Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: Thylakoid protein kinase deactivation by a single-turnover flash

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ABSTRACT Redox-controlled phosphorylation of thylakoid membrane proteins represents a unique system for the regulation of light energy utilization in photosynthesis. The molecular mechanisms for this process remain unknown, but current views suggest that the plastoquinone pool directly controls the activation of the kinase. On the basis of enzyme activation by a pH shift in the darkness combined with flash photolysis, EPR, and optical spectroscopy we propose that activation occurs when plastoquinol occupies the quinol-oxidation (Qo) site of the cytochrome *bf* complex, having its high-potential path components in a reduced state. A linear correlation between kinase activation and accessibility of the Qo site to plastoquinol was established by quantification of the shift in the g_y EPR signal of the Rieske Fe–S center resulting from displacement of the Qo-site plastoquinol by a quinone analog. Activity persists as long as one plastoquinol per cytochrome *bf* is still available. Withdrawal of one electron from this plastoquinol after a single-turnover flash exciting photosystem I leads to deactivation of the kinase parallel with a decrease in the g_z EPR signal of the reduced Rieske Fe–S center. Cytochrome *f*, plastocyanin, and P_{700} are rereduced after the flash, indicating that the plastoquinol at the Qo site is limiting in maintaining the kinase activity. These results give direct evidence for a functional cytochrome *bf*–kinase interaction, analogous to a signal transduction system where the cytochrome *bf* is the receptor and the ligand is the plastoquinol at the Qo site.

Light is not only the source for photosynthetic energy conversion but also an essential regulatory factor in plant signal transduction (1–5). The light-induced protein kinase activity of the chloroplast thylakoid membrane (1, 6, 7) connects light quality and intensity with the versatility of regulatory protein phosphorylation. This reversible post-translational modification regulates the balance of excitation energy between the two photosystems by means of phosphorylation of the light-harvesting chlorophyll (Chl) a/b–protein complex (LHC)II (7, 8), optimizing photosynthetic efficiency under ever-changing irradiance. The ensuing regulation of the reducing power produced by photosynthetic electron transfer controls the translation of key chloroplast-encoded proteins (3, 4). Moreover, phosphorylation regulates light-stress-induced turnover of photosystem (PS)II reaction center proteins (9–11). The redox-controlled signaling system for regulation of nucleus-encoded chloroplast *cab* gene expression was also proposed to be initiated by thylakoid protein phosphorylation (5).

Despite numerous studies aimed at the elucidation of this unique light-induced protein phosphorylation process, our understanding of the mechanism connecting light with kinase activation is still very limited. The activation/deactivation of the enzyme has been correlated to the redox state of the plastoquinone pool (7, 12), which depends on the excitation and electron flow of PSII relative to PSI. It has also been found that light-dependent phosphorylation of LHCII is abolished in cytochrome *bf* complex-deficient mutants of algae and higher plants (13–16), and specific inhibitors of cytochrome *bf* reduction deactivate thylakoid protein phosphorylation (13–19). Nevertheless, direct proof for interaction between the cytochrome *bf* complex and the protein kinase is still missing and, in particular, the specific redox component of the complex involved in the activation process remains to be identified.

Thylakoid protein phosphorylation has usually been induced *in vitro* by light, leading to the reduction of the plastoquinone pool by PSII, or by addition of reducing agents, which poise all the redox components of the membrane in their reduced state. Recently, we were able to show that a short incubation of thylakoids at low pH in darkness followed by neutralization (pH-shift) activates the typical protein phosphorylation of both the LHCII and the PSII protein subunits (20). We could demonstrate that such an acidic treatment transiently reduces the plastoquinone pool, which is rapidly reoxidized by ambient oxygen following the pH shift, while the kinase activity persists. Thus, in contrast to previous notions (1, 6, 7, 12), the kinase activity was not directly correlated to the redox state of the plastoquinone pool. It was therefore suggested that a plastoquinol "bound" to the cytochrome *bf* complex could be involved in the activation process (20).

Using the new kinase activation procedure combined with flash photolysis, EPR, and optical spectroscopy, we here present direct evidence for the role of plastoquinol at the quinol-oxidation (Qo) site of the reduced cytochrome *bf* complex in the process of kinase activation. The results are discussed in terms of a functional interaction between the

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kinase and the cytochrome *bf* complex as analogous to a signal transduction receptor system.

