described above, envelope membranes were solubilized at room temperature in a solution containing 50 mM Tricine-KOH buffer, pH 7.7, 300 mM β-mercaptoethanol, 125 mM sucrose, and either 4% SDS or 4% LDS. Electrophoresis was carried out in slab gels in four different ways: (i) at 4°C in LDS gels containing 20% polyacrylamide; (ii) at 4°C in LDS gels containing 10-15% polyacrylamide gradient; (iii) at room temperature in SDS gels containing 8 M urea and a 12-18% linear polyacrylamide gradient; and (iv) in two-dimensional gels developed (first direction) at 4°C in LDS gels containing 7.5-15% polyacrylamide and
(second direction) at room temperature in SDS gels containing 12–18% polyacrylamide and 8 M urea (9). The experimental procedures for gel preparation, sample solubilization, electrophoresis, and staining were those described extensively by Chua (10). 32P-labeled polypeptides were localized by autoradiography.

Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Cyanogenbromide-treated Paper—Polypeptides were transferred from polyacrylamide slab gels to cyanogenbromide-treated paper using procedures recently described (11). After transfer, the paper loaded with the proteins was treated with monospecific rabbit antibody raised against Rbu-Pz carboxylase/oxygenase (kindly provided by Prof. W. B. Taylor, Department of Genetics, University of California, Berkeley). 125I label was introduced by treatment with iodinated Staphylococcus aureus protein A (11). Autoradiography was used to visualize labeled zones which were cut out and counted in a scintillation counter equipped with the capability to discriminate the 32P from 125I radiation.

Protein Determination—Protein was estimated by the dye binding method of Bradford (12).

RESULTS

Demonstration of a Protein Kinase in Chloroplast Envelope Fraction—Chloroplast thylakoids are known to contain a bound protein kinase which, when activated by photoreduced plastoquinone, catalyzes the phosphorylation of a 24-kDa light-harvesting chlorophyll protein (13). Very recently, chloroplasts have been reported to contain two other membrane-bound protein kinases which are solubilized by treating the total membrane (thylakoid + envelope) fraction with detergents (14). To date, a protein kinase has not been reported to occur specifically in the envelope fraction.

Therefore, we have examined purified chloroplast envelope fractions for such an enzyme by testing their capability to incorporate 32P from [γ-32P]ATP into the trichloroacetic acid insoluble protein fraction. Under the assay conditions used (i.e. dark), there was no significant protein kinase activity associated with either the soluble protein (chloroplast extract) or thylakoid fraction, but there was active 32P incorporation by the isolated envelopes (Fig. 1). This activity was consistently present in fresh envelope preparations and was observed irrespective of the source of the parent leaves (greenhouse or market). Interestingly, in contrast to the thylakoid enzyme described earlier (13), the envelope-bound protein kinase was unaffected by added plastohydroquinone or plastoquinone, both at 0.5 mM. The activity of the envelope enzyme was also unaffected when assayed under illumination or in the presence of chloroplast extract (data not shown). These properties thus differentiate the envelope-bound protein kinase from the plastohydroquinone-linked thylakoid enzyme (13) and, in addition, show that the 32P acceptor(s) is bound in an accessible manner to an envelope membrane.

Some Properties of the Envelope-bound Protein Kinase—Chloroplast envelope-bound protein kinase activity was proportional to the concentration of ATP (Fig. 2) and, under optimal conditions, showed a Vmax of 56 pmol of 32P bound per mg of protein and an S0.5 for ATP of 37 μM. ADP inhibited the enzyme in a competitive manner, with respective inhibitions of 15, 25, and 45% being observed when the ATP:ADP ratio was 1:1, 1:2, and 1:5. As determined by a Dixon plot (15), the Ks for ADP was 255 μM (Fig. 3). Neither AMP (up to 0.2 mM) nor cAMP (up to 0.1 mM) had an effect on the enzyme irrespective of the presence of ADP (data not shown). Thus, of the adenosine nucleoside mono- and diphosphates tested, only ADP was found to alter activity of the protein kinase. A number of other compounds tested were also without effect on the enzyme, viz. NADP and NADPH (0.2 mM); NAD and NADH (0.4 mM); PPi (0.2 mM); Pi (5 mM); dithiothreitol (5 mM); thioredoxin f or m (100 μg) either oxidized or reduced by dithiothreitol; sodium fluoride (10 mM); and protein substrates, casein, phosphitin, and histone (250 μg). Thus, in short, the envelope fraction represented a self-contained protein phosphorylation system with both the protein kinase enzyme and its substrate being present. The envelope-bound protein kinase showed equal activities at pH 7.0, 7.4, and 7.7; its activity at pH 8.0 was about 90% that at the more acidic values.

Other than ATP, the only requirement displayed by the envelope-bound protein kinase was a divalent cation, i.e. Mg2+ or, less effectively, Mn2+ (Fig. 4). Ca2+ was without effect. EDTA or EGTA at 10 or 5 mM completely abolished the kinase activity, and only the inhibition at 5 mM could be partly (50%) overcome by adding Mg2+.

Localization of Envelope-bound Protein Kinase—The chloroplast envelope fraction used in the above experiments consisted of both the inner and outer membrane components. In initial attempts to localize components required for phosphorylation, we treated isolated intact chloroplasts with a protease, thermolysin, prior to purifying the envelope fraction. When subjected to this nonpenetrating proteolytic enzyme, the protein kinase lost about 70% of its activity, which was not restored by the addition of the protein substrates mentioned above (data not shown). This result suggested that the outer envelope membrane was required for activity, by supplying either the enzyme, the substrate, or both components.

