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PROTEIN KINASE AND PHOSPHATASE ACTIVITIES OF THYLAKOID MEMBRANES[1]

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ABSTRACT Dephosphorylation of the 25 and 27 kDa light-harvesting Chl a/b proteins (LHCII) of the thylakoid membranes is catalyzed by a phosphatase which differs from previously reported thylakoid-bound phosphatases in having an alkaline pH optimum (9.0) and a requirement for Mg^{2+} ions. Dephosphorylation of the 8.3 kDa psb H gene product requires a Mg^{2+} ion concentration more than 200 fold higher than that for dephosphorylation of LHC II. The 8.3 kDa and 27 kDa proteins appear to be phosphorylated by two distinct kinases, which differ in substrate specificity and sensitivity to inhibitors. The plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) inhibits phosphorylation of the 27 kDa LHC II much more readily than phosphorylation of the 8.3 kDa protein. A similar pattern of inhibition is seen for two synthetic oligopeptides (MRKSATTKKAVC and ATQTLESSSRC) which are analogs of the phosphorylation sites of the two proteins. Possible modes of action of DBMIB are discussed.

INTRODUCTION

The enzymes responsible for the phosphorylation and dephosphorylation of thylakoid proteins are poorly understood. Two kinases have been solubilized from

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thylakoids and purified [1] but do not show the redox control of activity which characterizes kinase operation in the membrane [2]. Prior to solubilization the kinases are sensitive to the redox state of the plastoquinone (PQ) pool [2-4], being activated by reduction of the pool by electron flow from photosystem II (in the light) [5] or from dithionite [2], duroquinol [4] and NADPH/ferredoxin [5] in the dark.

Recently we showed that the PQ antagonist DBMIB, in the presence of 5 mM ascorbate, inhibits thylakoid protein phosphorylation selectively [6]. The three most conspicuous phosphoproteins of thylakoids are the 25 and 27 kDa LHC II proteins and a 8.3 kDa protein identified as the product of the psb H gene of chloroplast DNA [7-9]. DBMIB (50 μM) inhibits phosphorylation of the 27 kDa LHC II by 90%, the 25 kDa LHC II by 60% and the 8.3 kDa protein by less than 5% [6]. If ascorbate is omitted, all protein phosphorylation is inhibited completely. Differential phosphorylation of LHC II and the 8.3 kDa protein in the presence of DBMIB was also reported earlier [10] but in that case there was no requirement for ascorbate.

The differential effect of DBMIB strongly supports the concept that thylakoids contain two redox-controlled kinases. This concept was put forward originally to explain the differential inhibition of the phosphorylation of LHC II and the 8.3 kDa protein by thiol reagents [11] and the adenosine derivative 5'-fluorosulfonylbenzyladenine [10]. The existence of multiple kinases was also suggested by the differential effects of salts and temperature on thylakoid protein phosphorylation [12] and by the isolation of two distinct kinases [13], although recent results [1] indicate that one of the two kinases was incorrectly identified.

Less is known about the phosphoprotein phosphatase(s) of thylakoids. Dephosphorylation of thylakoid proteins is not under redox control [14]. Two reports [15,16] indicate that thylakoids contain an acid phosphatase activity which may be assayed using nitrophenylphosphate as substrate. In one case [15] the phosphatase was solubilized by treatment of the membrane with hydroxylamine, while in the other case [16] solubilization was achieved with 0.33 M $MgCl_2$ or sodium deoxycholate. In neither case did the phosphatase require Mg^{2+} ions.

In this report we characterize further the protein phosphatase and kinase activities of thylakoid membranes. The effects of pH and Mg^{2+} ion concentration on phosphatase

activity are investigated, and the inhibitory effects of DBMIB on kinase activity are explored using synthetic peptide analogs of the 27 kDa LHC II and the 8.3 kDa protein. We also compare the effects of DBMIB with that of another PQ antagonist, the dinitrophenyl ether of iodonitrothymol (DNP-INT).

MATERIAL AND METHODS

Procedures for isolation and ^{32}P-labelling of intact pea (Pisum sativum L. var. Feltham First) chloroplasts [17], preparation of thylakoids [5], incubation of thylakoids under phosphorylating [5] or dephosphorylating [14] conditions, and analysis of phosphoproteins by SDS-PAGE [14] have been described. Specific phosphoproteins were quantified by counting of gel slices with liquid scintillant. Spinach (Spinacia oleracea L. cultiver hybrid 424) thylakoids were isolated and incubated with synthetic oligopeptides and inhibitors as described [6]. Phosphorylation of oligopeptides was assayed by an ion-exchange procedure [18].

