Activation/Deactivation Cycle of Redox-controlled Thylakoid Protein Phosphorylation

ROLE OF PLASTOQUINOL BOUND TO THE REDUCED CYTOCHROME b\(_{f}\) COMPLEX*

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Signal transduction via light-dependent redox control of reversible thylakoid protein phosphorylation has evolved in plants as a unique mechanism for controlling events related to light energy utilization. Here we report for the first time that protein phosphorylation can be activated without light or the addition of reducing agents by a transient exposure of isolated thylakoid membranes to low pH in darkness. The activation of the kinase after incubation of dark-adapted thylakoids at pH 4.3 coincides with an increase in the plastoquinol: plastoquinone ratio up to 0.25. However, rapid plastoquinol reoxidation (<1 min) at pH 7.4 contrasts with the slow kinase deactivation (t\(_{1/2}\) = 4 min), which indicates that the redox control is not directly dependent on the plastoquinone pool. Use of inhibitors and a cytochrome b\(_{f}\)-deficient mutant of Lemma demonstrate the involvement of the cytochrome b\(_{f}\) complex in the low-pH induced protein phosphorylation. EPR spectroscopy shows that subsequent to the transient low pH treatment and transfer of the thylakoids to pH 7.4, cytochrome f, the Rieske Fe-S center, and plastocyanin become reduced and are not reoxidized while the kinase is slowly deactivated. However, the deactivation correlates with a decrease of the EPR g\(_{2}\) signal of the reduced Rieske Fe-S center, which is also affected by quinone analogues that inhibit the kinase. Our data point to an activation mechanism of thylakoid protein phosphorylation that involves the binding of plastoquinol to the cytochrome b\(_{f}\) complex in the vicinity of the reduced Rieske Fe-S center.

Protein phosphorylation plays a major role in cellular signaling, developmental processes, and metabolism regulation of living cells (1–3). In chloroplasts a unique light mediated redox-controlled phosphorylation (4–7) of a number of proteins associated with the thylakoid membrane has evolved. Phosphorylation of the major light-harvesting chlorophyll a/b protein complex (LHCII)\(_{1}\) regulates the balance of excitation energy between the two photosystems (5, 6, 8). Phosphorylation of the D1 polypeptide in the light induced turnover and repair of photoinhibitory damage to the photosystem II reaction center (13–16).

The specific mechanism involved in the redox-mediated activation of the thylakoid kinase(s) is not yet understood. Activation of thylakoid protein phosphorylation is dependent on the redox state of the plastoquinone pool (5–7). In cytochrome b\(_{f}\)-deficient mutants of algae (17) and higher plants (18–20) the redox-controlled phosphorylation of the mobile subpopulation of LHCII enriched in the 25-kDa subunit (21) is abolished. Furthermore, a stable form of the LHCII kinase active in darkness in Acanthasteria thylakoids and the light-activated kinase of pea thylakoids are rapidly deactivated by specific inhibitors of cytochrome b\(_{f}\) reduction (22, 23). These results suggest that the cytochrome b\(_{f}\) complex is involved in LHCII kinase activation/deactivation. However, cytochrome b\(_{f}\) deficiency does not abolish redox-dependent phosphorylation of photosystem II proteins as revealed from studies on Lemma (18) and maize mutants (19, 20). These findings have been interpreted as evidence for the existence of more than one redox-controlled kinase or redox control mechanisms (11, 12). As opposed to that, based on redox titration of the phosphorylation of 13 proteins in pea thylakoids it was suggested that the redox control involves a single endogenous agent (24). Differential phosphorylation of LHCII and proteins of photosystem II under different light intensities (16) and the possibility that the kinase activation process affects also the substrate specificity of

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The abbreviations used are: LHCII, light-harvesting chlorophyll a/b proteins; D1 and D2, photosystem II reaction center polypeptides; CP43, chlorophyll a binding protein of photosystem II; HPLC, high pressure liquid chromatography; P700, reaction center in photosystem I; PQ, plastoquinone; PQH\(_{2}\), plastoquinod; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; HQNO, 2-(n-heptyl)-4-hydroxyquinoline-N-oxide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone.
the enzyme were considered as well (23). Moreover, a dual control of protein phosphorylation in intact chloroplasts by redox and energy states, including dissipation of a trans-thylakoid pH gradient, was previously proposed (25–27).