MATERIALS AND METHODS

Spinach (*Spinacea oleracea* Linnaeus) was grown hydroponically as described in ref. 21. Thylakoids were isolated from 6-week-old plants according to ref. 22 except that the last washing step and final suspension (3–4 mg of Chl per ml) were made in 10 mM sodium phosphate, pH 7.5/100 mM sorbitol/5 mM $MgCl_2$/20 mM NaCl. Chl was determined as in refs. 23 and 24.

Phosphorylation of Thylakoid Proteins. Protein phosphorylation was induced by the pH-shift procedure in darkness (20). Dark-adapted thylakoids were centrifuged and resuspended in 50 mM NaH_2PO_4, pH 4.3/100 mM sorbitol/5 mM $MgCl_2$ (acidic medium) to 1 mg of Chl per ml. The incubation at pH 4.3 was terminated after 3–5 min by centrifugation, and the thylakoids were resuspended (4 mg of Chl per ml) in phosphorylation medium containing 100 mM sodium phosphate at pH 7.6, 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM NaF, and 0.1 mM [γ-^{32}P]ATP (0.1 μCi/μl; 1 μCi = 37 kBq). Phosphorylation was continued for 10 min in darkness after the addition of [γ-^{32}P]ATP and was stopped by addition of 50 mM ice-cold EDTA followed by centrifugation and resuspension of the membranes in electrophoresis sample buffer.

For flash experiments, thylakoids after preincubation in acidic medium were resuspended (4 mg of Chl per ml) in 100 mM sodium phosphate, pH 7.6/100 mM sorbitol/5 mM $MgCl_2$/20 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU). After 105 s of incubation, 1 or 15 laser flashes (10-ns duration, 3 Hz, 200 mJ at 532 nm) were delivered to samples of 1-mm optical path. Aliquots were mixed with [γ-^{32}P]ATP (0.1 mM, 0.1 μCi/μl) and 10 mM NaF 30 s after the first flash (totally 135 s after pH shift). In the control experiments the thylakoids were mixed with [γ-^{32}P]ATP 135 s after the pH shift without the flash application. All phosphorylation reactions were continued for 10 min in darkness after the addition of [γ-^{32}P]ATP. For EPR measurements the membranes were frozen 135 s after the pH shift, 30 s after a single flash applied 105 s after the pH shift, or after 15 flashes applied 105 s after the pH shift.

Analysis of Thylakoid Phosphoproteins. Separation of the thylakoid proteins by SDS/PAGE was performed according to ref. 25. Quantification of the relative protein phosphorylation levels was done by scanning the LHCII as well as other phosphoprotein bands in autoradiograms by laser densitometry using the software package Image Quant from Molecular Dynamics. Autoradiograms were exposed for different times so as to ensure linearity of the film response.

Low-Temperature EPR Spectroscopy. For EPR measurements all incubation procedures were as described above but [γ-^{32}P]ATP was omitted in the resuspension buffer and thylakoids (4 mg of Chl per ml) were frozen in liquid nitrogen. When used, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) dissolved in ethanol was added to the thylakoid suspension at the end of preincubation in the acidic medium. The final ethanol concentration in the medium did not exceed 1%. The X-band EPR spectra were measured at 15 K using a Bruker ESP 300 spectrometer equipped with a helium flow cryostat and temperature controller. The EPR measurements were carried out using a microwave frequency of 9.24 GHz, 6.3 mW of microwave power, and a magnetic field modulation of 0.12 mT. All procedures were performed in darkness.

Optical Spectroscopy. Flash-induced absorbance changes of cytochromes *f* and *b* at 554 nm and 563 nm, respectively, were obtained by matrix deconvolution. The dark-adapted thylakoids after preincubation in acidic medium were resuspended to 75 μg of Chl per ml in 100 mM sodium phosphate, pH 7.6, containing 100 mM sorbitol, 5 mM $MgCl_2$, and 10 μM DCMU

in darkness. Six actinic red flashes (RG645 filter) at 10 Hz were provided with a xenon flash lamp (6-μs half-peak width). The first flash was applied 105 s after the pH shift. The flashes were delivered to both sides of a sample of 3-mm optical path, ensuring light saturation throughout the sample. From other measurements, these flashes are expected to induce about 1.5 turnovers per P_{700} due to double hits. Flash-induced absorbance changes were measured at 542, 554, 563, and 575 nm. Data are the average of four recordings at each wavelength, with an amplifier response time constant of 30 μs and a measuring beam bandwidth of about 1.5 nm. A new thylakoid sample was used for each transient. In thylakoids that had been subjected to low-pH treatment no carotenoid band shift (26, 27) was observed at 518 nm and a matrix for deconvolution of cytochromes, plastocyanin, and P_{700} was used (28, 29). To achieve the complete reduction of the plastoquinol pool in the control experiments, 280 μM duroquinol or light filtered through a red DC RG645 filter was used.