[^{32}P]P_i and [γ-^{32}P]ATP were obtained from New England Nuclear. The former was used undiluted, the latter as indicated in figure legends. DBMIB and DNP-INT were the generous gift of Dr. A. Trebst. CCCP was purchased from Sigma. The oligopeptide analog of the phosphorylation site of pea LHC II was synthesized manually as described [6], while the analog of the phosphorylation site of spinach 8.3 kDa protein [9] was synthesized automatically on a Biosearch 9500 Synthesizer. In both cases the chemistry was that described by Merrifield [19] and synthesis was initiated with cysteine bound to the resin. After synthesis the peptides were cleaved from the resin with HF, the cysteines were blocked with excess iodoacetamide and the peptides were purified to homogeneity by HPLC through a preparative C18 column with a water/acetonitrile gradient containing 0.1 M phosphate buffer and 0.1% hexane sulfonic acid [20].

RESULTS

When isolated intact pea chloroplasts were incubated in the light with [^{32}P]orthophosphate for 15 min, several thylakoid proteins became labelled (Fig. 1). They included

the psb H gene product (8.3 kDa) [9], apo-proteins of LHC II (25 and 27 kDa) [21], a 32 kDa protein (believed to be D2, the psb D gene product [22]), and the apo-protein of CPa-2 (psb C gene product [23]).

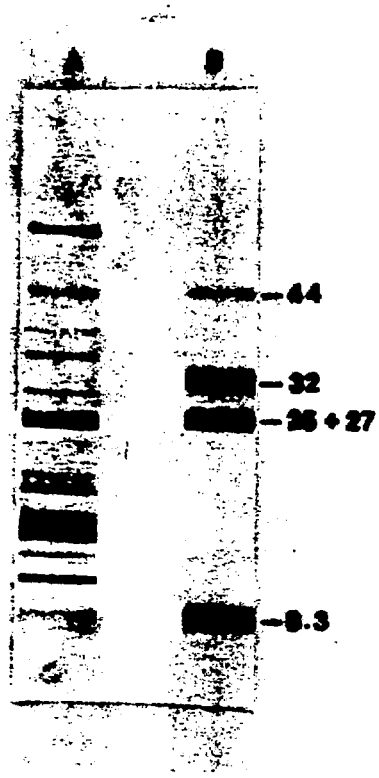

FIGURE 1. Thylakoid phosphoproteins. Isolated intact pea chloroplasts were incubated in the light for 15 min with [^{32}P]P_i. Thylakoid proteins were isolated and analyzed by SDS-PAGE. A, stained gel. B, autoradiogram.

To study dephosphorylation of thylakoid proteins, chloroplasts were incubated for 5 min in the light with [^{32}P]Pi and then treated in such a way as to inhibit either

labelling of ATP or kinase activity or both. Fig. 2 shows the results of treatment with CCCP, which inhibits ATP synthesis without inhibiting kinase activity [5]. Immediately after addition of the inhibitor, net protein dephosphorylation began. The time for 50% dephosphorylation was 7 min for LHC II (25 and 27 kDa) and 15 min for the 8.3 kDa protein. Dephosphorylation of both proteins followed approximately first-order kinetics. The 32 and 44 kDa proteins displayed intermediate kinetics of dephosphorylation (not shown).

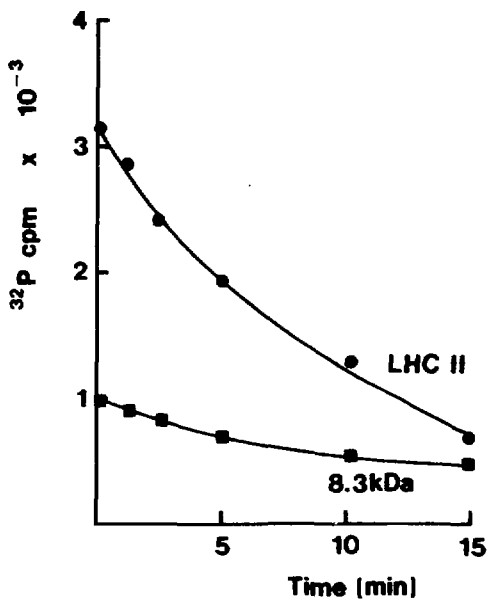

FIGURE 2. Dephosphorylation of thylakoid proteins in organello. Intact pea chloroplasts were incubated in the light for 5 min with [^{32}P]P_i, CCCP (10 μM) was added and the incubation was continued. At intervals, protein-bound ^{32}P was assayed by SDS-PAGE and scintillation counting.

