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Chloroplast thylakoid protein phosphorylation is influenced by mutations in the cytochrome *bf* complex

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The effects were studied of the plastoquinone analogs 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) and the 1,3-dinitrophenylether of iodonitrothymol (DNP-INT) on thylakoid protein phosphorylation activated either by light or by reduction in the presence of duroquinol or reduced ferredoxin. Light-activated phosphorylation in the presence of methyl viologen was increased by 0.5–1.0 μM DBMIB or DNP-INT as the reoxidation of plastoquinone and net linear electron flow were inhibited. The phosphorylations of the light-harvesting complex of Photosystem II, of a 32 kDa polypeptide and of the kinase itself (autophosphorylation) were progressively and selectively inhibited at tenfold higher concentrations of either inhibitory analog. Ascorbate, but not duroquinol or reduced ferredoxin, potentiated these selective inhibitions. If the membranes were reductively activated in the dark, however, DBMIB or DNP-INT progressively inhibited the phosphorylation of all thylakoid proteins (I_{50} = 10–15 μM). These observations appear to preclude a simple and direct regulation of protein kinase activity by the cytochrome *bf* complex at its plastoquinol oxidation (Q_Z) site. Mutants of *Zea* and *Lemna* lacking the cytochrome *bf* complex were tested for the ability to phosphorylate thylakoid proteins. In both cases, the redox-sensitive phosphorylation of light-harvesting chlorophyll a/b protein complex of Photosystem II (LHC-II) was abolished, whereas other PS II peptides were phosphorylated as in the wild types. Immune blot analysis showed that this lesion was not due to the absence of the 64-kDa protein kinase. Nor did the mutants possess defective LHC-II, as shown by its utilization as a phosphorylation substrate in a heterogeneous reconstitution assay.

Introduction

Spinach thylakoid membranes contain protein kinase activity which is activated by photochemical [1,2] or chemical [3,4] reduction of the plastoquinone pool. The major endogenous substrate for this activity is the light-harvesting chlorophyll *a/b* binding protein of Photosystem II (LHC-II), the reversible phosphorylation of which has been implicated in the control of energy transfer between the two photosystems [5–7]. Minor phosphorylation substrates within PS II include a

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 1,3-dinitrophenylether of iodonitrothymol; Fd, ferredoxin; FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine; LHC-II, light-harvesting chlorophyll-*a/b* protein complex of Photosystem II; MV, methyl viologen (1,1′-dimethyl-4,4′-bipyridiniumdichloride); SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; $TMQH_2$, duroquinol (2,3,5,6-tetramethyl-*p*-benzoquinol).

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9-kDa polypeptide [8], the D1 and D2 reaction centre polypeptides and a 44-kDa chlorophyll binding protein [9].

The actual number of thylakoid protein kinases responsible for these activities is still debated. Most data on the effects of electron transport inhibitors [6], kinase inhibitors [10], detergents [11], and reductive redox titrations [3,4] on thylakoid protein phosphorylation can be interpreted in terms of a single protein kinase. However, data on the differential sensitivity of thylakoid protein phosphorylation to thiol reagents [4], to the ATP-analogue FSBA [12], and to zinc ion [13] have been interpreted on the basis of two membrane-bound kinases. Biochemical evidence for the presence of three protein kinases [14,15] was questioned when later work [16,17] revealed the presence of only one thylakoid protein kinase that could phosphorylate LHC and endogenous thylakoid proteins in a reconstituted system [18].

The nature of the redox activation of the kinase or kinases also remains unknown. Recent work on the differential effect of DBMIB on light-dependent protein phosphorylation [19]; and the partial, and complete loss of LHC-II phosphorylation in mutants of *Chlamydomonas reinhardtii* [20], and *Lemna perpusilla* [21] lacking a functional cytochrome *bf* complex have indicated a possible role in the mechanism of redox activation for this complex. In the present report, we further test this hypothesis; both by extending the observations of the effects of the plastoquinone analogues DBMIB and DNP-INT on thylakoid protein phosphorylation; by examining the phosphorylation behaviour of corn mutants which lack a functional cytochrome *bf* complex; and by the use of a polyclonal antisera against spinach 64 kDa thylakoid protein kinase [18] to probe for the immunological presence of kinase activity in these mutants.

Materials and Methods

Spinach (*Spinacia oleracea* cv. Park Hybrid 424) was cultivated in a growth chamber as previously described [22].

Intact chloroplasts were obtained from 10–12-week-old deveined spinach leaves by the method of Walker [23]. Chlorophyll concentration was estimated by the method of Arnon [24]. Thylakoids were obtained by washing chloroplasts (1 mg Chl) with 2×1 ml of 10 mM NaCl, 10 mM Tricine-NaOH (pH 8.0), and centrifuging (30 s, Eppendorf minifuge). The final pellet was resuspended to 1 ml in 0.1 M sorbitol, 10 mM $MgCl_2$, 10 mM NaF, 50 mM Tricine-NaOH (pH 8.0).

Lemna perpusilla 6746 (wild type) and 1073 (a mutant lacking the cytochrome *bf* complex) were cultured at 20 °C on sterilized half-strength Hutners medium [25] supplemented with 1% (w/v) sucrose; illumination was provided by a 15 W daylight fluorescent lamp at 50 cm distance. Thylakoid membranes were isolated as for spinach.

Corn (*Zea mays* L. Missouri strain) wild type and cytochrome *bf* mutant *hcf*-6 were kindly supplied as seeds by Dr. D. Miles, University of Missouri [26,27]. Seeds were germinated in potting soil in a greenhouse under diffused sunlight. After 8–10 days, homozygous recessives carrying the lethal mutation were identified by excising leaf tips and directly measuring flash-induced cytochrome *f* and b_6 turnovers and the P518 electrochromic shift in a laboratory-constructed spectrophotometer [28]. Seedlings lacking these responses were decapitated and used for isolation of mutant thylakoids, using the above procedure for spinach.