RESULTS

A Single Flash-Induced Turnover of PSI Deactivates the Kinase. Using the pH-shift method for kinase activation, we were recently able to show that the kinetics of kinase deactivation is slower than that of the bulk of plastoquinol pool oxidation by ambient oxygen. On the basis of EPR measurements it was therefore proposed that the kinase deactivation may be related to the slow oxidation of a quinol interacting with the Qo site of cytochrome *bf* (20). Moreover, the components of the high-potential path of cytochrome *bf* as well as plastocyanin remained reduced during the kinase deactivation. The slowly oxidized residual plastoquinol may thus serve as the activating redox species as long as it is not oxidized at the Qo site, thereby affecting a kinase interacting with the cytochrome *bf* complex. This hypothesis predicts that the kinase should be deactivated by a single turnover flash causing oxidation of the reduced plastocyanin by PSI. As a result, a sequential cascade of electron flow will follow, whereby plastocyanin will oxidize cytochrome *f*, which in turn will oxidize the Rieske Fe–S center, which finally will take an electron from the plastoquinol at the Qo site. The high-potential path components of cytochrome *bf* will thus be rereduced after the flash. However, the residual plastoquinol will now be oxidized at the Qo site, and consequently the kinase should be deactivated.

To test this prediction we have exposed thylakoids in which the kinase was preactivated in darkness to a laser flash, 105 s after the pH shift, when the plastoquinol pool is already reoxidized (20). To prevent plastoquinone reduction by PSII during the flash, the inhibitor DCMU was added before the pH shift. As clearly shown by the autoradiogram of Fig. 1*A* illustrating the phospho-LHCII, the major phosphorylated component of the thylakoid membrane, a single flash inactivated its phosphorylation induced by the pH shift by about 70%. A similar degree of inactivation by the flash was observed for all the other phosphoproteins of the thylakoid membrane as summarized in Table 1.