Table 1 reports the $t_{0.5}$ for dephosphorylation of LHC II under four circumstances. In addition to treatment with CCCP, labelled chloroplasts were treated with 10 μM diuron or 5 mM sodium phosphate, or transferred to darkness. Diuron blocks electron flow from PS II to the PQ pool, thereby preventing activation of the protein kinase by PQH_2 [2-4]. It also prevents non-cyclic and pseudocyclic photophosphorylation and interferes with the poising of cyclic electron transport [24]. Unlabelled phosphate provided a chase, presumably by flushing ^{32}P out of the ATP pool. Transfer to darkness inhibited both kinase activation and photophosphorylation. In all four cases, 50% dephosphorylation occurred after about 7 min, establishing that dephosphorylation of LHC II is insensitive to light/dark changes, and to inhibition of electron transport or ATP synthesis.

TABLE 1

DEPHOSPHORYLATION OF LHC II IN ORGANELLO[a]

Treatment	$t_{0.5}$ (min)
5 min light then:	
Light + CCCP (10 μM)	7
Light + diuron (10 μM)	7
Light + P_i (5 mM)	7
Dark	6

[a]Isolated intact pea chloroplasts

Dephosphorylation In Vitro

It is known that the stromal pH rises by about 1 pH unit (from about 7.1 to about 8.0) when chloroplasts are transferred from dark to light [25]. The alkalinization of the stroma is a consequence of the pumping of protons into the lumen and is accompanied by an extrusion of Mg^{2+} ions into the stroma. The fact that LHC II dephosphorylation is

not markedly affected by light/dark changes suggests that the phosphatase is not very sensitive to pH changes in this range. To check this point directly, the pH optimum of the LHC II phosphatase was determined in vitro, using thylakoids that had been prelabelled in organello. The dephosphorylation rate was maximal at pH 9 (Fig. 3). The extent of dephosphorylation in 10 min increased by only 14% between pH 7.1 and pH 8.0. In contrast, the rate of dephosphorylation increased 9-fold between pH 6 and pH 7.

Thylakoid proteins differed markedly in their requirement for Mg^{2+} ions during dephosphorylation (Fig. 4). Thylakoids washed in EDTA showed no dephosphorylation over

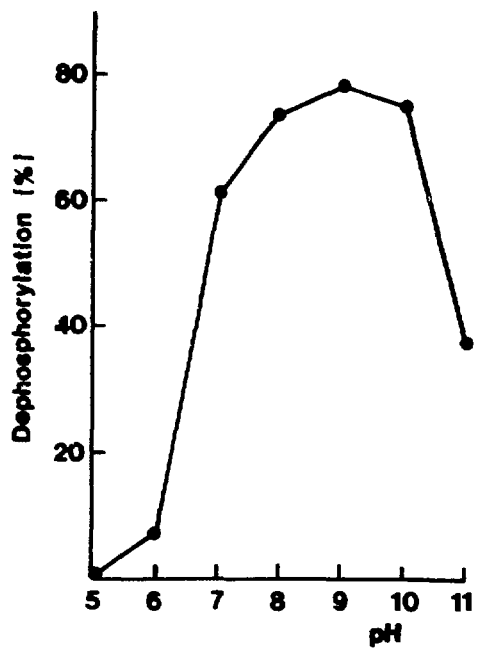

FIGURE 3. Dephosphorylation of LHC II in isolated thylakoids: pH dependence. Thylakoids were isolated from ^{32}P-labelled intact pea chloroplasts and incubated in 50 mM Mes-50 mM Tricine-50 mM glycine-1 mM $MgCl_2$ for 10 min at 20°C at the indicated pHs. ^{32}P level in LHC II was assayed by SDS-PAGE and scintillation counting.