Electron transport. Electron-transport rates were measured by means of a Yellow Springs O_2 electrode. The reaction vessel (2 ml volume) contained 400 μg Chl in resuspension medium supplemented with either 0.2 mM methyl viologen, 1 mM sodium azide and 0.2 mM ATP (whole-chain electron transport), or 2 mM potassium ferricyanide, 0.5 mM diaminodurene and 0.2 mM ATP (PS II-mediated electron transport). The reaction vessel was thermostatted at 20 °C and illuminated with red light (Schott RG 630, 200 W/m^2).

Thylakoid protein phosphorylation. thylakoids (20 μg Chl) were incubated in 90 μl of resuspension medium containing DBMIB or DNP-INT (0–50 μM, final ethanol concentration 1%, v/v), for 5 min in the dark on ice. The samples were either illuminated for 1 min at 20 °C in white light (400–700 nm, 100 W/m^2) or were incubated at 20 °C in the dark for 1 min under argon after adding 10 μM DCMU and 1 mM $TMQH_2$ [29], or 10 μM DCMU, 50 μM ferredoxin and 1 mM NADPH (final concentrations). Protein phos-

phorylation at 20 °C was initiated by addition of 10 μl of a 2 mM stock solution of ATP in resuspension medium containing 5 μCi [γ-^{32}P]ATP (specific activity, more than 10 mCi/mmol). The reaction was quenched by addition of 100 μl of 0.2 M EDTA on ice. The membranes were sedimented (15 s, Eppendorf Minifuge, 4 °C), rinsed quickly on ice in 250 μl of 10 mM NaF, 10 mM Tricine-NaOH (pH 8.0) and solubilised on ice with 30 μl of a solution containing 4% (w/v) lithium dodecyl sulfate, 25% (v/v) glycerol, 0.2 mM EDTA, 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue and 50 mM Tris-HCl (pH 8.0). Samples were heated (1 min, 80 °C) and then subjected to SDS-PAGE. Radioactive areas of the gel were detected and quantified as previously described [16–18].

Heterogeneous reconstitution assay. Thylakoids (100 μg Chl in 200 μl of 0.1 M NaCl) were frozen for 1 h at −20 °C, thawed (1 min, 25 °C), centrifuged and resuspended in 100 μl of resuspension medium. This treatment both uncouples electron transport and vesiculates the membranes [30]. Samples contained 1 μg of thylakoid kinase isolated as in Ref. 18, 10 μg Chl and 10 μM DCMU in 40 μl total volume. Protein phosphorylation was initiated by the addition of 10 μl of a 1 mM stock solution of ATP in resuspension medium containing 5 μCi [γ-^{32}P]ATP, and the suspension was incubated for 15 min at 20 °C in the dark. The reaction was quenched with EDTA and the membranes further processed as above.

Immunoblot analysis. Thylakoids (10–20 μg Chl) were solubilised in lithium dodecyl sulfate, heated as above and subjected to SDS-PAGE on a 15% (w/v) acrylamide/0.2% (w/v) bis-acrylamide resolving gel. Peptides were electrophoretically transferred to nitrocellulose as previously described [18]. The blot was sequentially probed with preimmune and anti-kinase mouse IgG, followed by rabbit IgG prepared against the four subunits of the cytochrome *bf* complex [31]. Positively reacting areas were visualised using ^{125}I protein A [31].

Materials. DBMIB and DNP-INT were the kind gift of Dr. Aachim Trebst. Stock solutions (10 mM in dry DMSO) were kept in darkened vials at −70 °C. Dilutions were prepared in dry ethanol and used immediately. [γ-^{32}P]ATP (more than 10 mCi/mmol) was purchased from New England Nuclear.

Results

The effects of the plastoquinone analogues DBMIB and DNP-INT on electron transport are well documented [32]. In agreement with previous work [6] we found that these compounds inhibit whole-chain electron transport (mediated by a terminal electron acceptor such as 0.2 mM methyl viologen) with an I_{50} of 0.5–1 μM; furthermore, conditions and components used to assay protein phosphorylation (200 μg Chl/ml, 0.2 mM ATP and in some cases, 5 mM ascorbate) did not affect inhibitor potency (Fig. 1a and b).

Inhibition of the cytochrome *bf* complex by plastoquinol antagonists [32] would be predicted to maintain a more reduced plastoquinone pool and a greater extent of kinase activation. Fig. 1a and b shows that this is indeed observed; the phosphorylation of thylakoid proteins is dramatically enhanced in the range 0.5–2.5 μM DBMIB or DNP-INT. Above 5 μM, these inhibitors have a secondary, inhibitory action which progresses up to 50 μM but is only approx. 80% complete [12,19]. This phase is remarkable in that the LHC-II component polypeptides, a 32 kDa polypeptide and autophosphorylation of the 64 kDa kinase are specifically affected (Fig. 1a and b and Fig. 2), whilst phosphorylation of the other PS II peptide (44 kDa, D1/D2, 9 kDa) was unaffected.

Other workers [6] have studied the effects of DBMIB in phosphorylation media lacking a terminal electron acceptor; the resulting slow turnover of the cytochrome complex would thus be already rate limiting, the kinase would be reductively activated in the light, and plastoquinone analogues would be unable to promote its activity further. This result is reproduced in Fig. 1C.