EPR Spectroscopy Shows That the Cytochrome *bf* High-Potential Path and Plastocyanin Are Rereduced After the Flash Deactivation of the Kinase. The cytochrome *bf* high-potential path components remain reduced in the dark-incubated thylakoids after the pH shift (20). Consequently these components should be only transiently oxidized after a single turnover flash if a plastoquinol is still available at the Qo site of the cytochrome *bf* complex. Rereduction in the dark following the flash should occur due to the withdrawal of one electron from this plastoquinol. Furthermore, the model predicts a decrease in the g_z EPR signal of reduced Rieske Fe–S center after the oxidation of the quinol by the first flash. To test these predictions we have recorded the EPR spectra of thylakoid samples after the pH shift with or without their
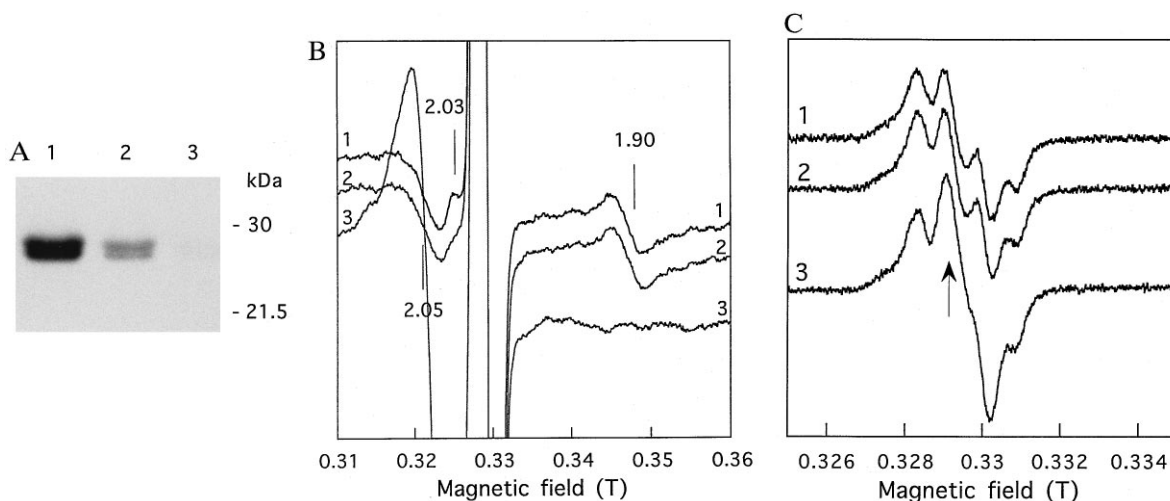
FIG. 1. Deactivation of the pH-shift-induced redox-dependent thylakoid protein phosphorylation in darkness by a single PSI turnover flash. (*A*) Autoradiogram of the SDS/PAGE-resolved thylakoid LHCII. Dark-adapted spinach thylakoids were subjected to the pH shift and [γ-^{32}P]ATP was added 135 s later (lane 1); one laser flash was applied 105 s after pH shift and [γ-^{32}P]ATP was added 30 s later (lane 2); 15 laser flashes were applied 105 s after pH shift and [γ-^{32}P]ATP was added 30 s latter (lane 3). All phosphorylation reactions were continued for 10 min in the dark after the addition of [γ-^{32}P]ATP. (*B* and *C*) Low-temperature EPR spectra. Traces 1, 2, and 3 correspond to the samples 1, 2, and 3 in *A*, but the membranes were frozen for EPR measurements 135 s after the pH shift without application of the laser flash (trace 1), 30 s after the single flash applied 105 s after the pH shift (trace 2), and after 15 flashes applied 105 s after the pH shift (trace 3). EPR signal assignments: g = 1.90 and g = 2.03, g_y and g_z signals of reduced Rieske Fe–S center; g = 2.05, oxidized plastocyanin; arrow, oxidized P$_{700}$.

exposure to the flash (Fig. 1 *B* and *C*). A single flash was applied 105 s after the pH shift. At this time point, according to our previous work (20), the plastoquinol pool is already oxidized. The P$_{700}^+$ signal is not seen in traces 1 and 2 of Fig. 1*C*. The EPR signal which is present in both traces originates from the tyrosine radical D$^+$ of PSII. For comparison the spectrum in Fig. 1*C*, trace 3, is included. This spectrum was obtained from a sample that had been exposed to 15 laser flashes after the pH shift. Here, a strong radical signal is superimposed on the spectrum shown in traces 1 and 2 due to the presence of the P$_{700}^+$ generated by the series of flashes. The Rieske Fe–S center (g_y = 1.90, trace 2 in Fig. 1*B*) and plastocyanin (g = 2.05, the same trace) are also reduced after the single flash. When comparing traces 1 and 2 in Fig. 1*B* it is seen that the g_y signal of Rieske Fe–S center at g = 1.90 recovers completely after the flash and it is identical to the signal in the control sample (Fig. 1*B*, trace 1). As the signal at g = 2.05 is not observed in traces 1 and 2, Fig. 1*B*, it is evident that plastocyanin is also rereduced. This signal of plastocyanin is, however, invoked by 15 flashes as shown in Fig. 1*B*, trace 3.

Table 1. Inactivation of thylakoid phosphorylation by single or multiple flashes

Protein	Phosphorylation, % of control		
	1 flash	15 flashes	Dark control
62-kDa	29	6	8
54-kDa	33	7	6
CP43	33	13	14
D1, D2	28	6	7
LHCII	39	11	10
12-kDa	25	3	3
9-kDa	32	3	2

Same experimental conditions as in Fig. 1*A*. Phosphorylation was determined as described in the text and was normalized to that in the control experiment, in which [γ-^{32}P]ATP was added 135 s after the pH shift, and no flash was applied. The "dark control" represents redox-independent phosphorylation in the dark without a low pH-shift treatment. The values are the mean of four identical experiments and the SEM is in the range of 10%.

Notably, the only change which coincides with the kinase deactivation after application of the single flash (Fig. 1*A*) is a decrease in the g_z EPR signal of the reduced Rieske Fe–S center at g = 2.03 (compare traces 1 and 2 in Fig. 1*B*). The decrease in this signal intensity indicates a decrease in the occupancy of this site by plastoquinol due to its oxidation. This interpretation is based on the dependence of this signal on ligands binding at the Qo site as well as on the appearance of a strong g_z signal at g = 2.03 when the Qo site is occupied by plastoquinol-9 (30, 31).