20 min at 40°C, but washing in Mg^{2+}-free buffer followed by resuspension in 10 μM Mg^{2+} resulted in rapid dephosphorylation of LHC II, significant dephosphorylation of D2 and no dephosphorylation of the 8.3 kDa protein. Rapid

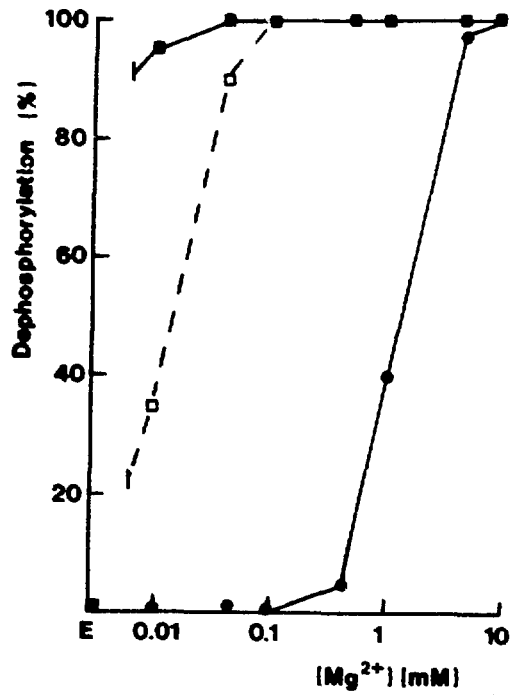

FIGURE 4. Dephosphorylation of proteins in isolated thylakoids: Mg^{2+} ion dependence. Thylakoids were isolated from ^{32}P-labelled intact pea chloroplasts, washed once in 50 mM Tricine (pH 8.0) and incubated in 50 mM Tricine-NaOH (pH 8.0) for 20 min at 40°C with 10 mM EDTA (E) or the indicated Mg^{2+} ion concentration. ^{32}P levels in LHC II (■), D2 (□) and 8.3 kDa protein (●) were assayed by SDS-PAGE and scintillation counting. The incubation temperature of 40° C was chosen to enhance overall dephosphorylation rate and so permit extensive dephosphorylation of 8.3 kDa protein.

dephosphorylation of D2 and 8.3 kDa protein required 50 μM and 5 mM Mg^{2+} ions, respectively. It is not clear whether these differences in Mg^{2+} requirement reflect the existence of several phosphatases or are the result of changed interactions between the different substrates and one phosphatase.

LHC II Phosphorylation In Vitro

Thylakoids were incubated with [γ-^{32}P]ATP in the presence of NaCl or the phosphatase inhibitor NaF. Incorporation was 40-50% higher with NaF, whether the kinase was activated in the light by electron flow from PS II

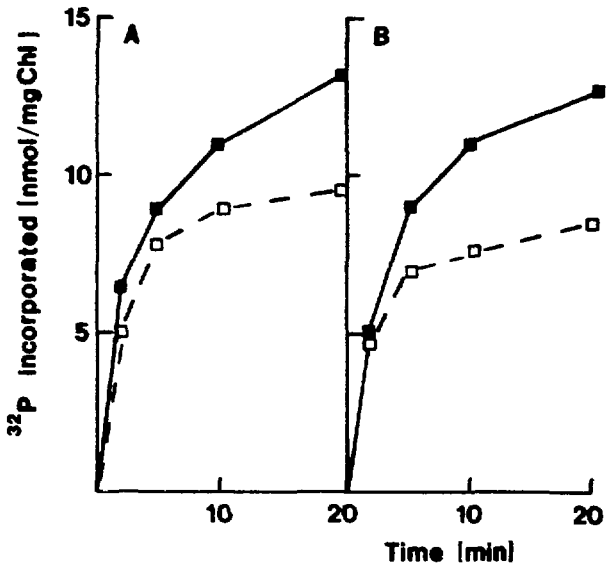

FIGURE 5. Phosphorylation of LHC II by isolated pea thylakoids. The incubation was in 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (200 μCi/μmole) at 20°C in the presence of 10 mM NaCl (□) or NaF (■). A: Light B: dark and dithionite

(Fig. 5A) or in darkness by electron flow from dithionite (Fig. 5B). This result shows the substantial effect of concomitant dephosphorylation on yield of phosphorylated LHC II. To obtain initial rates of phosphorylation, very short incubation times are required in the absence of NaF.