In contrast to a published report [19] ascorbate did not abolish light-dependent protein phosphorylation. However, it did potentiate the inhibitory effect of both DBMIB and DNP-INT on LHC-II phosphorylation (I_{50} = 2–4 μM with ascorbate) whilst leaving phosphorylation of the other PS II polypeptides unaffected (Fig. 1c and d). This effect was not due to reduction of DBMIB by ascorbate, since the T_{50} for inhibition of elec-

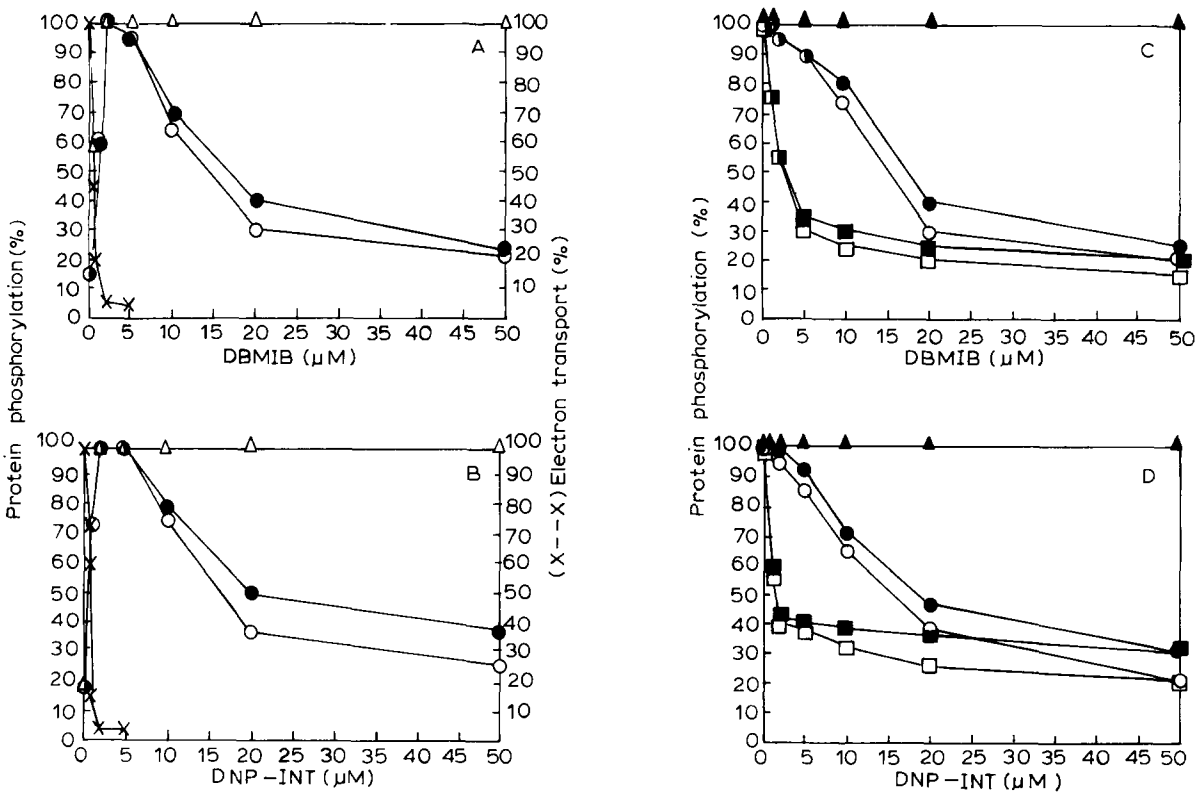

Fig. 1. The effect of DBMIB and DNP-INT on light-induced thylakoid protein phosphorylation and linear electron transport. Conditions were as described in Materials and Methods. Control (basal) rates of electron transport ($H_2O \rightarrow$ methyl viologen) were 15–20 μmol O_2 consumed per h per mg Chl. Control values for protein phosphorylation were 25–30 pmol per 5 min per 20 μg Chl for the 27-kDa and 25-kDa peptides of LHC-II, and 5–10 pmol per 5 min per 20 μg Chl for the 9-kDa peptide. All values were the mean (s.d. = less than ±10%) of six independent determinations, and were normalised to control (100%) values. (A) The effect of DBMIB on light-induced thylakoid protein phosphorylation in the presence of methyl viologen (0.2 mM). ○———○, Phosphorylation of 27-kDa polypeptide; ●———●, phosphorylation of 25-kDa polypeptide; △———△, phosphorylation of 9-kDa polypeptide, ×——— ×, linear electron transport. (B) The effect of DNP-INT (0–50 μM) on light-induced protein phosphorylation in the presence of methyl viologen (0.2 mM). Symbols have the same significance as in (A). (C) The effect of DBMIB on light-induced thylakoid protein phosphorylation. DBMIB was varied over the range of 0-50 μM in the presence or absence of 5 mM ascorbate. ○———○ and □———□, phosphorylation of 27-kDa polypeptide (no ascorbate present and ascorbate present, respectively); ●———● and ■———■, phosphorylation of 25-kDa polypeptide (no ascorbate present and ascorbate present, respectively); △———△ and ▲———▲ phosphorylation of 9-kDa polypeptide (no ascorbate present and ascorbate present, respectively). (D) The effect of DNP-INT on light-induced protein phosphorylation. Symbols have the same significance as in (C).

tron transport was unaffected by the presence or absence of ascorbate (data not shown), and DBMIB is in any event photoreduced in the light [32]. Furthermore, the effect was also apparent with DNP-INT, which does not undergo reversible reduction and oxidation in this E_h range [32].

The reported midpoint potential of DBMIB, +170 mV, is sufficiently close to that of plastoquinone (+55 mV) to mediate plastoquinone reduction by ascorbate in the dark [33,34]. The partial restoration of membrane phosphorylation (Fig. 2) following addition of DBMIB and ascorbate to DCMU-poisoned thylakoids [19] is presumably a reflection of this fact. The addition of DBMIB or ascorbate separately (Fig. 2), or DNP-INT plus ascorbate (data not shown), did not activate the kinase in the dark.