Protein kinases isolated from thylakoid membranes have not shown redox-dependent activity (28). Attempts to obtain preparations enriched in kinase activity still exhibiting redox control resulted in a partial copurification of the LHClI kinase with the cytochrome b f (29). However, the redox-controlled kinase(s) and the corresponding phosphatase enzymes still remain unidentified.

Identification of the specific regulatory component(s) involved in the activation/deactivation of the thylakoid protein phosphorylation has been hampered by the fact that the activation process was so far achieved only under conditions in which all thylakoid electron carriers were reduced following either illumination or excess addition of reducing agents in darkness. In this work we introduce a new experimental system in which thylakoid kinase activation is induced in darkness by a short preincubation of the membranes at low pH in the absence of added reducing agents. Studies on the kinetics of the kinase activation and deactivation using this new experimental approach combined with low temperature EPR spectroscopy indicate that control of the thylakoid protein phosphorylation is related to the maintenance of a plastocyanin bound to a cytochrome b complex in which the Rieske Fe-S center is in the reduced state.

**EXPERIMENTAL PROCEDURES**

**Plant Growth and Preparation of Thylakoid Membranes—Spinach (Spinacia oleracea L.) was grown hydroponically in the nutrient solution (30) at 25 °C and light intensity of 475 μmol of photons m⁻² s⁻¹ using a 10/14-h light/dark period. Thylakoids were isolated from 6-week-old plants according to Andersson et al. (31) except that the last washing step and final suspension (3–4 mg of chlorophyll/ml) were made in 10 mM sodium phosphate, pH 7.5, 100 mM sorbitol, 5 mM MgCl₂, and 20 mM NaCl. Lepus perpusilla strain 6746 (wild type) and mutant strain 1073 were grown at 22 °C under dim light in sterilized medium (32). Plants were washed with 25 mM Tris/HCl, pH 7.6, 0.4 mM sorbitol, 10 mM NaCl, 0.5 mg/ml ascorbate, collected by filtration on a porcelain Buchner funnel, and homogenized in the same buffer using a Waring blender (3 × 10 s, 15 ml of the buffer per 1 g of wet weight). The homogenate was filtered through eight layers of nylon mesh and centrifuged at 1,000 × g for 2 min prior to addition of glucose oxidase (0.4 mg/ml, Sigma) and catalase (4,000 units/ml, Sigma). For assay of deactivation of the light-activated protein kinase a thylakoid suspension (0.4 mg of chlorophyll/ml) in 50 mM sodium phosphate, pH 7.4, 100 mM sorbitol, and 5 mM MgCl₂ was illuminated (white light, 100 μmol of photons m⁻² s⁻¹) for 2 min and transferred to darkness (time 0). Aliquots of the suspension were taken at the indicated time points and mixed with 5 μl of 0.6 mM [γ-³²P]ATP (2 μCi) and 60 μM NaF at times as indicated. All phosphorylation reactions were continued for 20 min in the dark after the addition of [γ-³²P]ATP and stopped by the addition of 10% trichloroacetic acid.

Analysis of Thylakoid Phosphoproteins—Electrophoretic separation of the thylakoid proteins by SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (36). Thylakoid membranes suspended in electrophoresis sample buffer were heated to 70 °C for 3–4 min, and electrophoresis was performed using 15% acrylamide slab gels. The gels were stained with Coomasie Brilliant Blue or with silver, dried, and autoradiographed using x-ray film. Quantification of the protein phosphorylation level was done by scanning the autoradiograms by laser densitometry using the software package Image Quant from Molecular Dynamics.

