Evidence for different kinases in thylakoid protein phosphorylation

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Thylakoid protein phosphorylation was facilitated in darkness by using the ferredoxin–NADPH system. CoCl_2 and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) were potent inhibitors of LHCP (light-harvesting chlorophyll-binding protein) phosphorylation, but 3-(3,4-dichlorophenyl)-1,1-dimethylurea and atrazine had no significant effect. Differential effects on phosphorylation of the 9 kDa polypeptide and LHCP were observed in darkness with DBMIB and certain other inhibitors specific for Photosystem-II electron transport. Similarly, during illumination of intact chloroplasts or of the reconstituted chloroplast system, a differential action of bicarbonate was observed on the relative phosphorylation of the two proteins. The degree of phosphorylation of the 9 kDa polypeptide was increased in the presence of bicarbonate compared with its absence, whereas that of LHCP was relatively unchanged. Changes in the degree of phosphorylation of the 32 kDa polypeptide in these experiments did not correlate consistently with changes in phosphorylation of either LHCP or the 9 kDa polypeptide, although changes in the 32 kDa polypeptide more often paralleled phosphorylation of the 9 kDa polypeptide rather than the phosphorylation of LHCP. These observations suggest that the protein kinase that phosphorylates LHCP is distinct from that which phosphorylates the 9 kDa polypeptide.

INTRODUCTION

The chloroplasts of higher plants contain discrete and clearly distinct protein kinase enzymes in the thylakoids [1], in the stroma [2] and on the chloroplast envelope [3]. The thylakoid membranes contain protein kinase activity, which is regulated by the redox state of plastoquinone [4–6] such that the kinase is activated when the plastoquinone pool becomes reduced by light [1]. The kinase may also be activated in the dark with strong reducing agents and reduced ferredoxin [7]. The substrate proteins that become phosphorylated are the light-harvesting chlorophyll-*a/b*-binding protein (LHCP) and other thylakoid proteins associated with Photosystem II (PSII), the most prominent of which has a molecular mass of approx. 9 kDa. The identity of the 9 kDa polypeptide remains to be established, but it has been shown not to be cytochrome 599*b* [8] or the dicyclohexylcarbodi-imide-binding CF_0 component [9].

In order to phosphorylate thylakoid membrane polypeptides, the protein kinase requires ATP ($K_m = 90 \mu\text{M}$), Mg^{2+} (3–5 mM) and reduced plastoquinone (for activation). An increasing amount of evidence supports the view that there are at least two distinct thylakoid protein kinase isoenzymes [9–12]. The protein kinase that phosphorylates LHCP has been distinguished from the protein kinase that phosphorylates the 9 kDa polypeptide, in several ways. The enzymes differ in the degree of protection against inhibition by the thiol reagent *N*-ethylmaleimide afforded by pre-incubation with ATP [13], the ability of Zn^{2+} ions to stimulate phosphorylation [11] and the differential inhibition of phosphorylation of LHCP and the 9 kDa polypeptide by the nucleotide

affinity inhibitor 5'-*p*-fluorosulphonylbenzoyladenine [9]. However, the case for multiple thylakoid protein kinase isoenzymes is not generally accepted [14], and recently the basis for regulation of distribution of absorbed excitation energy between the photosystems has been discussed in terms of simultaneous phosphorylation of all thylakoid polypeptides by a single protein kinase [15]. The present study examines the nature and properties of thylakoid protein kinases.

MATERIALS AND METHODS

Intact chloroplasts were prepared from spinach (*Spinacia oleracea* var. *virtuosa*) grown in hydroponic culture [16] by the method of Walker [17]. Intact maize mesophyll chloroplasts were prepared as described previously [18]. Stromal and thylakoid protein fractions were isolated from intact spinach or maize mesophyll chloroplasts (by centrifugation at 10000 *g* for 5 min) after osmotic lysis in a medium containing 10 mM-Hepes/KOH buffer (pH 7.5), 1 mM-EDTA and 10 mM-dithiothreitol. CO_2 -dependent O_2 -evolution measurements and ^{32}P incorporation in intact chloroplasts were performed in paired Hansatech oxygen electrodes in red light (300 $\text{W} \cdot \text{m}^{-2}$) at 20 °C.

Preparation of CO_2 -free media

Acidified distilled water was boiled for 1 h and bubbled continuously with N_2 until required. Media were made up in this water and bubbled with N_2 overnight at acid pH. As required, the media were adjusted to the

Abbreviations used: LHCP, light-harvesting chlorophyll-binding protein; PSII, Photosystem II; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; RuBP, ribulose 1,5-bisphosphate.

appropriate pH values with KOH pellets that had been washed with the CO₂-free water immediately before use.