In unreported work, isoascorbate was found to substitute for ascorbate in potentiating the inhibition of LHC-II phosphorylation by DBMIB or

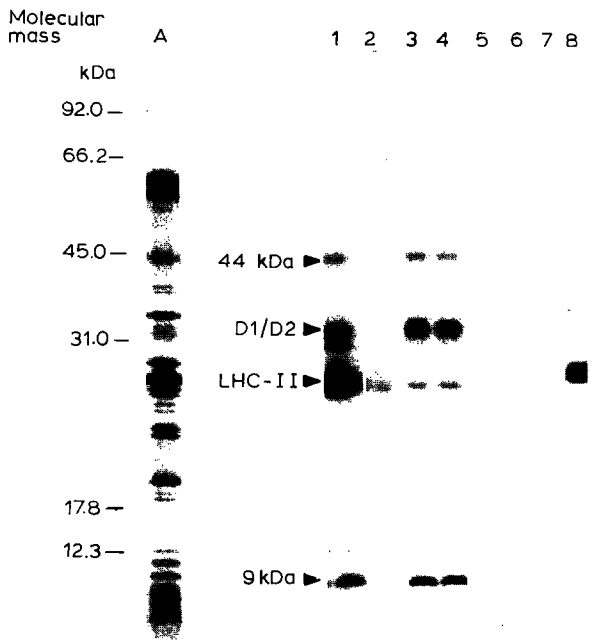

Fig. 2. The differential effect of DBMIB and DNP-INT on light-induced thylakoid protein phosphorylation in the absence of electron acceptor: autoradiogram of polyacrylamide gel. All conditions are described in Materials and Methods. Lane A: Coomassie stain of spinach thylakoid membranes; lanes 1–8 autoradiogram (4 h, −70 °C). Lane 1, control membranes, light; lane 2, 10 μM DCMU, light; lane 3, 50 μM DBMIB, light; lane 4, 50 μM DNP-INT, light; lane 5, 10 μM DCMU, dark; lane 6, 50 μM DBMIB, dark; lane 7, 5 mM ascorbate, dark; lane 8, 50 μM DBMIB plus 5 mM ascorbate, dark. The molecular-mass markers used were: phosphorylase *b* (92.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (17.5 kDa), and cytochrome *c* (12.8 kDa).

DNP-INT. However, reduction of the plastoquinone pool by the ferredoxin/NADPH couple in the dark, gives a contrasting result (Fig. 3) in which all protein phosphorylations were inhibited in parallel. Reductive activation by $TMQH_2$ seemed to make the phosphorylation of PS II components somewhat less sensitive to inhibitor (Fig. 3a and c).

Photosynthetic mutants present an alternative approach to locate regulatory mechanisms [26]. *Lemna perpusilla* mutant 1073 has recently been reported to have no redox-dependent LHC-IIphosphorylation, whilst retaining phosphorylation of the other PS II peptides [21]. Corn mutants lacking a functional cytochrome *bf* complex yield similar data (Fig. 4). Lanes 1–5 of the autoradiogram show the normal redox-dependent phosphorylation of the *Zea* heterozygote; the homozygous recessive, however, lacked all LHC-II phosphorylation whether activated by light or reductant. The other Photosystem II peptides were phosphorylated following light activation, though this process appeared rather insensitive to DCMU inhibition. They were also labelled in the dark on addition of dithionite; however, $TMQH_2$ did not seem to promote reductive activation (lanes 6–10).

A monospecific polyclonal antiserum to the spinach kinase [18] was used to probe immunologically for the presence of the kinase in mutant and wild type plants (Fig. 5). The cross-reactivity of the kinase antiserum with *Zea* and *Lemna* enzyme was adequate (Fig. 5), allowing recognition of a 64 kDa protein kinase in both mutant and wild-type tissues of the two organisms. In contrast, there was no cross reactivity with *Chlamydomonas reinhardtii* (Fig. 5, lane 7). Probing the blot with antiserum to cytochrome *f* (Fig. 5) and the other three subunits of the *bf* complex (data not shown) revealed a total absence (less than 1%) in Lemna mutant 1073, as reported in Ref. 35, and about 5% residual immune reactivity in *Zea* mutant *hcf*-6, as found by Barkan et al. [36] in a related *hcf*-2 mutant. The Coomassie stained gels of *Zea* (Fig. 4) and *Lemna* (Fig. 6) mutants lack a 34 kDa peptide which is cytochrome *f*. The deletions of cytochrome *b*-563 (23 kDa) and the Rieske protein (20 kDa) are not obvious, but a 17 kDa peptide was also missing in the mutant. Polypeptide profiles of the wild type and mutant strains are somewhat different in the 10–12 kDa region of the gel: the mutant has a large Coomassie staining band at 12 kDa which is absent in the wild type. Similar data were obtained from *Lemna* mutant 1073, which lacked components of 17 and 15 kDa and possessed lower molecular-weight peptides. These results are in general agreement with published polypeptide patterns of corn [27] and *Lemna* [35].