**Lipid Extraction and Determination of the Plastocyanin/Plastoquinone Ratio—**Lipid extraction was performed according to Abeg et al. (35). All procedures were carried out in darkness. The thylakoid suspension (0.5 ml; 0.1 mg of chlorophyll/ml) was extracted with 4 ml of methanol and 4 ml of petroleum ether (boiling point 40–60 °C) by extensive vortexing for 30 s. After phase separation the upper phase was removed and evaporated under nitrogen in the dark. The residue was redissolved in 100 μl of chloroform:methanol (2:1 v/v) and washed once with water. The samples were injected directly into the HPLC column. Reverse phase HPLC was performed on a Hewlett-Packard Hypersil ODS 3-mm column with a linear gradient of methanol:H₂O (91, v/v) to methanol:2-propanol (2:1, v/v) for 20 min at a flow rate of 1.5 ml/min. The absorbance of the eluate was monitored at 210 nm. The elution times of plastocyanin and plastoquinone were determined using reference compounds.

**Low Temperature EPR Spectroscopy—**Samples of dark-adapted spinach thylakoids containing 3.8 mg of chlorophyll/ml were frozen in liquid nitrogen at various stages of the activation and deactivation of the protein kinase. Directly afterwards, the X-band EPR spectra were measured at 15 K on a Bruker ESP 300 spectrometer equipped with a helium flow cryostat and temperature controller (Oxford Instruments). All measurements were performed using the same membranes. EPR conditions for measurement of signals from reduced Rieske Fe-S center, plastocyanin, and oxidized cytochrome f were microwave power 6.3 milliwatts and modulation 0.12 millitesla.
RESULTS

Activation of Thylakoid Protein Phosphorylation by Transient Low pH Treatment—Preincubation of spinach thylakoid membranes in a solution of monosubstituted phosphate, pH 4.3, and subsequent transfer to the phosphorylation buffer, pH 7.4 or 8.0, results in a significant activation of the protein kinase in darkness (Fig. 1). The pattern of protein phosphorylation induced by the transient low pH preincubation is identical to that induced by light. However, the extent of the phosphorylation is lower, varying between 30 and 60% of that induced by continuous illumination. Notably, the level of phosphorylation induced in darkness by preincubation at acidic pH is 100-fold higher than that of the basal phosphorylation at pH 4.3 or that at pH 7.4 without prior preincubation at low pH as judged by densitometry of autoradiograms (Fig. 1). The activation of protein phosphorylation could not be elicited in the dark by preincubation of the thylakoids at pH between 5.8 and 8.0 (not shown). This suggests that the low pH (pH 4.3) treatment before transfer of the membranes to a higher pH is required for the kinase activation. Among the phosphorylated polypeptides the most conspicuous are those of 25 and 27 kDa of LHCII. Considering the molar ratio of these two LHCII subunits (1:4), the relative phosphorylation of the mobile component, the 25-kDa protein is significantly higher than that of the stationary LHCII 27-kDa protein subunit (see also Ref. 21).

Addition of nigericin (0.2 μM or 2 μM) either in the presence or absence of KCl (10 mM), NaCl (10 mM), or FCCP (10 μM) has no effect on the extent or rate of the low pH-induced phosphorylation in the time range between 0.5 and 30 min (not shown). Thus, the activation of the kinase by the above procedure is not due to the formation or dissipation of a transmembrane pH gradient.

The Activation of Protein Phosphorylation by Low pH Preincubation Is Related to a Change in the Membrane Redox Potential—Low pH incubation may alter the redox potential by affecting the equilibrium [PQH−][PQH2] and activate the kinase via reduction of plastoquinone. To test this possibility the oxidizing effect of ferricyanide on both the low pH- and light-induced activation of protein phosphorylation was studied. The results show that the induction of phosphorylation by both low pH treatment and by light is completely inhibited by ferricyanide (Fig. 2A). This indicates that plastoquinol and/or an additional reduced redox component are involved in the process of kinase activation by transient low pH in darkness.