As a control, 15 consecutive flashes were applied to the sample, thus ensuring that all the high-potential path components of the cytochrome *bf*, plastocyanin, and P$_{700}$ can be oxidized when the plastoquinol is completely oxidized (Fig. 1 *B* and *C*) and the kinase is deactivated (Fig. 1*A* and Table 1).

Time-Resolved Optical Spectroscopy of Cytochrome *f* Oxidoreduction Following Flash Deactivation of the Kinase. As described previously, a short incubation of thylakoids in the dark at pH 4.3 followed by raising the pH to 7.4 results in the reduction of a fraction of the plastoquinol pool as well as of all the high-potential path components of cytochrome *bf* and activates the thylakoid protein phosphorylation. Further incubation of the thylakoid suspension in the dark after the pH shift for 2 min causes oxidation of most of the plastoquinol by ambient oxygen and only a small residual amount of reduced quinone is present while all the high-potential path of the cytochrome *bf* components remains reduced and the kinase is still active (20).

To further prove that the single flash inactivates the kinase by the oxidation of the residual plastoquinol interacting with the cytochrome *bf* Qo site, we have also performed optical measurements of flash-induced cytochrome *f* oxidoreduction kinetics during a series of saturating flashes. In such an experiment we expected that after oxidation of cytochrome *f* by the first flash, applied 105 s after the pH shift, the residual plastoquinol at the Qo site would rapidly rereduce cytochrome *f*. Indeed, almost complete oxidation of cytochrome *f* is induced by the first flash followed by a rapid partial rereduction in the dark (Fig. 2, trace A), while no rereduction of the cytochrome *f* is measurable in a control experiment in which the flashes were applied to dark-adapted thylakoids without a pH shift (Fig. 2, trace B). The difference between the traces A
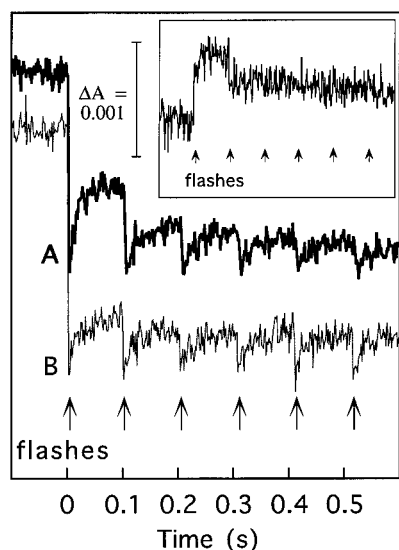
FIG. 2. Flash-induced absorbance changes of cytochrome *f*. Absorbance changes at 554 nm were obtained by matrix deconvolution as described in the text. The first flash was applied 105 s after the pH shift (trace A). As a control, thylakoids were exposed to the flash after incubation in darkness for 30 min without pH shift (trace B). (*Inset*) Subtraction of traces (A − B).

and B in Fig. 2 shows that cytochrome *f* is rereduced only after the first saturating flash, as expected if all the residual plastoquinol has been oxidized by the flash (Fig. 2 *Inset*). The partial rereduction after such a flash (about 40%; Fig. 2, trace A) is explained by the fact that the flash duration induces some double hits per PSI (about 1.5 turnovers). The ratio of cytochrome *f* to PSI as calculated from optical measurements was 0.9. Thus, about 1.65 electrons may be withdrawn from cytochrome *f* per flash, while less plastoquinol remains available at the Qo site to rereduce cytochrome *f* in the dark. The initial rate of cytochrome *f* rereduction after the first flash applied 105 s after the pH shift was in the range of 70–100 s^{-1}. This rate is comparable to 120 s^{-1} in control experiments in which the plastoquinol pool was completely reduced by 280 μM

duroquinol or by exposure to continuous red DC RG645-filtered light, if one takes into account the double hits of PSI by the saturating flash and ensuing turnovers of the cytochrome *bf* during the flash. Optical measurements also revealed that the cytochrome *b* hemes of the complex are already oxidized before the flash exposure (not shown). Since the oxidation of the cytochrome *b* by electron transfer at the Qi site to plastoquinone is very rapid, cytochrome *b* appeared oxidized throughout the experiment (not shown). Consequently, oxidoreduction of cytochrome *b* does not play a direct role in the control of the kinase activity. Thus, the results of optical spectroscopy measurements are in agreement with the EPR data described above and support the concept that oxidation of a residual plastoquinol at the Qo site of cytochrome *bf* complex having the high-potential path in the reduced form results in the kinase deactivation.