Immunological evidence for the presence of the kinase in both wild type and mutant thylakoids, their essentially identical kinase activities toward exogenous substrates (Ref. 21, and unreported data from *Zea*), but the inability of the mutants

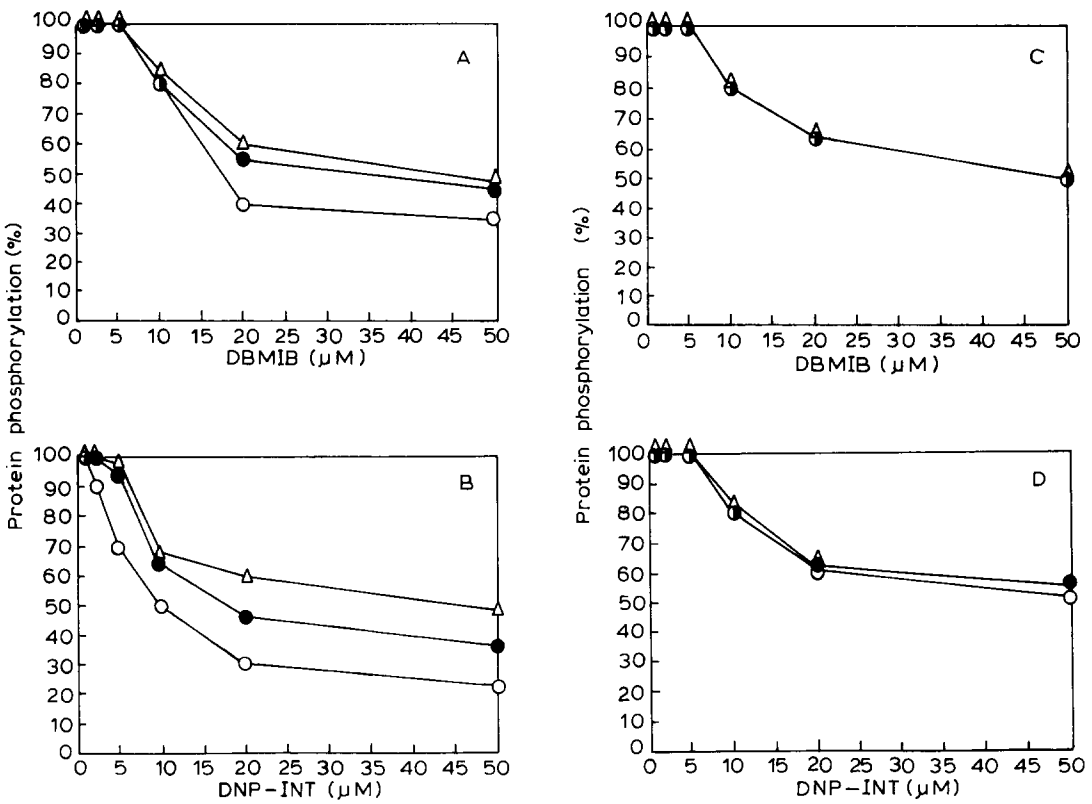

Fig. 3. The differential effect of DBMIB and DNP-INT on thylakoid protein phosphorylation activated by the addition of $TMQH_2$ or Fd/NADPH, in the presence of 10 μM DCMU. Conditions were as in Fig. 1, except that 10 μM DCMU was present in all samples and membrane-bound protein kinase activity was reductively activated in the dark by either $TMQH_2$ or by Fd/NADPH as described in Materials and Methods. Control values for protein phosphorylation were as in Fig. 1. All plotted data (normalized to zero addition values) were the mean (s.d. was less than ±10%) of four independent determinations. (A and B) Phosphorylation of membrane proteins (activation by $TMQH_2$); (C and D) phosphorylation of membrane proteins (activation by Fd/NADPH). ○———○, Phosphorylation of 27-kDa polypeptide; ●———●, phosphorylation of 25-kDa polypeptide; △———△, phosphorylation of 9-kDa polypeptide.

TABLE I

RELATIVE RATES OF PHOSPHORYLATION OF LHC-II AND 9-kDa POLYPEPTIDES BY PURIFIED KINASE ADDED TO WILD-TYPE AND MUTANT THYLAKOIDS

All assays were performed in the dark at 20 °C on thylakoids vesiculated by a freeze/thaw cycle. A sample volume of 50 μl contained 20 μg chlorophyll, 20 mM sodium tricine (pH 8.0), 10 mM $MgCl_2$, 10 μM DCMU, 1.0 μg kinase protein and 0.2 mM ATP containing 5 μCi [γ-^{32}P]ATP. After 15 min incubation, samples were processed as in Materials and Methods. Control rates for intrinsic kinase activity in spinach membranes activated with 1 mM duroquinol in the dark were: 50–70 pmol (LHC-II) and 5–10 pmol (9-kDa component) of phosphate incorporated/20 μg chlorophyll in 5 min. Reconstituted rates for the conditions given were 20–30 pmol (LHC-II) and 1–2 pmol (9-kDa) phosphate incorporated. In the absence of enzyme and $TMQH_2$, residual intrinsic and extrinsic rates were less than 1.0 pmol total phosphate incorporated/20 μg chlorophyll in 15 min.

Phosphoprotein	Thylakoid preparation tested as phosphorylation substrate					
	spinach (control)	pea	corn wild type	corn *hcf*-6	*Lemna* wild type	*Lemna* 1073
LHC-II	100 [a]	26	42	41	32	38
9-kDa	100	21	40	45	40	36

[a] Radioactivity incorporated into phosphoprotein.