³²P incorporation

The reaction mixture for intact chloroplasts was similar to that routinely used for optimal CO₂ assimilation [2,16,17] and consisted of chloroplasts (100 µg of chlorophyll/ml), 0.3 mM-P_i, [³²P]P_i (150 µCi/ml), 3 mM-Na₂P₂O₇, 0.33 M-sorbitol, 1 mM-EDTA, 1 mM-MgCl₂, 50 mM-Hepes/KOH buffer (pH 7.6) and catalase (200 units/ml) with the stated concentrations of added NaHCO₃.

The reconstituted chloroplast system [19] was similar to that used previously for the measurement of protein phosphorylation [2,20] and consisted of 50 mM-Hepes/KOH buffer (pH 7.9), 12.5 mM-MgCl₂, catalase (1500 units/ml), 1 mM-ADP, 0.5 mM-P_i, [³²P]P_i (200 µCi/ml), 0.4 mM-NADP⁺, ferredoxin (200 µg/ml), 0.33 M-sorbitol, 1 mM-EDTA, 10 mM-KCl, 4 mM-ascorbic acid, 1 mM-dithiothreitol and thylakoids and stromal protein equivalent to 100 µg of chlorophyll/ml, incubated in the light at 25 °C for 5 min.

Incorporation of ³²P into thylakoid membrane polypeptides was facilitated by incubating a reaction medium containing 50 mM-Hepes/KOH buffer (pH 7.9), 5 mM-MgCl₂, catalase (200 units/ml), 500 µM-[γ-³²P]ATP (20 Ci/mol), 1 mM-NADPH, ferredoxin (100 µg/ml), 10 mM-NaF and thylakoids (50 µg, of chlorophyll/ml) in the dark at 25 °C for 15 min, as in [2].

All reactions were terminated by the addition of trichloroacetic acid (to 5%, w/v). ³²P incorporation into specific polypeptides was determined in the excised bands of specific apoproteins after SDS/polyacrylamide-gel electrophoresis as described previously [2,16]. In Fig. 2 the values given are single point estimations. Autoradiograms of thylakoid protein phosphorylation were allowed to develop for 24 h.

Fluorescence measurements

The thylakoids were phosphorylated as for ³²P incorporation, except that the radioisotope was omitted, and at the end of the 15 min incubation period 0.5 mM-Methyl Viologen and 1.0 µM-nigericin were added. After 30 s, 20 µM-DCMU was added, and after a further 20 s the fluorescence induction curves were measured.

Chlorophyll assay

Chlorophyll was measured in 80% (v/v) acetone extracts by the method of Arnon [21].

RESULTS

Effect of PSII inhibitors

Phosphorylation of LHCP and the 32 kDa and 9 kDa polypeptides occurred in darkness in the presence of NADPH and ferredoxin. The effects of the presence of PSII inhibitors during phosphorylation of thylakoid proteins on the thylakoid protein kinase activity were measured directly by ³²P incorporation into specific polypeptides (Table 1), and also by fluorescence measurements (Table 2) in this system, in which the added complication of effects on light-driven electron transport was not present. The extent of total chloroplast and thylakoid protein phosphorylation varied between batches of chloroplasts, but extents of phosphorylation

Table 1. Effects of PSII electron-transport inhibitors on the phosphorylation of spinach thylakoid membrane polypeptides in the dark

Thylakoid protein kinase activation was facilitated in the dark by the addition of 1 mM-NADPH and ferredoxin. PSII inhibitors were added before addition of NADPH and ferredoxin. Reactions were terminated after 15 min incubation.

Addition to assay	Protein phosphorylation (c.p.m.) in:		
	9 kDa poly-peptide	LHCP	32 kDa poly-peptide
None	50	396	110
25 µM-DCMU	54	394	143
25 µM-Atrazine	51	320	122
0.5 µM-DBMIB	51	201	143
10 mM-CoCl ₂	40	47	32
None	62	296	61
1 mM-Hydroxylamine	68	310	96
20 µM-CCCP	64	370	56
1 mM-Tetraphenylboron	136	419	64

Table 2. Effect of PSII electron-transport inhibitors on fluorescence induction curves obtained from spinach thylakoid membranes phosphorylated in darkness

Protein phosphorylation was carried out as for Fig. 1. After 15 min incubation 0.5 mM-Methyl Viologen, 1.0 µM-nigericin and 20 µM-DCMU were added. Fluorescence induction measurements were then made to calculate the decrease in F_m .