Titration of the Qo-Binding Sites Involved in Kinase Activation/Deactivation. Since plastoquinol interaction with the Qo site appears to be directly related to the control of thylakoid protein phosphorylation, we have quantified the relation between its occupancy at the Qo site and the kinase activation, using DBMIB as an antagonist to the plastoquinol binding at this site (29–32). The effect of DBMIB concentration on the extent of protein phosphorylation was studied previously (14, 16, 19). DBMIB can be reduced to the quinol form; however, even in its reduced form it remains a potent inhibitor of the kinase (19). Moreover, the advantage of using this inhibitor as opposed to other more potent inhibitors such as stigmatellin (17, 19, 31) resides in the fact that when bound to the Qo site, DBMIB gives a characteristic and quantifiable shift of Rieske Fe–S center EPR resonance lines (30- 32). While it is known that at high concentrations (10 μM) DBMIB may bind also to the Q$_B$ site of PSII, such interaction was prevented by adding DCMU, which binds at this site with high affinity. Taking advantage of the method of kinase activation by the pH shift in darkness and using EPR spectroscopy, we could for the first time use DBMIB to titrate the number of Qo sites required for the kinase activation/deactivation.

When DBMIB was added at the end of the acidic preincubation of the thylakoids, the g_y signal of the Rieske Fe–S center was shifted from $g = 1.90$ to $g = 1.94$ (Fig. 3A). One should note that the maximum value of the $g = 1.94$ signal amplitude
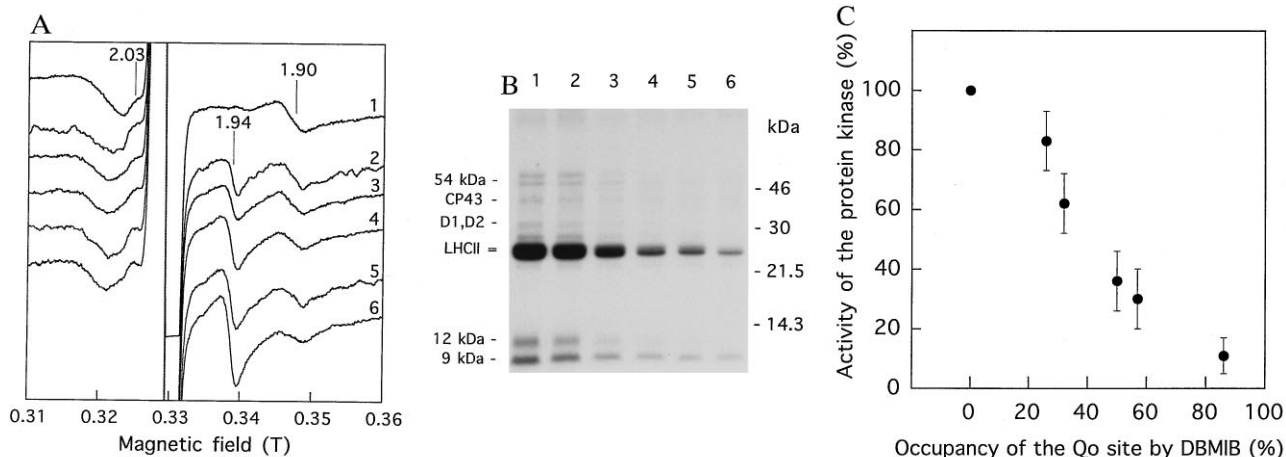