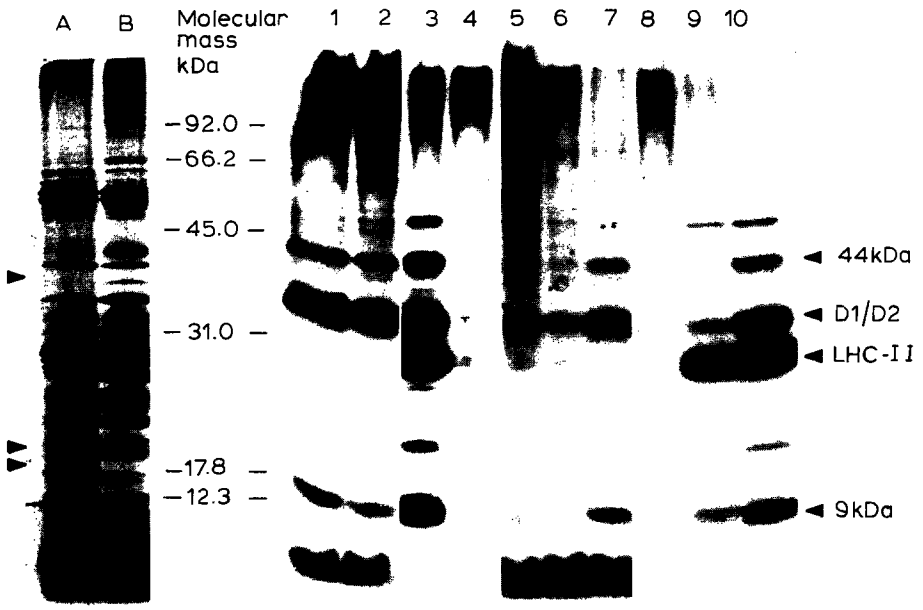

Fig. 4. Membrane protein phosphorylation in wild type and a cytochrome *bf*-minus (*hcf*-6) mutant of corn. Autoradiogram of polyacrylamide gel. All conditions were as in Materials and Methods. Lanes A and B: Coomassie-stained polypeptide pattern of mutant and wild type, respectively (10 μg Chl). Lanes 1–4, light-activated protein phosphorylation: lanes 1 and 2 mutant; lanes 3 and 4, wild type; in the absence (lanes 1 and 3), or presence (lanes 2 and 4) of 10 μM DCMU. Lanes 5–10, reductant-activated protein phosphorylation in the presence of 10 μM DCMU: lanes 5–7 mutant, lanes 8–10 wild type; no reductant (lanes 5 and 8), 1 mM $TMQH_2$ (lanes 6 and 9), 5 mM dithionite (lanes 7 and 10). The positions of the major phosphoprotein groups are indicated on the right side of the autoradiogram, and molecular weight markers on the left. Arrows on the left side of the Coomassie-stained gel denote differences in the polypeptide pattern between mutant and wild type.

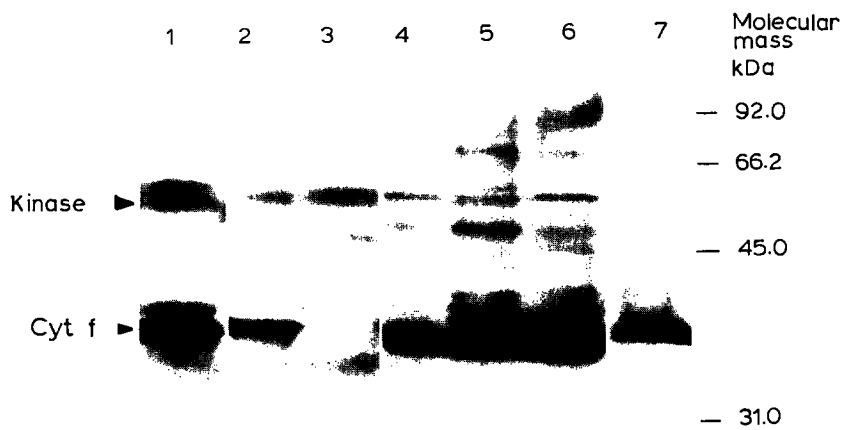

Fig. 5. Immunoblot analysis of thylakoid membranes. All conditions were as in Materials and Methods. The blot was probed sequentially with anti-kinase IgG (1/100 dilution), and anti-cytochrome *f* (1/1000 dilution). The developed blot was autoradiographed for 12 h, −70 °C using Cronex X-ray film and a Lightning Plus Intensifier screen. Lane 1, spinach; lanes 2 and 3, *Lemna* wild-type and mutant; lanes 4 and 5, corn mutant and wild type; lane 6, spinach; lane 7, *Chlamydomonas reinhardtii*. Molecular weight markers are denoted on the right edge of the blot, the positions of the kinase and cytochrome *f* are denoted on the left edge.

to phosphorylate endogenous LHC-II made it necessary to establish that LHC-II in the mutant plants can serve as a kinase substrate. A heterogeneous reconstitution assay [18] was performed using kinase isolated from spinach and a variety of membranes as substrate. As shown in Table I, the phosphorylation of in situ LHC varied between different organisms, from *Chlamydomonas reinardtii* which showed no phosphorylation (data not shown), to pea and *Lemna* which showed about 25% of control (spinach) activity, and corn which gave about 50% of this control. In each organism studied the degree of LHC-II phosphorylation was not significantly different between wild type and mutant thylakoids deficient in cytochrome *bf* complex. Thus both the substrate and the enzyme are potentially functional in the mutants, but the redox-dependent activation mechanism has been disrupted directly or indirectly by the loss of the cytochrome *bf* complex.

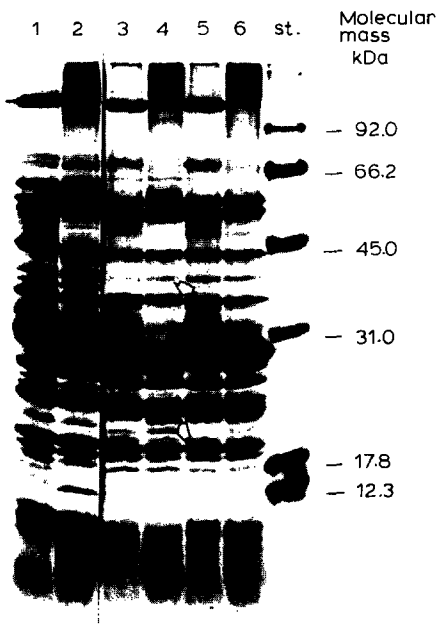

Fig. 6. SDS-polyacrylamide gel of *Lemna* membranes. All conditions are as in Methods. Lanes 1 and 2, spinach; lanes 3 and 4, *Lemna* wild-type; lanes 5 and 6, mutant; lane 7, molecular weight markers as in Figure 2. Lanes 1, 3 and 5, membranes dissolved at 4° C. Lanes 2, 4 and 6, samples heated 1 min, 80° C prior to separation. The gel was run at 4° C and stained with Coomassie Blue as in Materials and Methods.