In the presence of NaF, both incubations in Fig. 5 yielded phosphorylated LHC II of about 13 nmoles/mg Chl after 20 min. CPa-2, D2 and the psbH protein were also phosphorylated. Table 2 lists the yields for the three PS II phosphoproteins, which together accounted for 8 nmoles phosphate/mg Chl. On the assumption that there are approximately 3 nmoles of PS II reaction center per mole Chl in thylakoids [26], we calculate that the 8.3, 32 and 44 kDa proteins together contribute about 2.4 phosphate molecules per PS II, with LHC II contributing another 4.0 molecules. If we assume that LHC II accounts for about 50% of total Chl and contains about 7 Chl molecules per molecule of protein [27], there are about 24 molecules of LHC apo-protein per PS II, with about 17% in phosphorylated form. If we assume one molecule each of 8.3, 32 and 44 kDa proteins per PS II, the two larger proteins are more than 50% phosphorylated, while

TABLE 2

PHOSPHORYLATION OF PHOTOSYSTEM II PROTEINS

Protein	Mol. wt.	Assumed Abundance[a]	Phosphate Content I[b]	II[c]	III[d]
LHC II	25-27,000	24	13.0	4.0	16.7
CPa-2	44,000	1	2.0	0.60	60
D2	32,000	1	1.7	0.51	51
psbH protein	8,300	1	4.3	1.31	131

[a] mol protein/mol PSII
[b] nmol P/mg Chl
[c] mole P/mol PSII, assuming 3 nmol PSII/mol Chl
[d] % protein molecules phosphorylated

the 8.3 kDa protein contains more than one phosphate per molecule. However, as analysis of the phosphorylated 8.3 kDa protein from spinach reveals only a single phosphorylation site [9], there may be two molecules of 8.3 kDa protein per PS II reaction center or a second phosphoprotein of that molecular weight in the thylakoids.

A major problem with the purification of thylakoid-bound protein kinases is to relate the kinase activities recovered after solubilization to those detected in the intact membrane. The fact that DBMIB gives differential inhibition of the 27 kDa LHC II and the 8.3 kDa psb H protein [6] implies the existence of two redox-controlled kinases differing in substrate specificity and interaction with PQ analogs. To maximize the probability of successfully assaying and distinguishing between these two enzymes after solubilization, we have synthesized oligopeptide

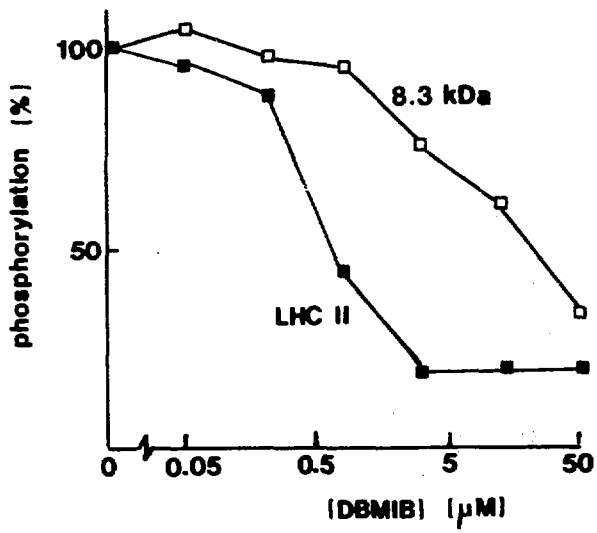

FIGURE 6. Differential inhibition by DBMIB of phosphorylation by isolated spinach thylakoids of phosphorylation site analogs of LHC II and 8.3 kDa protein. The incubation mixture was illuminated for 20 min at 20°C in 25 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (87 Ci/mole). Chl was 67 μg/ml and the peptides were 333 μg/ml.

analogs of the phosphorylation sites of the two proteins. The synthesis of the LHC II analog (MRKSATTKKAVC) was described previously [6]. The synthesis of the analog (ATQTLESSSRC) of the 8.3 kDa protein, whose phosphorylation site has been sequenced [9], is reported here. Fig. 6 shows that spinach thylakoids phosphorylate both peptides. Phosphorylation of the peptides exhibits differential sensitivity to DBMIB: inhibition of phosphorylation reaches 50% at 0.7 μM for the LHC II analog and at 25 μM for the 8.3 kDa protein analog. Similar differential inhibition is seen *in situ* for phosphorylation of the corresponding proteins.