FIG. 3. Deactivation of thylakoid protein phosphorylation due to displacement of plastoquinol from the Qo site by DBMIB. (*A*) Low-temperature EPR spectra of spinach thylakoid membranes frozen for the EPR measurements after the pH shift in darkness. Preincubation of thylakoids in the acidic medium was done in the absence of DBMIB (trace 1) or in the presence of 3, 6, 9, 12, or 18 μM DBMIB (traces 2–6, respectively); same EPR signal assignment as in Fig. 1. (*B*) Autoradiogram of SDS/PAGE-resolved thylakoid phosphoproteins. Phosphorylation by [γ-^{32}P]ATP was induced by the pH shift in darkness after preincubation of the membranes in the acidic medium in the absence of DBMIB (lane 1) or in the presence of DBMIB (same concentrations and lane numbering as above, respectively). The concentration of Chl in the final thylakoid suspensions for both *A* and *B* was 4 mg/ml. The major phosphoproteins are indicated on the left side of the autoradiogram. (*C*) Dependence of deactivation of protein kinase (calculated from *B*) on the occupancy of the Qo site of the cytochrome *bf* complex by the inhibitor DBMIB as quantified by the shift of the g_y Rieske Fe–S center signal from $g = 1.90$ to $g = 1.94$ (calculated from *A*).

is 3.3 times higher than that of the $g = 1.90$ signal (32). The shift of the g_y signal parallels the deactivation of the kinase as shown by the decrease in the ^{32}P-radioactive labeling of the various thylakoid phosphoproteins (Fig. 3*B*). Notably, a proportionality is evident between the deactivation of the protein kinase and occupancy of the Qo site by DBMIB as revealed by quantification of the above data of the EPR signals at $g = 1.94$ and $g = 1.90$ (Fig. 3*C*). No change was detected by EPR in the redox state of plastocyanin, Rieske Fe–S center (Fig. 3*A*), and cytochrome *f* (data not shown) due to the addition of DBMIB.

DISCUSSION

The activation of the redox-controlled thylakoid protein phosphorylation has been ascribed to the interaction between the kinase and plastoquinol at high plastoquinol-to-plastoquinone ratios (1, 6, 7, 12). Considering the evidence for involvement of the cytochrome *bf* complex in the activation process (13–16) and our recent data based upon the kinase activation in the darkness by the pH-shift method (20), we have proposed a detailed alternative model according to which the kinase activation is related to occupancy of the Qo site of the cytochrome *bf* complex by plastoquinol. The results presented here give strong support to this alternative mechanism for the thylakoid kinase activation.

Presence of plastoquinol at the Qo site without its immediate oxidation is possible only if the cytochrome high-potential path is already reduced. Thus, under physiological conditions, generation of this "activating mode" of the cytochrome *bf* complex occurs when the plastoquinol reduction rate by PSII is faster than its oxidation by the cytochrome *bf* complex. We have been able to demonstrate that the "activating mode" of the complex can be achieved by use of the pH shift in the dark (20). Inactivation of the kinase by a single-turnover flash, under conditions preventing rereduction of the plastoquinone pool, leading to the withdrawal by PSI of one electron from the plastoquinol "bound" at the Qo site, served as a crucial test of our model. Using EPR and optical spectroscopy, we also show here that upon the flash, the high-potential path of the cytochrome *bf* and plastocyanin are rereduced. However in the absence of "bound" plastoquinol this reduced state of the cytochrome *bf* is no longer sufficient to activate the kinase. Furthermore, the free plastoquinol in the pool, prevented from interacting with the Qo site of cytochrome *bf*, cannot directly activate the kinase. This conclusion is supported by the strong correlation between the shift in the g_y EPR signal of the Rieske Fe–S cluster induced by the plastoquinol analog DBMIB bound at the Qo site and the kinase deactivation.

It is also important to note that only one plastoquinol molecule per cytochrome *bf* complex in its "activating mode" is indeed sufficient to induce and maintain protein phosphorylation. This is evidenced by the fact that the flash-induced kinase deactivation correlated well with the loss of the g_z EPR signal of the Rieske Fe–S center, indicating the oxidation of a quinol at the Qo site. Furthermore, a significant part of cytochrome *f* was rereduced following a single-turnover flash excitation of PSI that also deactivated most of the kinase. After additional flashes, the cytochrome *f* as well as all the high-potential path of cytochrome *bf* remained oxidized as expected if no plastoquinol was further available as an electron donor.