Discussion

Progress has been made in recent years on the biochemical characterisation [16–18] and location in the membrane [37] of the thylakoid protein kinase responsible for phosphorylating LHC-II. However, the mechanism linking the redox state of the plastoquinone pool to the activation of the kinase or kinases responsible for phosphorylating endogenous proteins remains unknown. A notable difficulty is that the redox sensing mechanism is sensitive to detergents [18] and does not survive membrane fractionation by other than the Yeda press and phase partition methods [11].

In the low concentration range (1–3 μM), DBMIB or DNP-INT will inhibit the reoxidation of plastoquinol by blocking the Q_Z site on the cytochrome *bf* complex [32]. We have shown that the tendency of terminal acceptors such as methyl viologen to promote oxidation of the PQ pool with consequent inhibition of the kinase can be blocked by these inhibitors, which then appear to stimulate protein phosphorylation.

In the original work describing redox-controlled phosphorylation of thylakoid proteins, Allen et al. [6] showed that at tenfold higher concentration, DBMIB also inhibits phosphorylation; this was attributed to an inhibition of PS II [32]. DNP-INT, however, is here shown to give a similar result at high concentration, while having only a minimal effect on the activity of PS II.

As previously noted [12,19], a striking differential effect of DBMIB or DNP-INT is seen on light-activated phosphorylation of thylakoid proteins. The addition of ascorbate further increases the potency of both DBMIB and DNP-INT toward LHC-II phosphorylation, though there persists a discrepancy between the I_{50}'s for inhibition of linear electron transport and LHC-II phosphorylation. Although we do not yet understand the ascorbate effect, we conclude that the inhibition of LHC-II phosphorylation by DNP-INT and DBMIB occurs at a site distinct from the Q_Z binding site, or involves a fundamentally different mechanism.

The inhibition of mitochondrial electron transport by 20–40 μM DBMIB has been attributed to a decrease in membrane fluidity [38]. It seems reasonable to assume by analogy that the intro-

duction of bulky lipophilic molecules such as DBMIB and DNP-INT into the thylakoid membrane might inhibit the association of kinase with its substrates and/or the dissociation of reaction products. In the latter case, the additive could prove especially effective toward phosphorylation of substrates such as LHC that migrate as phosphoproteins away from the grana stacks to the stromal lamellae [6]. The phosphorylation of immobile core components of PS II would be correspondingly insensitive to changes in membrane fluidity. This hypothesis can be tested by measurement of membrane fluidity [39] in the presence and absence of DBMIB, and by studying protein phosphorylation in membranes in which the fluidity has been altered by incorporation of exogenous cholesterol [40], by the use of certain herbicides [41], or by low temperature [42,43]. Indeed, Haworth [44] has shown that the incorporation of cholesterol hemisuccinate into thylakoid membranes inhibits state transitions whilst leaving protein phosphorylation relatively unaffected. At present we are further characterizing the influence of thylakoid membrane fluidity on the interplay between protein phosphorylation and state transitions.

The mechanism whereby ascorbate potentiates the differential effect of both DBMIB and DNP-INT on light-activated protein phosphorylation is also under investigation. The violaxanthan cycle [45], which is activated in the light by ascorbate, may have a role in the inhibition of state transitions as discussed elsewhere [46].

Evidence of a role for the cytochrome *bf* complex in the redox-dependent phosphorylation of thylakoids has come from recent studies on *Lemna* [21] and *Zea* (this work). The data show that although the 64-kDa protein kinase is constitutively present in mutants lacking the *bf* complex, LHC-II phosphorylation is absent. Data obtained from a *Chlamydomonas* mutant lacking the cytochrome *bf* complex were less clear cut [20]. Although the PS II polypeptides were phosphorylated in the mutant, they no longer appeared to be under redox control and LHC-II phosphorylation was not totally abolished. Unfortunately, the spinach anti-kinase IgG does not cross-react with *Chlamydomonas* membranes on immune blotting (Fig. 5, lane 7), hence it was not possible to probe them for the presence of kinase.

These results seem indicative of either two different mechanisms for redox activation of a single kinase, or two different kinases. It must be emphasized, however, that detailed comparisons of the Coomassie-stained polypeptide profiles of the *Lemna* and *Zea* mutants and their corresponding wild types reveals differences that cannot be attributed to known components of the cytochrome *bf* complex. The lipid composition of the mutant membranes may also be altered [26,35]. Furthermore, although electron microscopy of the *Lemna* 1073 mutant revealed no obvious ultrastructural changes [21], a more detailed freeze-fracture analysis of *Chlamydomonas reinhardtii* mutants lacking a functional cytochrome *bf* complex found numerous ultrastructural differences [47].

Thus the lesion in LHC-II phosphorylation could be directly due to the absence of the assembled cytochrome *bf* complex, or to the absence of some other minor polypeptide, or indirectly due to changes in membrane ultrastructure caused by the lack of one of the four principal protein complexes. It would be instructive to screen all the cytochrome *bf* mutants of *Chlamydomonas* described by Bendall et al. [48] and Lemaire et al. [10], and other corn mutants lacking the *bf* complex [26], then compare the phosphorylation patterns with those obtained from Photosystem I and II mutants in the hope of reaching a consensus from all of the mutant data.

