The Redox-controlled Light-harvesting Chlorophyll a/b Protein Kinase

DEACTIVATION BY SUBSTITUTED QUINONES*

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The deactivation of the redox-controlled light-harvesting chlorophyll a/b protein kinase of Acetabularia acetabulum and pea thylakoids was studied. Substituted benzoquinone, napthoquinone, and anthraquinone analogs including mono-, di-, and trihalogenated and/or alkylated quinones, which are known to inhibit the cytochrome bs/f activity, deactivate the kinase in the dark, and prevent its activation in the light. Analog halogenated at positions 2- or 3- are the most effective deactivators. Increasing the size of the alkyl side chain and/or the number of rings lowers the deactivation effect. The activated state of the pea kinase decays with a t1/2 of 15 min, while the Acetabularia enzyme retains its active state for at least 2 h. The midpoint potential for Acetabularia kinase activity in the dark is 120 ± 10 mV and is compatible with the involvement of plastoquinone in the kinase activation via reduction of the cytochrome complex. Deactivation of kinase by the analogs inhibiting cytochrome bs/f complex activity and the kinase copurification with the cytochrome bs/f fraction obtained from the Acetabularia thylakoid further support this conclusion. These results indicate that the process of kinase activation/deactivation includes the binding of plastoquinol or quinone analogs by the cytochrome complex and its interaction with the kinase. We propose that the latter process may constitute the rate-limiting step controlling the kinase activation/deactivation kinetics.

Presently, a large body of information is available on the role of LHCII1 phosphorylation in the regulation of light energy distribution between the two photosystems in oxygenic organisms (for review, see Refs. 1 and 2). The activation of the redox-controlled thylakoid-bound LHCII kinase by reduced plastoquinol is not yet well understood. Although progress toward the elucidation of the mechanism of kinase activation has been made during the last years, there is practically no information on the process of LHCII kinase deactivation following the oxidation of the plastoquinol pool. This situation stems from the fact that most of the work so far was carried out with isolated spinach or pea thylakoids, systems that impose severe limitations on the experimental design, since LHCII kinase is inactive in the isolated thylakoids and requires activation by light or reducing agents, and its deactivation in the dark is rather fast.

Experimental results obtained with cytochrome bs/f-less mutants of both algae (3, 4) and higher plants (5-7) as well as by use of specific inhibitors of cytochrome bs/f complex reduction in vitro (7-9) indicate that the plastoquinol pool itself is not the sole mediator involved in the LHCII kinase activation. Thus, it was proposed that the cytochrome bs/f may act as a redox sensor and mediate the activation/deactivation of the LHCII kinase (6-7, 9).

Recently, it was reported that isolated LHCII kinase system can exhibit redox control in vitro (10). Using this experimental system, it was possible to demonstrate that a putative quinone binding site may be directly involved in the inhibition of the basal kinase activity. The process of kinase activation by interaction with the cytochrome bs/f complex increased the affinity of the kinase toward LHCII (10). However, the degree of activation exhibited by this system was relatively low as compared with intact thylakoids.

We have previously reported that the LHCII kinase present in prochlorophytes, Prochloron and Prochlorothrix hollandica, retains its activity in the dark in vivo or in isolated thylakoids in vitro (11, 12). A similar situation was also found in chloroplasts of Acetabularia acetabulum (previously referred to as A. mediterranea), a unicellular green alga of the Dasycladales. The LHCII kinase of these cells is redox-controlled (9). The activity of Acetabularia LHCII kinase persisted in isolated thylakoids in the dark significantly longer than that of higher plants' thylakoids, such as pea or spinach. In the present work, we have used this experimental system, which offers the possibility of testing the role of cytochrome bs/f in the redox control of the enzyme by use of different cytochrome bs/f inhibitors.

The results of these experiments demonstrate the role of cytochrome bs/f in the deactivation of LHCII kinase and characterize the properties of the quinone analog(s) binding to the site(s) involved in the deactivation process.

MATERIALS AND METHODS

Biological Material

Acetabularia cells were grown at the Max-Planck Institute as previously described (13). Cells 2-3 months old were transferred to plastic bottles (10,000-40,000 cells/transport) and were carried to the biological chemistry department in Jerusalem within 6-8 h. The cells

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1 The abbreviations used are: LHCII, light-harvesting chlorophyll a/b protein; MES, 4-morpholinioethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DCIP, 1,2-dichlorophenol-indophenol; PSII, photosystem II; DDBB, 2,3-dibromo-5-t-butyl-p-benzoquinone; DIBB, 2,3-dioido-5-t-butyl-p-benzoquinone; DBMIB, 2,3-dibromo-5-methyl-6-isopropyl-p-benzoquinone; cm, midpoint redox potential.
were transferred to deep Petri dishes containing 500–1000 cells in 200 ml of artificial seawater (13) and kept at 22 °C with a 12-h light, 12-h dark regime for up to 2 weeks. During this period, experiments on living cells were performed, and from the remaining cells, thylakoids were prepared and frozen until further use. A total of 150,000 cells were used for each experiment.

Pea plants (Pisum sativum, Dan hybrid) were grown under a similar light regime as previously described (14).

