CHLOROPLAST PHOSPHOPROTEINS. THE PROTEIN KINASE OF THYLAKOID MEMBRANES IS LIGHT-DEPENDENT

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1. Introduction

Isolated intact pea chloroplasts incorporate [^{32}P]orthophosphate into several polypeptides in the 7000–70 000 mol. wt range [1]. The three most conspicuous of the phosphoproteins are bound to the thylakoid membrane and comprise a 9000 M_r polypeptide and two polypeptides of about 26 000 M_r which form a closely-spaced doublet on SDS–polyacrylamide gels. The doublet is derived from the light-harvesting chlorophyll *a/b* binding protein complex of the thylakoid [2]. Labelling of the phosphoproteins with [^{32}P]orthophosphate is light-dependent in intact chloroplasts [1]. In this paper I show that the protein kinase responsible for the phosphorylation is also bound to the thylakoids and is itself light-dependent.

Protein kinases generally utilize [γ-^{32}P]ATP as phosphoryl group donor in in vitro assays. An obvious light-dependent step in chloroplast phosphoprotein labelling from [^{32}P]orthophosphate is the generation of [γ-^{32}P]ATP by photophosphorylation within the intact organelles. The fact that the uncoupler CCCP inhibits such phosphoprotein labelling [1] supports the notion that photophosphorylation is involved. However, when protein kinase activity is assayed in osmotically-shocked chloroplasts or in isolated thylakoids, using exogenous [γ-^{32}P]ATP as phosphoryl group donor, protein phosphorylation is insensitive to CCCP but still light-dependent.

Abbreviations: SDS, sodium dodecyl sulphate; CCCP, carbonyl cyanide M-chlorophenylhydrazone; DCMU, dimethyl dichlorourea

2. Materials and methods

Intact chloroplasts were isolated from pea *(Pisum sativum)* leaves as described [1]. Chloroplasts were either incubated in vitro with [^{32}P]orthophosphate to label phosphoproteins [1], or osmotically shocked with 10 ml buffer A (10 mM Tris, 5 mM 2-mercaptoethanol, adjusted to pH 8.0 with HCl) per mg chlorophyll. Some of the shocked organelles were fractionated by centrifugation (2800 × *g* for 5 min) into a thylakoid pellet and a dilute stromal supernatant. The pellet was resuspended to the same chlorophyll concentration with either stromal supernatant or buffer A. Protein kinase assays were performed on the shocked chloroplasts and derived subfractions by making aliquots of each fraction 50 mM in Tris–HCl (pH 8.0), 5 mM in 2-mercaptoethanol, 0–20 mM in $MgCl_2$, 0.1 mM in [γ-^{32}P]ATP (200 μCi/μmol) and 100 μg/ml in chlorophyll. Incubations were performed at 20°C in an illuminated waterbath (photosynthetically-active radiation: 500 μE .m^{-2} .s^{-1}). Tubes for dark incubations were wrapped in aluminium foil. Reactions were terminated by addition of 4 vol. acetone. Precipitated proteins were fractionated by SDS–polyacrylamide slab gel electrophoresis and phosphoproteins were located by autoradiography of the stained and dried slab gel [1,2]. Quantitative results on the incorporation of radioisotope into the light-harvesting chlorophyll *a/b* binding protein doublet (26 000 M_r) were obtained by Cerenkov counting of gel slices [1].

3. Results

Protein kinase activity is detectable in osmotically-

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Table 1
Phosphorylation of endogenous light-harvesting chlorophyll *a/b* binding protein by shocked chloroplasts and by isolated thylakoids

Source of photosynthetic membrane	Protein kinase activity[a]
Osmotically-shocked chloroplasts	4.8
Thylakoids in buffer A	9.4
Thylakoids in stromal supernatant	4.0

[a] Activity was assayed for 10 min in the light in buffer containing 50 mM Tris–HCl (pH 8), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP and 100 μg chlorophyll/ml. Measured as pmol phosphate incorporated into the light-harvesting protein doublet μg chlorophyll^{-1} . 10 min incubation^{-1}