Note added in proof

A modified pattern of thylakoid phosphorylation was recently reported for intact leaves of the *Zea* mutant *hcf*-6 by Bennett et al. (Bennett, J., Shaw, E.K. and Michel, H. (1988) Eur. J. Biochem. 171, 95–100).

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References

1 Bennett, J. (1977) Nature 269, 344–347.
2 Bennett, J. (1979) FEBS Lett. 103, 342–344.
3 Horton, P., Allen, J.F., Black, M.T. and Bennett, J. (1981) FEBS Lett. 125, 193–196.
4 Millner, P.A., Widger, W.R., Abbott, M.S., Cramer, W.A. and Dilley, R.A. (1982) J. Biol. Chem. 257, 1736–1742.
5 Bennett, J., Steinback, K.E. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5253–5257.
6 Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature 291, 21–25.
7 Horton, P. and Black, M.T. (1981) Biochim. Biophys. Acta 635, 53–62.
8 Widger, W.R., Farchaus, J.W., Cramer, W.A. and Dilley, R.A. (1984) Arch. Biochem. Biophys. 233, 72–79.
9 Millner, P.A., Marder, J.B., Gounaris, K. and Barber, J. (1986) Biochim. Biophys. Acta 852, 30–37.
10 Lemaire, C., Girard-Bascou, J., Wollmann, F.A. and Bennoun, P. (1986) Biochim. Biophys. Acta 851, 229–238.
11 Whitelegge, J.P., Millner, P.A., Gounaris, K. and Barber, J. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 809–812, Martinus Nijhoff, Dordrecht.
12 Farchaus, J., Dilley, R.A. and Cramer, W.A. (1985) Biochim. Biophys. Acta 809, 17–26.
13 Markwell, J.P., Baker, N.R., Bradbury, M. and Thornber, J.P. (1984) Plant Physiol. 74, 348–354.
14 Lin, Z.-F., Lucero, H.A. and Racker, E. (1982) J. Biol. Chem. 257, 12153–12156.
15 Lucero, H.A., Lin, Z.-F. and Racker, E. (1982) J. Biol. Chem. 257, 12157–12160.
16 Coughlan, S.J. and Hind, G. (1986a) J. Biol. Chem. 261, 11378–11385.
17 Coughlan, S.J. and Hind, G. (1986b) J. Biol. Chem. 261, 14062–14068.
18 Coughlan, S.J. and Hind, G. (1987) J. Biol. Chem. 262, 8402–8408.
19 Bennett, J., Shaw, E.K. and Bakr, S. (1987) FEBS Lett. 210, 22–26.
20 Lemaire, C., Girard-Bascou, J. and Wollman, F.A. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. IV, pp. 655–658, Martinus Nijhoff, Dordrecht.
21 Gal, A., Shahak, Y., Schuster, G. and Ohad, I. (1987) FEBS Lett. 221, 205–210.
22 Clark, R.D. and Hind, G. (1983) J. Biol. Chem. 258, 10348–10354.
23 Walker, D.A. (1980) Methods Enzymol. 69, 94–104.
24 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
25 Shahak, Y., Posner, H.B. and Avron, M. (1976) Plant Physiol. 57, 577–579.
26 Miles, D. (1980) Methods Enzymol. 69, 3–23.
27 Metz, J.G., Miles, D. and Rutherford, A.W. (1983) Plant Physiol. 66, 18–24.
28 Crowther, D. and Hind, G. (1980) Arch. Biochem. Biophys. 204, 568–577.
29 Allen, J.F. and Horton, P. (1981) Biochim. Biophys. Acta, 638, 290–295.
30 Coughlan, S.J. and Pfanz, H. (1986) Biochim. Biophys. Acta 849, 32–40.
31 Coughlan, S.J., Matthijs, H.C.P. and Hind, G. (1985) J. Biol. Chem. 260, 14891–14893.
32 Trebst, A. (1980) Methods Enzymol. 69, 675–715.
33 Chain, R.K. and Malkin, R. (1979) Arch. Biochem. Biophys. 197, 52–56.
34 Malkin, R. (1982) Biochemistry 21, 2945–2950.
35 Lam, E. and Malkin, R. (1985) Biochim. Biophys. Acta 810, 106–109.
36 Barkan, A., Miles, D. and Taylor, W.C. (1986) EMBO J. 5, 1421–1427.
37 Coughlan, S.J. and Hind, G. (1987) Biochemistry 26, 6515–6521.
38 Siedow, J.N., Huber, S.C. and Moreland, D.E. (1979) Biochim. Biophys. Acta 547, 282–295.
39 Ford, R.C. and Barber, J. (1980) Photobiochem. Photobiophys. 1, 263–270.
40 Yamamoto, Y., Ford, R.C. and Barber, J. (1981) Plant Physiol. 67, 1069–1072.
41 St. John, J.B. (1976) Plant Physiol. 57, 38–40.
42 Moll, B.A., Eilmann, M. and Steinback, K.E. (1987) Plant Physiol. 83, 428–433.
43 Huner, N.P.A., Krol, M., Williams, J.P., Maissan, E., Low, P.S., Roberts, D. and Thompson, J.E. (1987) Plant Physiol. 84, 12–18.
44 Haworth, P. (1983) Arch. Biochem. Biophys. 226, 145–154.
45 Yamamoto, H.Y. (1979) Pure Appl. Chem, 51, 639–648.
46 Meck, E. and Strasser, R.J. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 709–712, Martinus Nijhoff, Dordrecht.
47 Olive, J., Vallon, O., Wollman, F.A., Recouvreur, M. and Bennoun, P. (1986) Biochim. Biophys. Acta, 851, 239–248.
48 Bendall, D.S., Sanguansermsri, M., Girard-Bascou, J. and Bennoun, P. (1986) FEBS Lett. 203, 31–35.