If there are indeed two kinases, how does DBMIB cause differential inhibition? To investigate this question in more detail we examined the effect of another PQ antagonist the dinitrophenyl ether of iodonitrothymol (DNP-INT) on kinase activity in thylakoids. Both DBMIB and DNP-INT inhibit the cytochrome b_6-f complex by binding at the PQH_2 oxidase site [28], although DBMIB interacts more directly with the Rieske FeS protein than does DNP-INT [29]. DBMIB, with its reactive bromines, is a potential lipophilic thiol reagent [30]. It is also a quinone capable of undergoing redox reactions [31]. DNP-INT is neither a potential thiol reagent nor a quinone.

Table 3 shows that DBMIB and DNP-INT inhibit phosphorylation of the 27 kDa LHC II in the light in the presence of ascorbate and in the dark in the presence of diuron when duroquinol (DQH_2) is used to reduce PQ pool. Much higher concentrations of inhibitors are required to inhibit phosphorylation of the 8.3 kDa protein. These results suggest that the selective inhibition by DBMIB of LHC II phosphorylation is not due to its abilities to act as a thiol reagent or a redox-active quinone but is due either to its binding at the PQH_2 oxidase site of the cytochrome b_6-f complex or to a structurally similar site on the LHC II kinase.

DISCUSSION

Through the use of artificial substrates, an acid phosphatase has been detected on thylakoid membranes [15,16]. There was no evidence for an alkaline phosphatase, even when phosphorylated casein was used as substrate. However, the data presented here establish clearly that LHC II is dephosphorylated by a membrane-bound alkaline phosphatase

which has a requirement for Mg^{2+} ions. The activity against all thylakoid phosphoproteins *in vitro* is accelerated by increased Mg^{2+} ion concentration. This is especially true for the 8.3 kDa protein.

PS II units and LHC II units are usually found mainly in appressed granal membranes rather than exposed stromal membranes [32]. In a recent study [33] it was found that a 1 min exposure of thylakoids to a temperature of about 40°C in the presence of 10 mM Mg^{2+} ions led to the separation of PS II units from LHC II and the migration of the former to exposed membranes, while LHC II remained in the appressed regions. This pattern of movement is exactly opposite to that observed when thylakoids become phosphorylated [34]; in the latter situation, phosphorylated LHC II migrates from appressed to exposed regions and phosphorylated PS II units remain in the appressed regions. The data presented here may provide insight into the temperature-induced separation of LHC II and PS II. As the high temperature incubation was performed in the absence of ATP, net dephosphorylation would have occurred. Furthermore, the combination of high temperature and high Mg^{2+} ion concentration would have caused rapid dephosphorylation of LHC II and all PS II proteins, including the 8.3 kDa protein. Since dephosphorylated LHC II would be expected to remain in the appressed membranes, its behavior at high temperature is readily understood. To explain the migration of PS II units it is necessary to propose that dephosphorylation might allow PS II to leave the appressed regions.

Phosphorylation of thylakoids *in vitro* in the presence of the phosphatase inhibitor NaF leads to almost total phosphorylation of PS II proteins. In fact, the percentage phosphorylation of the 8.3, 32 and 44 kDa proteins (51-131%) is much higher than that for LHC II (16.7%). This result raises the possibility that phosphorylation of PS II proteins has an important role in photosynthesis. It is known that phosphorylation alters several aspects of PS II structure and function, including the redox equilibrium between Q_A and Q_B [35], the light-saturated rate of PS II electron transport [36], the rate of cyclic electron flow around PS II [37] and possibly herbicide binding [38]. However, it is not clear whether these responses are due to the phosphorylation of specific proteins or to the general accumulation of negative charge [39] in the close confines of the appressed membranes.