Addition to assay	Decrease in F_m (%)
None	22.0
25 µM-DCMU	22.0
25 µM-Atrazine	16.0
0.5 µM-DBMIB	3.1
1 mM-Hydroxylamine	20.0
20 µM-CCCP	23.0
10 mM-CoCl ₂	2.5

and trends were consistent within a given preparation of spinach or maize mesophyll thylakoids. The results of individual experiments are therefore used as representative of a series of experiments in which a consistent trend was observed.

The maximum yield of fluorescence is achieved when Q_A (the primary electron acceptor to PSII) is reduced and electron transfer is blocked (for example by DCMU). LHCP phosphorylation causes a decrease in the absorption cross-section of PSII and therefore produces a decrease in maximum fluorescence yield (F_m). The decrease in F_m , which is taken as a measure of the degree of the fraction of absorbed excitation energy reaching PSII (accompanying the phosphorylation of LHCP) in these experiments, is shown in Table 2. CoCl₂, which has been shown to inhibit irreversibly electron donation to the PSII reaction centre in a manner that prevents hydroxylamine acting as an electron donor [22], was also a potent inhibitor of LHCP phosphorylation (Table 1). This was

associated with a decrease in F_m relative to controls incubated in the absence of ATP (Table 2). CoCl_2 also inhibits the phosphorylation of the 9 kDa and 32 kDa polypeptides, and therefore Co^{2+} may be competitive with Mg^{2+} .

DBMIB reduced the decrease in fluorescence yield and inhibited LHCP phosphorylation to a much greater extent than atrazine in all experiments, whereas DCMU had no effect at all (Tables 2 and 3). This is of particular interest, because all three compounds are plastoquinone antagonists, yet DBMIB is the only one which appears effectively to prevent LHCP phosphorylation and its associated function of spill-over. The concentration of atrazine required to show an inhibitory effect on LHCP protein phosphorylation was exceptionally high (25 μM in Tables 1 and 2), and at this concentration it may be interacting with other binding sites in addition to the Q_B site (the 32 kDa herbicide-binding polypeptide). Although measurement of fluorescence in the presence of DBMIB can be complicated by non-photochemical quenching by oxidized quinones, these results suggest that DBMIB inhibits the protein kinase which phosphorylates LHCP. Conversely DBMIB had no effect on the degree of phosphorylation of the 9 kDa or 32 kDa polypeptides. The concentration of DBMIB in these experiments was 0.5 μM , and, since this substantially inhibited LHCP phosphorylation in the presence of 1 mM-NADPH, it was therefore unlikely to be acting primarily as an oxidant. It was evident from these experiments that the effects of the various inhibitors on the degree of LHCP phosphorylation were not always consistent with that on the phosphorylation of the 9 kDa and 32 kDa polypeptides. Throughout this series of experiments, the presence of DBMIB decreased phosphorylation of LHCP, whereas the phosphorylation of the 9 kDa and 32 kDa polypeptides was unchanged or even increased relative to controls. For example, Table 1 shows that DBMIB decreased LHCP phosphorylation by approx. 50 %, but phosphorylation of the 9 kDa and 32 kDa polypeptides was unchanged relative to controls. Similarly, in the presence of 10 mM- CoCl_2 , phosphorylation of LHCP was inhibited by 88 % and that of the 32 kDa polypeptide was inhibited by 71 %, but that of the polypeptide was inhibited by only 20 %. These observations show that there was a differential response in the phosphorylation state of the various polypeptide substrates to the presence of certain PSII inhibitors, and this supports the hypothesis that the thylakoids contain more than one protein kinase enzyme, as suggested previously [9]. Hydroxylamine, CCCP and tetraphenylboron, which block electron flow at the O_2 -evolving complex, generally slightly increased thylakoid protein phosphorylation (Table 1). The presence of fluoride in the reaction media (as an inhibitor of protein phosphatase activity) increased the total radioactivity incorporated, but did not affect the relative extents of phosphorylation between polypeptides.

Effect of bicarbonate

The reconstituted chloroplast system [2,17] was used to study the effects of stromal metabolites on light-dependent thylakoid and stromal protein phosphorylation (Table 3). In the absence of bicarbonate, the presence of 1 mM-RuBP inhibited both thylakoid and stromal protein phosphorylation. In this situation, the addition of 1 mM-RuBP inhibited thylakoid protein

Table 3. Effect of bicarbonate and RuBP on spinach thylakoid and stromal protein phosphorylation in the reconstituted chloroplast system

HCO_3^- and RuBP were added to reaction mixtures which had been depleted of CO_2 . Reactions were allowed to proceed for 5 min in saturating light. Stromal and thylakoid proteins were then separated and protein phosphorylation was measured as described by Foyer [2].