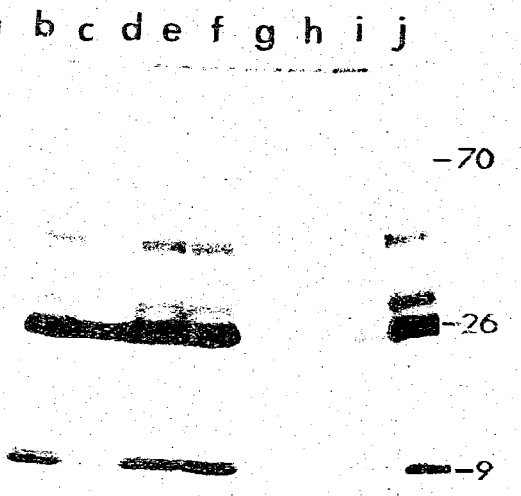

Fig.1. Demonstration of a light-dependent protein kinase in thylakoids. Isolated pea thylakoids were incubated at 20°C and 100 μg chlorophyll/ml in a buffer containing 50 mM Tris–HCl (pH 8), 5 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP and the following additions:
a, 10 mM $MgCl_2$ (0 min, light);
b, 0 mM $MgCl_2$ (10 min, light);
c, 5 mM $MgCl_2$ (10 min, light);
d, 1 mM $MgCl_2$ (10 min, light);
e, 10 mM $MgCl_2$ (10 min, light);
f, 10 mM $MgCl_2$ + 10 μM CCCP (10 min, light);
g, 10 mM $MgCl_2$ + 10 μM DCMU (10 min, light);
h, 10 mM $MgCl_2$ (10 min, dark);
i, 20 mM $MgCl_2$ (10 min, dark). The incubation times and conditions are given in parentheses.
j, Isolated intact chloroplasts incubated in light for 10 min at 20°C with [^{32}P]orthophosphate. All reactions were stopped by acetone treatment. Delipidated proteins were fractionated by gel electrophoresis and phosphoproteins detected by autoradiography of the dried slab gel. The apparent molecular weights of polypeptides are given × 10^3.

shocked chloroplasts and in isolated thylakoids (table 1). Thylakoids resuspended in buffer A are more active than shocked chloroplasts or thylakoids resuspended in dilute stroma, suggesting that most, if not all, of the protein kinase which phosphorylates the light-harvesting protein and other thylakoid polypeptides is bound to the membrane.

The polypeptides labelled in isolated thylakoids incubated with [γ-^{32}P]ATP are largely identical in apparent molecular weight and relative degree of labelling to those labelled in intact chloroplasts incubated with [^{32}P]orthophosphate (fig.1, tracks e, j). The minor phosphoproteins in the 60 000–70 000 M_r range that are labelled in intact chloroplasts but not in thylakoids are not thylakoid components [1]. The minor phosphoproteins labelled in thylakoid preparations but not in chloroplasts probably represent phosphoproteins of contaminating chromatin.

Figure 1 establishes that the thylakoid protein kinase is dependent on Mg^{2+}. In the absence of added $MgCl_2$ (track b) there is no detectable protein kinase activity. Maximal phosphorylation is achieved at 5–10 mM $MgCl_2$ (tracks c,e).

The protein kinase also requires light for activity (fig.1, tracks e,h). This is not due to an involvement of photophosphorylation because 10 μM CCCP is not inhibitory in the thylakoid assay (track f), an expected result as the addition of exogenous [γ-^{32}P]ATP renders photophosphorylation superfluous. However, 10 μM DCMU completely inhibits the light-dependent (thylakoid) protein kinase without affecting the light-independent (presumably chromatin) protein kinase (track g). The inhibitory effect of DCMU indicates that photosynthetic electron transport is required for activation of the protein kinase.

Activation may involve reduction of a particular component of the electron transport chain. Can activation be reproduced in darkness by adding a physiological reducing agent? Table 2 shows that ferredoxin stimulates the protein kinase activity of washed thylakoids either in darkness in the presence of NADPH or in the light. As NADPH is known to reduce ferredoxin in the presence of thylakoids carrying ferredoxin:NADP reductase (EC 1.6.7.1)

Table 2
Activation of thylakoid protein kinase by light and reducing agents

Assay conditions[a]	Protein kinase activity (%)
Light	100
Light + 10 μM ferredoxin	195
Dark	12
Dark + 10 μM ferredoxin	12
Dark + 1 mM NADPH	25
Dark + 1 mM NADPH + 10 μM ferredoxin	50

[a] Isolated thylakoids were washed once in buffer A and assayed as described in the footnote to table 1, except that $MgCl_2$ was 10 mM

[3], it appears that activation of the protein kinase is a direct or indirect consequence of the reduction of ferredoxin.

4. Discussion

These results enable several conclusions to be reached.

1. The thylakoid phosphoproteins are the substrates of a light-dependent protein kinase that is itself membrane-bound. Although membrane-bound protein kinases are common in animal cells [4], this is the first report of a membrane-bound kinase in plants [5].
2. The light dependence of the thylakoid protein kinase is not directly analogous to that shown by the other known light-dependent protein kinase, opsin kinase [6]. Opsin is not phosphorylated until a light-dependent configurational change in rhodopsin reveals a phosphorylation site on the protein. The protein kinase is not directly affected by light. If the absorption of light by chlorophyll molecules associated with the light-harvesting proteins were necessary and sufficient to activate phosphorylation, then activation should not be inhibited by DCMU in the light or promoted by NADPH and ferredoxin in the dark.
3. Phosphorylation is not dependent on either the establishment of a proton gradient across the membrane or ATP synthesis. Both would be abolished in the presence of CCCP [7]. The effect of light is also not mediated by an efflux of Mg^{2+} from thylakoids because incubation in the dark with $\leqslant 20$ mM $MgCl_2$ cannot replace the photorequirement (fig.1, track i).

Finally, all the thylakoid protein phosphorylation reactions are equally light-dependent. This indicates either that the protein kinase molecules are photoactivated irrespective of their particular protein substrate, or that all substrates are subjected to electron transport-dependent conformational changes that reveal sites for phosphorylation. To distinguish between these possibilities it will be necessary to understand more fully the role of photosynthetic electron transport in stimulating protein kinase activity, especially the significance of reduced ferredoxin in the process. Reduced ferredoxin is known to be involved in the light-activation of certain stromal enzymes [8].

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