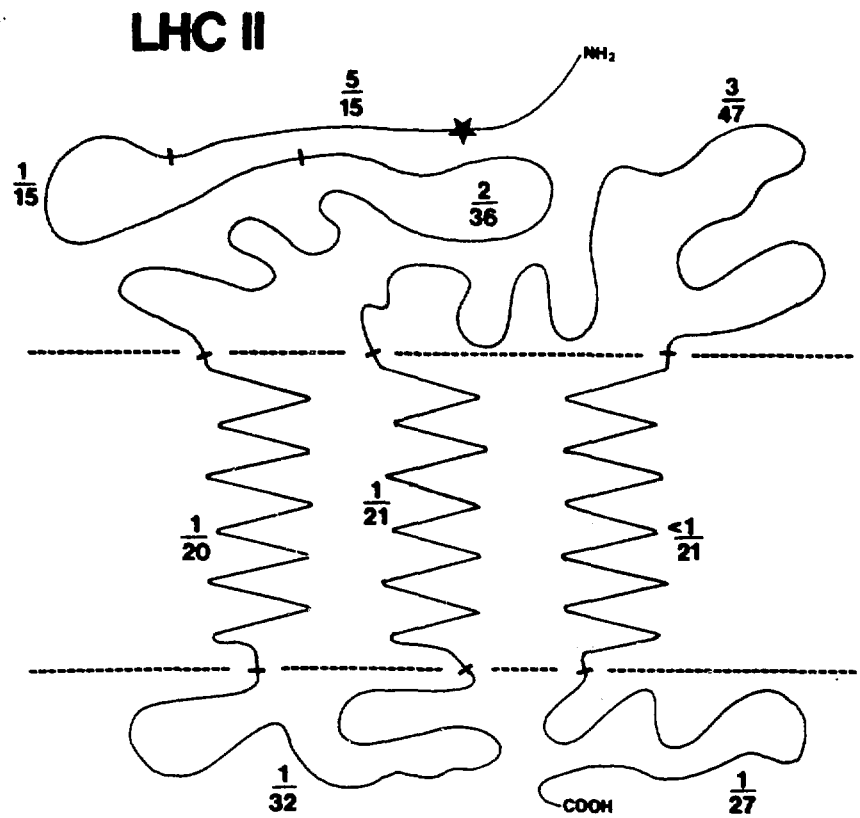

FIGURE 7. Schematic representation of LHC II in thylakoid membrane. Based on a model in [45], the scheme shows three transmembrane α-helices, the N-terminus exposed at the outer surface of the membrane and the C-terminus exposed in the lumen. The phosphorylation site is shown by the star. The fractions refer to the average number of amino acid replacements in twelve LHC II proteins (compared segment by segment with the A30 LHC II of Lemna gibba [43]) divided by the number of residues per segment.

TABLE 3
CONCENTRATIONS[a] OF DBMIB AND DNP-INT REQUIRED FOR 50% INHIBITION OF PHOSPHORYLATION OF 27 kDa LHC II AND 8.3 kDa PROTEIN

Incubation Conditions[b]	DBMIB		DNP-INT	
	LHC	8.3	LHC	8.3
Light + 5 mM ascorbate	0.25	1.5	2.3	50
Dark + 10 μM diuron + 0.5 mM DQH_2	0.1	28	0.1	50

[a] μM
[b] With isolated spinach thylakoids (50 μg Chl/ml)

One possible role of PS II phosphorylation is to ensure adequate buffering in the partitions, the narrow spaces between appressed membranes. The need for buffering in this space arises from the fact that it is presumably the source of protons taken up by Q_B^{2-} prior to emergence from PS II as PQH_2. The flow of protons in photosynthesis may be summarized as follows. Protons are deposited into the lumen by oxidation of H_2O in PS II and oxidation of PQH_2 by the cytochrome b/f complex. The flow of protons through ATP synthase and into the stroma results in ATP formation. The cycle is completed by the diffusion of protons from the stroma to the partition gap between appressed membranes [40]. Proteins buffer poorly in the pH range 6-9 but phosphorylation adds an ionizable group with a pK_a of about 7.2.

Most higher plants that have been examined appear to have at least 10 genes for LHC II, although it is not clear that all these genes are expressed. The base sequences indicate that LHC II is generally a highly conserved protein both within and between species. However, one region of the mature LHC II apo-protein is hypervariable: the first 15 amino acids (Fig. 8). The data for this analysis are sequences of LHC II deduced from selected genes in Pisum [41], Arabidopsis [42], Lemna [43], Petunia [44] and Lycopersicon [45]. Twelve sequences were compared with the AB30 sequene of Lemna gibba, the proteins being divided into segments as indicated. Whereas all the segments of LHC II showed an average of 1 amino acid change per 15 or more residues compared with AB30, the N-terminal segment averaged 5 changes per 15 residues. Since this segment contains the phosphorylation site, it may be that a major reason for multiple LHC II genes is to provide a variety of N-termini differing in ease of phosphorylation/dephosphorylation. We are investigating this possiblity in the case of tomato by synthesizing oligopeptide corresponding to known LHC II N-termini [45]. We believe that synthetic oligopeptides will prove to be important tools in characterizing both the enzymes and the substrates involved in thylakoid protein phosphorylation.

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