Conditions	Phosphorylation (c.p.m.) in:		
	Total stromal protein	Total thylakoid protein	LHCP
No HCO_3^-	313	4463	1434
No HCO_3^- + 1 mM-RuBP	147	2454	723
1 mM- HCO_3^- + 1 mM-RuBP	371	10059	1989

phosphorylation by 45 % (Table 3), and in similar circumstances the addition of 1 mM-3-phosphoglycerate inhibited by 21 % and 1 mM-ribose 5-phosphate by 61 % (results not shown). This inhibition cannot be explained solely in terms of relative demand for assimilatory power imposed by the various Calvin-cycle metabolites, as suggested previously [20], since RuBP was a more effective inhibitor than phosphoglycerate, although the ATP and NADPH requirement would be the same. When bicarbonate was omitted from the reconstituted chloroplast system, phosphorylation of LHCP in the light accounted for 32 % of the total thylakoid protein phosphorylation in the light. In the presence of RuBP and 1 mM-bicarbonate, LHCP phosphorylation increased, but in this case accounted for only 20 % of the total thylakoid protein phosphorylation (Table 3). Thus it appeared that the presence of bicarbonate stimulated the phosphorylation of thylakoid polypeptides, particularly the PSII-associated species.

The differential effect of bicarbonate on the phosphorylation of LHCP and other thylakoid polypeptides was confirmed with observations of thylakoid protein phosphorylation in intact chloroplasts. Fig. 1 shows thylakoid polypeptides phosphorylated *in situ*, in intact chloroplasts in the absence or presence of NaHCO_3 (0.1 and 0.25 mM). In all cases phosphorylated species were clearly evident at 9 kDa, 26 kDa and 32 kDa. Phosphorylated stromal polypeptides cannot be distinguished in this autoradiogram, which was developed overnight; autoradiograms showing stromal protein phosphorylation take relatively longer (4–5 days). Fig. 2 shows the rates of phosphorylation of LHCP and of 32 kDa and 9 kDa polypeptides in intact chloroplasts in the absence and presence of NaHCO_3 . At all NaHCO_3 concentrations, the maximum protein phosphorylation was achieved rapidly (within 5 min). The steady-state extent of phosphorylation attained after 5 min was therefore taken as a measure of the relative degree of phosphorylation in individual polypeptides (Fig. 3 and Table 4). In the intact chloroplast system, when the CO_2 concentration of the medium was increased, the steady-state extent of LHCP phosphorylation was relatively constant, whereas that of the 9 kDa polypeptide increased considerably (Figs. 2 and 3). In the experiment

shown in Table 4, the steady-state ratio of LHCP phosphorylation to that of the 9 kDa polypeptide decreased from 2.61 in the absence of added bicarbonate

to 0.87 in the presence of 1 mM- NaHCO_3 . These results show that bicarbonate can influence phosphorylation of thylakoid proteins, especially that of the 9 kDa polypeptide, and may indicate a role for bicarbonate in the activation of the protein kinase which phosphorylates this polypeptide.

Fig. 1. Autoradiogram of ^{32}P -labelled polypeptides from intact spinach chloroplasts

Chloroplasts were illuminated for 5 min in the absence (a) or in the presence of 0.1 mM- NaHCO_3 (b) or 0.25 mM- NaHCO_3 (c). Molecular masses of the major phosphorylated species are given.

DISCUSSION

The data presented here support the view that the thylakoid membranes contain more than one protein kinase. The phosphorylation state of the 9 kDa polypeptide can be modulated independently from that of LHCP, suggesting that these polypeptides are phosphorylated by the activities of different thylakoid protein kinases. This is evidenced by differences in the catalytic capacity to phosphorylate the two polypeptides in darkness in the presence of compounds which block electron transfer at different sites around PSII and also by the action of bicarbonate in the light. DBMIB selectively inhibited phosphorylation of LHCP, while having no effect on the phosphorylation of the other thylakoid polypeptides. DCMU and atrazine had little effect on thylakoid protein phosphorylation in the dark. The major site of action of atrazine and DCMU is different from that of DBMIB; the former bind to the Q_B protein, whereas DBMIB blocks electron flow from the plastoquinone pool at the cytochrome *b/f* complex. DBMIB is therefore a much stronger plastoquinol 'antagonist', acting much more strongly at the Q_z site of the cytochrome *b/f* complex than at the Q_B site of PSII. Although the presence of DBMIB would slow the rate of reduction of the plastoquinone pool, which has been suggested to be involved in the activation mechanism for the thylakoid protein kinase [6], DBMIB also decreases the extent of phosphorylation of LHCP in the light. This would suggest that DBMIB inhibits the activation of the protein kinase which phosphorylates LHCP. Tetraphenylboron was found to stimulate phosphorylation of both the 9 kDa polypeptides and LHCP. This might suggest the presence of a common kinase. However, in the light of the remaining data in Tables 1, 2 and 4, which strongly suggest the presence of distinct kinases for these proteins, it is more likely that the presence of tetraphenylboron stimulates the activity of both the individual kinases for these proteins.