To elucidate further the role of the outer membrane, we applied recently devised methods to separate the inner and outer envelope membranes (8) and found protein kinase activity to be associated mostly with the outer envelope fraction (Fig. 5). The low (15% of total) activity recovered with the inner envelope fraction was ascribed to contaminating outer membrane which, based on SDS-polyacrylamide gel electrophoresis analysis, represented about 15% of the protein of the

FIG. 1 (left). Protein kinase activity of the envelope, thylakoid, and soluble (chloroplast extract) fractions of spinach chloroplasts. Protein kinase activity tested with 3.2 mg of chloroplast extract, 55 μg of envelope protein, and 1.25 mg of thylakoid chloroplast.

FIG. 2 (center). Effect of [ATP] and [ADP] on chloroplast envelope-bound protein kinase.

FIG. 3 (right). Dixon plot showing competitive inhibition of chloroplast envelope-bound protein kinase by ADP (●, 50 μM; ▲, 200 μM; ●, 500 μM ATP).
inner membrane fraction (cf. Ref. 8). Under our conditions, the purity of the isolated outer envelope membrane was about 95%. Significantly, there was no synergistic increase in activity when the inner and outer envelope fractions were combined for assay. These findings thus corroborated the need for the outer membrane in phosphorylation and, furthermore, demonstrated that both the protein kinase and an appropriate substrate are bound to this structure.

Identification of Protein Substrate for Envelope-bound Protein Kinase—In accordance with the conclusion that both substrate and enzyme are envelope-bound, a 14-kDa polypeptide associated with the outer membrane (8, 9) became highly labeled following incubation with [γ-32P]ATP (Fig. 6). Other laboratories have established that this component is the mature form of the small subunit of Rbu-P2 carboxylase that is tightly bound to the envelope membrane (9) and is present even after treatments that removed other supposedly bound proteins such as fructose-1,6-bisphosphatase (8, 9). We detected the bound 14-kDa polypeptide in the four different gel electrophoresis systems identified under “Experimental Procedures” and confirmed its identity as the Rbu-P2 carboxylase small subunit by a Western blotting technique in which the isolated phosphorylated polypeptide was transferred from 8 M urea slab gels to cyanogen bromide-treated filter paper. After transfer, the filter paper was treated, first with rabbit Rbu-P2 carboxylase antibody and then, following a wash, with 125I-labeled S. aureus protein A. If the 14-kDa substrate for the envelope-bound protein kinase was identical with the Rbu-P2 carboxylase small subunit, then it should be labeled with both 32P and 125I following treatment with S. aureus protein A. That was found to be the case. When the 14-kDa protein spot, located by autoradiography, was cut out and its radioactivity analyzed by multichannel scintillation counting which could discriminate between 32P and 125I, we observed radioactivity corresponding to both isotopes. 32P and 125I were detected following correction for possible radioactivity introduced by antibody and protein A nonspecifically adhering to the treated paper determined by counting pieces of equal size and adjacent to the transferred protein.

Interestingly, the mature form of the Rbu-P2 carboxylase small subunit was identified as the reaction product when the envelope was prepared and assayed in the presence of protease inhibitors (0.1 mM l-chloro-3-tosylamido-7-amino-2-heptanone or 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl acetone). It is also noteworthy that the presence of Rbu-P2 antibody during assay had no effect on phosphorylation of the envelope-bound small subunit. These results suggest (i) that the nature, not the precursor, form of the Rbu-P2 carboxylase small subunit was the substrate for the envelope-bound protein kinase, and (ii) that the small subunit undergoing phosphorylation was firmly bound to the envelope membrane. The nature of the 24-kDa polypeptide that was a minor phosphorylated product in our experiments remains to be determined (Fig. 6).

DISCUSSION

The present results provide evidence for a cAMP-independent type of protein kinase that is bound to be outer envelope
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membrane of spinach chloroplasts. The new kinase was activated by Mg\(^{2+}\) and was inhibited (sluggishly) by ADP. It seems possible that the relative concentration of ADP, together with the availability of ATP, could regulate the catalytic activity of the enzyme.

The phosphorylation product of the envelope-bound protein kinase was the mature form of the Rbu-P\(_2\) carboxylase small subunit which, like the kinase, was bound to the outer envelope membrane. The relationship of the outer envelope protein kinase to other membranous protein kinases of chloroplasts, especially to the detergent-solubilized enzyme that phosphorylates Rbu-P\(_2\) carboxylase small subunit (14), remains to be determined. This enzyme had an estimated molecular weight of 25-kDa and was autophosphorylated (14). It seems possible that this autoprophosphorylated enzyme is identical to the 24-kDa outer envelope polypeptide phosphorylated in our studies.

Aside from describing a new protein kinase, the present findings are of interest from the standpoint of protein transport in chloroplasts. From the work of Grossman et al. (3), it now seems certain that the transport of the precursor form of Rbu-P\(_2\) carboxylase small subunit, as well as of other proteins, is dependent on ATP. Furthermore, based on the recent description of special envelope receptors that recognize these higher molecular weight precursors, binding to the envelope is seemingly independent of ATP (16). One possibility raised by the present results is that phosphorylation takes place following binding of the precursor to a receptor site either while or after it is converted to the mature form by proteolysis. Further experiments will be required to determine the exact time of phosphorylation and also to elucidate whether protein phosphorylation is an obligatory step in the transport process.

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REFERENCES