The question arises as to what is the nature of the Qo site occupancy by the residual plastoquinol, required for the kinase activation. Two possibilities can be considered: (*i*) a fast turnover of plastoquinol at the Qo site as it occurs during its oxidation at steady-state electron flow; (*ii*) a high occupancy—i.e., a state compatible with the notion of "bound plastoquinol" at the Qo site. Redox titration of the activation of thylakoid proteins phosphorylation ranges between 40 and 120 mV (12, 19, 33), while the midpoint potential of the plastoquinone pool

reduction is estimated to be about 100 mV. The possibility that a quinol associated with the Qo site of cytochrome *bf* is involved in the kinase activation is thus not excluded by the redox titration data presently available. Previous experiments involving redox titration of the cytochrome reduction following a single-turnover flash and measurements of the electrochromic shift at 518 nm (27, 34) did not provide evidence to suggest that plastoquinol may be "bound" or sequestered at the Qo site. On the other hand, the plastoquinol pool autooxidation exhibits a biphasic kinetics indicating that a small part of the pool is only very slowly oxidized (P.R.R., unpublished data). The possibility that a bound quinone serves as an intermediate in the reduction of cytochrome *bc1* has recently been demonstrated (35). Considering the similarity of this cytochrome complex with the cytochrome *bf*, one should not exclude a similar possibility for the cytochrome *bf* as well. Indeed, we have demonstrated a correlation between maintenance of the kinase activity and presence of a residual reduced plastoquinol that is only slowly oxidized by ambient oxygen (20). The residual plastoquinol may indicate a high plastoquinol occupancy of the Qo site of cytochrome *bf* in its "activating mode." The kinetics of the cytochrome *f* rereduction following the first flash is rather fast (Fig. 2, trace A). This is not expected if the reducing plastoquinol is a residual free molecule in the quinone pool, lending weight to the possibility of a "bound" plastoquinol. Further direct investigation of the thermodynamic properties of a possible bound quinol at the Qo site of the cytochrome *bf* complex in the kinase "activating mode" seems now warranted.

The results presented here also demonstrate that indeed the redox activation/deactivation process of the kinase is related to a functional interaction between the kinase and the cytochrome *bf* complex. Furthermore, the titration of the Qo sites available for interaction with plastoquinol thus activating the kinase imply that any cytochrome *bf* complex may be found in the "activating mode." The interaction between the cytochrome *bf* and the kinase may be based on a stable association whereby the redox poise of the plastoquinol-cytochrome *bf* controls the enzyme activity. Alternatively, the kinase may only transiently interact with any cytochrome *bf*, "read" its redox state, and accordingly be activated or deactivated. Presently we cannot distinguish between these two mechanistic possibilities. Subfractionation analysis has revealed a partial copurification of the cytochrome *bf* and the kinase which may indicate a stable functional interaction between these two entities (18). However, from a physiological point of view, a transitional interaction seems more attractive. Kinase diffusion in the membrane plane should occur more rapidly than that of the large cytochrome *bf* complex and the substrate LHCII–PSII supercomplex and consequently in an alternating way interact with both. In light of recent data (36, 37), one should also consider the possibility that the kinase–cytochrome *bf* interaction may be influenced by the transition between a dimer and a monomer state of the cytochrome complex.

An essential remaining question to be addressed is how the redox sensing around the Qo site is transmitted across the membrane to the active site of the kinase exposed at the stromal side of the membrane where phosphorylation of substrate threonines takes place (1, 6). The understanding of this problem is hampered by the fact that presently we have no knowledge on the molecular properties of the kinase (38) besides that it is tightly bound to the thylakoid membrane (1, 6).

The new concept put forward here with regard to the mechanism of kinase activation highlights the kinase–cytochrome *bf* system as being analogous to a signal transducing system connecting light with a physiological response. In this context the cytochrome *bf* in its "activating mode" acts as the receptor, the plastoquinol pool as the mediator or agonist, and the plastoquinol at the Qo site as the ligand. The unique-

ness of this signal transduction system resides in the fact that all its components are permanently membrane bound and acting only within the membrane. Unlike the majority of the membrane-bound receptor systems, including the phytochrome and blue light receptors in plants, which exhibit only a specialized communication function, the receptor-like component in this system is a major electron transfer complex.

In conclusion, the experimental data presented in this work support the concept that the signal transduction system responsible for the regulation of the redox-controlled protein kinase is based on the interaction of the enzyme with cytochrome *bf* in which the high-potential path components are reduced and a plastoquinol persists in its reduced state while interacting with the cytochrome Qo site. The sensitivity of the system is manifested by the fact that light-dependent withdrawal of a single electron from this plastoquinol is sufficient to lead to desensitization or response-inactivation of the signal transduction system.

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