The kinase activation induced by transient low pH treatment may involve a mechanism different from that of the light-induced process. If this is the case, the light-dependent reduction of the plastoquinone pool could have an additive effect to the low pH-induced kinase activation. Therefore, the extent of thylakoid protein phosphorylation was assayed in the light subsequent to the preincubation of the membranes at low pH in darkness. Moreover, the experiments were done either in the presence or absence of DCMU, an inhibitor of light-dependent plastoquinone reduction by photosystem II (Fig. 2B). Light was found to have no additive effect on the phosphorylation of thylakoid proteins after preincubation of the membranes at low pH in the dark. (Fig. 2, A and B). DCMU completely inhibited the phosphorylation in the light but had no effect on the kinase activation by low pH in darkness. However, DCMU inhibited the phosphorylation performed in the light subsequent to the dark preincubation of thylakoids at low pH (Fig. 2B). These results can be explained by photosystem I-mediated oxidation of plastoquinol formed during the transient low pH treatment under conditions when electron transfer from photosystem II to the plastoquinone pool is inhibited by DCMU.

Kinetics of Activation/Deactivation of the Thylakoid Kinase Induced by Low pH Treatment: Relation to the Redox State of the Plastoquinone Pool—The protein kinase activity in darkness in the presence of reducing agents has previously been shown to be optimal in the range between pH 6.0 and 9.5 (37). Thus, one could expect a dual effect of the transient low pH treatment, a possible irreversible inactivation due to the acidic
The kinetics of protein kinase activation by transient low pH incubation is shown in Fig. 3. The activation is rather rapid and is maximal in less than 2 min. However, an initial limited loss of activity occurs during the first 10 min of incubation at low pH.

Notably, the kinase activated by transient low pH treatment phosphorylates the thylakoid proteins in the dark for up to 20 min. Fig. 4 shows the kinetics of LHCII phosphorylation in darkness after the low pH and light induction of the kinase activity. The kinase activated by light phosphorylates LHCII only for about 4 min after transfer of the thylakoids to darkness. This difference in the deactivation kinetics of the kinase induced by preincubation at low pH and by light was further analyzed. The thylakoids were incubated either in the light at pH 7.4 or in darkness at pH 4.3 for 2 min and then transferred in darkness and at pH 7.4 for different times prior to the addition of \( \gamma^{-32P}\)ATP. The phosphorylation was then continued for 20 min, allowing the reaction to proceed to completion (see Fig. 4). The kinase deactivation proceeds similarly with respect to all thylakoid substrate polypeptides in both the light- and low pH-activated enzyme(s) (Fig. 5, A and B). However, the kinase activity induced by the low pH treatment is deactivated considerably more slowly in the dark as compared to the deactivation of the light-activated enzyme, with a half-life time of 4 and 1 min, respectively (Fig. 5C). The deactivation of the low pH-induced phosphorylation was further retarded under anaerobic conditions, while the electron acceptor ferricyanide deactivated the kinase completely in 1 min (Fig. 5C).

To test directly whether the reduction state of the plastoquinone pool is involved in the low pH activation/deactivation process, we have determined the relative levels of plastoquinol and plastoquinone in thylakoids under the experimental conditions used for the low pH-induced kinase activation. The ratio of \( \text{PQH}_2 \) to PQ in control thylakoids incubated in the dark at pH 7.4 was in the range of 0.02–0.07 (Fig. 6). After 2 min of incubation at pH 4.3 this ratio increased to 0.20–0.27. Transfer of thylakoids from pH 4.3 to 7.4 resulted in reoxidation of plastoquinol in only 1 min (Fig. 6). Therefore, it should be noted that at the time when the kinase activity is maximal (see Fig. 5) the plastoquinone pool is already oxidized to a level incompatible with the activation process. These results, therefore, strongly suggest that the active state of the kinase, once achieved, is only indirectly related to the redox state of the “free pool” of plastoquinone.