Fig. 2. Time course of phosphorylation of LHCP and the 32 kDa and 9 kDa polypeptides in intact spinach chloroplasts in the absence or presence of 1 mM- NaHCO_3

Fig. 3. Effects of NaHCO_3 concentration on CO_2 -dependent O_2 evolution (●) and phosphorylation of the 9 kDa polypeptide (▲) and LHCP (○) in intact spinach chloroplasts

Chloroplasts were incubated with $[\text{}^{32}\text{P}]\text{P}_i$ and measurements were made after 5 min illumination.

Table 4. Effect of bicarbonate on the rate of CO_2 -dependent O_2 evolution and the relative phosphorylation of LHCP and the 9 kDa polypeptide in intact spinach chloroplasts supplied with $[\text{}^{32}\text{P}]\text{P}_i$

Protein phosphorylation in intact chloroplasts was allowed to proceed at the stated bicarbonate concentrations for 5 min. Phosphorylation of LHCP and the 9 kDa polypeptide was then determined after SDS/polyacrylamide-gel electrophoresis.

NaHCO_3 concn. (mM)	CO_2 -dependent O_2 evolution ($\mu\text{mol/h}$ per mg of chlorophyll)	Phosphorylation of LHCP
		Phosphorylation of 9 kDa polypeptide
0.00	8	2.61
0.05	16	1.01
0.10	80	0.95
1.00	148	0.87

The LHCP of PSII and the 9 kDa polypeptide share several common features. Both are membrane-spanning proteins with sites of phosphorylation that are threonine residues residing in the *N*-terminal regions that are exposed to the stroma [23,24,25]. In addition, there appears to be some degree of homology between the protein sequences of the *N*-terminal region of the LHCP associated with PSII and that of the 9–10 kDa phosphoprotein [23]. Allen & Holmes [15] have proposed that these proteins are phosphorylated by a common kinase and that, like LHCP, the 9 kDa phosphoprotein may

also be involved in the regulation of excitation-energy distribution. The protein-sequence data [23] would support this view, and Hird *et al.* [23] have suggested that differences in the lateral mobility of the two proteins might explain differences in the rates of dephosphorylation [14,23]. A more simple explanation might be that, although both polypeptides could perform a similar function in relation to the regulation of excitation-energy distribution, modulation through phosphorylation/dephosphorylation changes is catalysed by distinct protein kinase and protein phosphatase isoenzymes.

Bicarbonate has a central role in photosynthesis, since CO_2 is the substrate for RuBP carboxylase and bicarbonate itself is the substrate for phosphoenolpyruvate carboxylase. However, the presence of bicarbonate ions is also required for electron flow through PSII. Bicarbonate depletion results in inhibition of the Hill reaction [26]. Two types of bicarbonate-binding sites on the thylakoid membranes have been described, but electron flow through PSII depends on a small pool of tightly bound bicarbonate at the DCMU-binding site alone [27]. Electron flow from PSII to Photosystem I can be stimulated by bicarbonate, which has been also suggested to cause an inhibition of pathways of cyclic electron flow around PSII [28]. In these experiments the effects on protein phosphorylation may be due to either NaHCO_3 or CO_2 . At 1.0 mM- NaHCO_3 the CO_2 concentration in the chloroplasts was $63 \mu\text{M}$, and at 0.1 mM- NaHCO_3 was $6.3 \mu\text{M}$ (by using a pK' of 6.407 at 20°C).

The phosphorylation state of the 9 kDa polypeptide was increased in the presence of bicarbonate. The function of the 9 kDa polypeptide remains to be elucidated, but, if it is a component of a protective dissipative cycling pathway around PSII [29], then it is possible to speculate on the role of bicarbonate stimulation of the phosphorylation of this protein. We may postulate that phosphorylation results in a decrease in the activity of this pathway [28] in a situation where carbon assimilation and energy utilization were facilitated by the presence of substrate. LHCP is often found to be the most predominantly ^{32}P -labelled thylakoid protein [1,7]. However, Figs. 1–3 show that this is not always the case, since more label may be incorporated at the 9 kDa polypeptide than in LHCP. This was also found to be true in developing wheat leaf tissue [30].

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