To explore this possibility, we have assayed the effect of DBMIB, a cytochrome b inhibitor, on the low pH activation of thylakoid phosphorylation. This inhibitor binds to the cytochrome complex at a site permitting interaction with the Rieske Fe-S protein and the low potential cytochrome b, thereby preventing reduction of the cytochrome complex (38, 39). As shown in Table I, DBMIB inhibits the low pH-induced phosphorylation of the thylakoid proteins to about 50% at a concentration of 1 \( \mu \)M, and complete inhibition is obtained at 5 \( \mu \)M.

It was reported before that DBMIB inhibits preferentially the light-induced LHCII phosphorylation relative to that of the photosystem II proteins (20, 40, 41), and consequently it was postulated that there are at least two thylakoid protein kinases with different sensitivity to DBMIB inhibition (11). Thus, it was of interest to analyze the effect of DBMIB on the degree of phosphorylation of various thylakoid polypeptides by the low pH-activated kinase in darkness. The results show a similar degree of inhibition of the transient low pH-induced phosphorylation for all of the thylakoid phosphoproteins by DBMIB (Table I).

Oxidation of reduced cytochrome b via NADP and ferredoxin inhibits to a similar extent the radioactive labeling of all the phosphoproteins. Moreover, this inhibitory effect is completely reversed by the addition of 5 \( \mu \)M HONO (Table I). At this concentration HONO is known to block electron transfer from cytochrome b to plastoquinone, preventing the oxidation of the cytochrome complex (22, 42–44). These results, therefore, provide evidence supporting the involvement of reduced cytochrome complex in the activation of the thylakoid protein phosphorylation by transient low pH treatment.

This conclusion was further supported by analysis performed on the thylakoids of the cytochrome b-deficient mutant 1073 of Lemma perpusilla, in which the phosphorylation of the LHCII polypeptides is not induced by light or by the addition of reducing agents in the dark. In contrast, redox-controlled phosphorylation of other thylakoid proteins can be induced in this
mutant (18). As shown in Fig. 7, in the wild type thylakoids (Fig. 7).

mass range of about 100 kDa appeared to be enhanced in the mutant as compared with the wild type.

pH treatment induces a lower level of phosphorylation of other polypeptides in the mutant as compared with the wild type.

of the cytochrome bf-deficient thylakoids. Moreover, the acidic pH treatment induces a lower level of phosphorylation of other polypeptides in the mutant as compared with the wild type thylakoids. The labeling of a polypeptide(s) in the molecular mass range of about 100 kDa appeared to be enhanced in the mutant thylakoids (Fig. 7).

FIG. 7. Reversible reduction of plastoquinone induced by low pH treatment of the dark-adapted thylakoids. Plastoquinone and plastoquinol were extracted from spinach thylakoids after incubation in the dark at pH 7.4 or 4.3 (2 min) or 1 min after transfer from pH 4.3 back to pH 7.4 (4, 5, 8A). The content of the both compounds was determined by HPLC. Left panel, example of HPLC elution profile for the sample preincubated at pH 4.3; right panel, PQH2/PQ ratios calculated from HPLC elution profiles.

TABLE I

Effect of cytochrome bf complex inhibitors and electron acceptors on thylakoid protein phosphorylation induced by transient low pH treatment

Low pH-induced phosphorylation of spinach thylakoid proteins was performed for 20 min in darkness either in the presence or absence of various additions. The concentration of NADP was 5 mM, and that of ferredoxin and HQNO was 5 μM. The level of phosphorylation of each polypeptide was determined by scanning of autoradiograms and normalized to that in the control experiment (no additions). Autoradiograms were exposed for various times to permit quantitative estimation of polypeptides phosphorylated to different extents. The values are the mean of four identical experiments, and the standard deviation is ±8%.

| Protein       | DBMIB (1 μM) | DBMIB (5 μM) | NADP and ferredoxin – HQNO | + HQNO |
|---------------|--------------|--------------|---------------------------|--------|
| 9 kDa         | 48           | <5           | 58                        | 97     |
| 12 kDa        | 46           | <5           | 61                        | 114    |
| 18 kDa        | ND           | ND           | 65                        | 98     |
| LHCI          | 56           | <5           | 59                        | 100    |
| D1,D2         | 47           | <5           | 55                        | 93     |
| 39 kDa        | ND           | ND           | 62                        | 96     |
| CP43          | 63           | <5           | 57                        | 91     |
| 54 kDa        | 61           | <5           | 52                        | 101    |
| 62 kDa        | 58           | <5           | 75                        | 103    |

*ND, not determined.

Changes in the Redox State of High Potential Path Components of Cytochrome bf during Activation/Deactivation of the Kinase as Determined by EPR Spectroscopy—The experimental system described in this work permits a transient reduction of plastoquinone and change in the membrane redox potential without light excitation of the two photosystems. Taking advantage of this new approach we used EPR spectroscopy to identify the reduced redox component of the cytochrome bf involved in the activation as well as in the slow deactivation of the protein phosphorylation.

The slow protein kinase deactivation occurring after the reoxidation of the plastoquinol pool, which is another merit of this system, implies that the component involved in maintaining the kinase in the active state could not be in the low potential path of the cytochrome bf complex, since following reduction, cytochrome b is promptly reoxidized on a millisecond time scale (45–47).

In the dark-adapted control thylakoids, kept at pH 7.4, cytochrome f is oxidized as indicated by the characteristic EPR signal (48) at g = 3.53 (Fig. 8A). This signal disappears after incubation of the thylakoids for 2 min at pH 4.3 in darkness, indicating reduction of cytochrome f (Fig. 8A). Moreover, the plastocyanin also becomes reduced at pH 4.3 as indicated by
the EPR signal at \( g = 2.05 \) (Fig. 8B), which is equal to that of a control sample reduced by 10 mM ascorbate (not shown). Transfer of the membranes from pH 4.3 back to pH 7.4, when the kinase is activated, coincides with the reduction of the Rieske Fe-S center as evidenced by the appearance of the characteristic EPR signal at \( g = 1.90 \) (50, 51), (Fig. 9, compare with Fig. 8B).

Unexpectedly, following subsequent incubation for up to 10 min, during which the kinase becomes slowly deactivated (see Fig. 5C), cytochrome f (not shown), plastocyanin (\( g = 2.05 \)), and the Rieske Fe-S center (\( g = 1.90 \)) remain reduced (Fig. 9). However, the spectra in Fig. 9 show a decrease of the EPR signal around \( g = 2.03 \) occurring as a function of incubation time from 1 to 10 min, which reflects a decrease in the \( g \) signal of the reduced Rieske Fe-S center (50, 51). The \( g \) signal is known to be affected by displacement of bound plastoquinol from the reduced Rieske Fe-S center when DBMIB or other quinone analogues compete for this binding site (39). As shown in Fig. 9 (lower trace), addition of DBMIB induces the appearance of a signal at \( g = 1.94 \) arising from the interaction of the reduced Rieske Fe-S center with the quinone analogue (39) as well as a shift in the \( g \) signal at \( g = 2.03 \). Notably, binding of DBMIB inhibits the activation of the kinase induced by low pH treatment (Table I).

Thus, during deactivation of the protein kinase in the dark all redox components of the high potential pathway of the cytochrome b-f complex remain in their reduced state. However, the correlation between the changes in the EPR \( g \) signal and the kinase deactivation in darkness as well as the change of this signal in presence of DBMIB indicate a connection between the kinase active state and occupancy of a quinol binding site in the vicinity of the reduced Rieske Fe-S center.

**DISCUSSION**

In this work we demonstrate that thylakoid protein phosphorylation ascribed to activation of the redox-controlled thylakoid kinase(s) can be induced by transient exposure of the thylakoid membranes to low pH in the absence of light and without the addition of reducing agents. The kinase activation by the transient low pH treatment can be explained in terms of a pH-dependent shift of the membrane redox potential, causing the reduction of plastoquinone, cytochrome f, plastocyanin, and the Rieske Fe-S center. Transfer of dark-adapted thylakoids from pH 7.4 to pH 4.3 leads to the increase in the ratio of 

The kinase activation by the transient low pH treatment can be explained in terms of a pH-dependent shift of the membrane redox potential, causing the reduction of plastoquinone, cytochrome f, plastocyanin, and the Rieske Fe-S center. Transfer of dark-adapted thylakoids from pH 7.4 to pH 4.3 leads to the increase in the ratio of PQH2 to PQ, from an average of 0.05 to 0.25, as measured by the total quinone extraction and HPLC quantification. One can therefore consider that basically all the plastoquinone pool is oxidized at pH 7.4 in darkness. The level of reduction of the plastoquinone pool after incubation of the thylakoids at pH 4.3 is comparable with the level that was determined to be sufficient for activation of thylakoid protein phosphorylation in experiments with single-turnover flashes (6). Furthermore, one should take into account that part of the plastoquinone present in the thylakoids is localized in plastoglobuli and therefore not accessible to the redox-mediated reactions (52). The fast reoxidation of the plastoquinol after the transfer of the thylakoid membranes from pH 4.3 back to pH 7.4, while the kinase is still in its active state, demonstrate that the free plastoquinol pool could not be responsible for maintaining its activity.

In the experimental system we have introduced here, the low

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**FIG. 7.** Transient low pH treatment activates phosphorylation of LHCII in thylakoids of Lenma wild type but not in those of cyt chrome b deficient mutant. The thylakoids of Lemna wild type (Wt) or mutant (Mt) were phosphorylated by \([\text{32P}]\text{ATP} \) at pH 8.0 for 15 min either in the light (+) or in the dark (−). Thylakoids were either preincubated in the dark at pH 4.3 and then transferred to the phosphorylation medium, pH 8.0 (pH 4.3 → 8.0, +), or preincubated and phosphorylated at pH 8.0 only (−).

**FIG. 8.** Low temperature EPR spectra of dark-adapted spinach thylakoids at pH 7.4 and 4.3. The thylakoids (3.8 mg of chlorophyll/m) were incubated in the dark at pH 7.4 for 1 h and frozen (pH 7.4) or transferred to pH 4.3 for 2 min and frozen afterwards (pH 4.3). The EPR spectra in the region of cytochrome f (A) and plastocyanin and Rieske Fe-S center (B) were measured. EPR conditions were as follows: 15 K; microwave frequency 9.24 GHz; microwave power 6.3 milliwatts; magnetic field modulation 0.12 millitesla. The \( g \) values 2.05 (49) (Fig. 8B), which is equal to that of a control sample reduced by 10 mM ascorbate (not shown). Transfer of the membranes from pH 4.3 back to pH 7.4, when the kinase is activated, coincides with the reduction of the Rieske Fe-S center as evidenced by the appearance of the characteristic EPR signal at \( g = 1.90 \) (50, 51), (Fig. 9, compare with Fig. 8B).

**FIG. 9.** Changes in the low temperature EPR spectra of the Rieske Fe-S center during kinase deactivation. Dark-adapted spinach thylakoids were preincubated at pH 4.3 for 2 min, transferred back to pH 7.4 (time 0), and frozen at times as indicated (upper traces). DBMIB (5 μM) was added to the thylakoid suspension after 2 min of preincubation at pH 4.3, and thylakoids were transferred to the buffer, pH 7.4, containing DBMIB in the same concentration and frozen after 1 min (lower trace). All procedures were performed in the dark. The samples contained 3.8 mg of chlorophyll/ml. The EPR conditions were as in Fig. 8. The \( g \) values 2.05 and 1.90 signals of the reduced Rieske Fe-S center (46, 47) are indicated. The \( g \) values 1.94 signal is attributed to the shift in the \( g \) signal by DBMIB (39). DBMIB did not saturate all centers at the chlorophyll concentration used.
pH treatment activates the phosphorylation of all the thylakoid phosphoproteins as in the case of light activation. Moreover, experiments using inhibitors and the cytochrome b-f deficient mutant demonstrate that the cytochrome b-f complex is involved in the control of the phosphorylation induced by the transient low pH treatment.

While it is generally accepted that cytochrome b-f complex is involved in the process of the kinase activation (for review, see Refs. 11 and 12) the molecular mechanism of this process has so far not been elucidated. It was previously proposed that binding of a reduced quinone to the cytochrome b-f complex may be responsible for the kinase activation (22). This hypothesis was based on an extensive study of the Acetabularia thylakoid LHClI kinase, which retains its activity in the dark for very long periods of time but could be rapidly inactivated by the binding of quinone analogues to the cytochrome b-f quinol oxidizing site (23). The data of the present work further resolve the process of plastoquinol/cytochrome b-f involvement in the kinase activation. Thus the kinase active state is induced and maintained as long as the high potential path of the cytochrome b-f complex is reduced and plastoquinol is bound to the quinol-oxidizing site (23). Halogenated quinone analogues including DBMIB displace plastoquinol from the quinol binding site and induce a shift in the g_eff EPR signal (g = 2.03) (39) as also shown in this work. Replacement of the plastoquinol by DBMIB prevents the kinase activation. We interpret the decrease in the EPR signal at g = 2.03 paralleling the kinase deactivation as a change in the interaction between the reduced Rieske Fe-S center and a plastoquinol bound to a site in its vicinity. A slow dissociation of the bound plastoquinol from this site may allow its oxidation by ambient oxygen and/or exchange with the oxidized plastoquinone pool. This interpretation is supported by the slower rate of the kinase deactivation under anaerobic conditions.

The observed persistence in the reduction state of the cytochrome b-f high potential path in darkness and the related active state of the kinase after reduction of part of the plastoquinone pool by the low pH treatment can be explained by the fact that the reduced plastoquinol, the high potential cytochrome b-f components, and plastocyanin cannot be oxidized by photosystem I in the absence of light excitation. The observation that the deactivation of the light-activated kinase in darkness is significantly faster than that of the kinase activated by the transient low pH exposure can be explained by the rapid re-reduction of P700" in the dark after illumination. Consequently, one electron from the high potential path of the cytochrome b-f complex is consumed. This corresponds to withdrawal of the first electron from plastoquinol bound to the quinol oxidizing site of cytochrome b-f complex (47). The second electron of semiquinone reduces cytochrome b-f, which in turn is very rapidly reoxidized (45–47). Thus, the bound plastoquinol is rapidly oxidized when the thylakoids are transferred from light to dark, causing the observed rapid kinase deactivation.

The molecular identity of the redox-controlled thylakoid kinase(s) is not yet established. Involvement of the cytochrome b-f complex in the kinase(s) activation would suggest a physical interaction between the two entities. Indeed, protein kinase activity was found to be associated with the purified cytochrome b-f complexes (29, 53). On the other hand, phosphorylation of some of the thylakoid polypeptides can be induced by light in cytochrome b-f-deficient mutants (Refs. 18–20 and this work) but only partially by the transient low pH treatment. This could be explained if the kinase involved has a putative plastoquinol binding site with a low affinity for plastoquinol or readily susceptible to oxidation in the absence of cytochrome b-f complex.

Despite the close interaction between the plastoquinol and the Rieske Fe-S center, there is no evidence for quinol binding directly to the Rieske protein (petC). Possible involvement of subunit IV (petD) of the cytochrome b-f complex in close connection with the Rieske protein should also be considered (for review see Ref. 54).

We conclude that the activation of thylakoid protein phosphorylation requires reduction of the cytochrome b-f complex only inasmuch as to maintain a plastoquinol bound in the quinol oxidizing site of the complex. The deactivation of the kinase is consequently due to the release or oxidation of this bound plastoquinol. The binding of plastoquinol to the cytochrome b-f complex in the vicinity of the reduced Rieske Fe-S center could correspond to a ligand-receptor interaction in a signal transduction system of the photosynthetic membrane.

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