Preparation of Thylakoids

Acetabularia thylakoids were prepared as described (9). The cells (approximately 4.5-cm long) were washed in fresh medium and aligned in parallel bundles of 100 cells. The thylakoids were separated by pulling a sewing string close above the nucleus. The “hats” were removed by cutting while the cell bundles were submersed, and the bundles were hung with the cut stalks pointing down in test tubes containing 2 ml of an isotonic buffer consisting of 20 mM potassium phosphate, 1 mM MgCl₂, 2 mM EDTA, 0.8 mM sorbitol, 20 mM NaCl, 0.25% w/v Ficoll (Pharmacia LKB Biotechnology Inc.), and 50 mM MES buffer, pH 6.1. The tubes were spun at 3,000 × g for 10 min at 4 °C to release the cell content. The chloroplast pellet was resuspended in hypotonic buffer (5 mM MgCl₂, 50 mM Tricine, pH 8.0), frozen in liquid nitrogen, and thawed to ensure chloroplast breaking. After centrifugation (3,000 × g for 10 min), the thylakoid membrane pellet was resuspended in the same buffer to a final chlorophyll concentration of 0.4 mg ml⁻¹ and stored in ice until use.

Pea thylakoids were prepared as previously described (14) and were resuspended at a final chlorophyll concentration of 0.4 mg ml⁻¹ in 50 mM NaCl, pH 7.5, 0.5 mM MgCl₂, and 2 mM dithiothreitol. Thylakoid pellets were stored in dark at 4 °C for up to 2 h. For long-term storage, both Acetabularia and pea thylakoids were immediately frozen in liquid nitrogen and stored for up to 3 months at −80 °C without significant loss of kinase activity.

Assays

Phosphorylation of Thylakoid LHCII and Histone III-s-LHCII

Phosphorylation in isolated thylakoids of Acetabularia was assayed as previously reported (9). The assay mixture in a final volume of 100 µl consisted of 50 mM HEPES buffer, pH 7.5, 10 mM MgCl₂, 5 mM NaCl, 0.1 mM γ-[³²P]ATP (0.1 mCi ml⁻¹), and thylakoids equivalent to 2 µg of chlorophyll. Incubation was in white light (100 watts m⁻²) or in the dark at 25 °C. Duroquinol (0.1 mM) was reduced with sodium dithionite to 2 µg of chlorophyll. Incubation was in white light (100 watts m⁻²) or in the dark at 25 °C. Duroquinol (0.1 mM) was reduced with sodium dithionite as an electron donor and methyl viologen as an electron acceptor as previously described (18). The light-dependent DCIP reduction was followed by spectrophotometric recording using a Kentron UV-visible spectrophotometer. The reaction mixture contained 5 µg of chlorophyll, 20 mM MES buffer, pH 6.5, 10 mM NaCl, and 5 mM MgCl₂ at 25 °C. Light excitation was provided through a Schott 665 cut-off filter, and the photomultiplier was protected by a Corning 6-90 blue filter. The Mehler reaction using duroquinol reduced by ascorbate as an electron donor and methyl viologen as an electron acceptor was recorded by White et al. (19).

Variable fluorescence kinetics was measured using a home-built fluorometer as previously described (20).

Redox titration of the Acetabularia LHCII kinase activity was carried out basically as reported by Millner et al. (21). Redox measurements were done in the dark at 25 °C under a N₂ stream, using an Ingold electrode, Pt-4800-M5, connected to a Radiometer pH meter. Thylakoids (50 µg of chlorophyll ml⁻¹) were incubated in a final volume of 200 µl containing HEPES buffer (50 mM, pH 8.0), 5 mM NaF, and 10 mM MgCl₂ in the presence of the following redox mediators at 25 µM final concentration (emₚ, given in parentheses): DCIP (+217); 1,2-naphthoquinone (+135); phenazine ethosulfate (+80); phenazine methosulfate (+55); 2,3,5,6-tetramethylbenzoquinone (+5); 2,3,5,6-tetramethyl-1,4-naphthoquinone (+9); pyocyanine (+54); 2-hydroxy-1,4-naphthoquinone (+138); anthraquinone 2,3,4-trihydroxy- (184); anthraquinone 2-sulfonate (−225). The redox potential was adjusted to and maintained at the desired potential by additions of small volumes of sodium dithionite or potassium ferricyanide solutions. Prior to the measurements, the electrode was calibrated with potassium ferricyanide/potassium ferrocyanate at different ratios. After poising the desired potential, the kinase reaction was initiated by addition of γ-[³²P]ATP (0.1 mM, 10 µCi ml⁻¹, final concentration). Phosphorylation assays were carried out for 10 min while monitoring the redox potential (±20 mV). The reaction was stopped by drawing 100-µl samples into Eppendorf tubes containing 100 µl of 10% (w/v) trichloroacetic acid and 10 µl of NaCl and dithionite sample buffer. SDS-PAGE was performed as described above. Quantitation of LHCII phosphorylation was done either by excising the dried radioactive LHCII bands and counting them in a Beckman scintillation counter using the Cerenkov method or by densitometry scanning of the autoradiogram as above.

Isolation and Quantitation of Cytochrome b₅ Complex

Cytochrome b₅ complex was isolated from Acetabularia cells using the method described by Wynn et al. for isolation of the complex from green algae (22). For spectrophotometric quantitation, extinction coefficients of 1 mmol l⁻¹ cm⁻¹ for cytochrome f and 1 mmol l⁻¹ cm⁻¹ for cytochrome b₅ were used, respectively (23). Heme staining was carried out as described (24).

Protein and chlorophyll concentrations were determined according to Lowry et al. (25) and Arnon (26), respectively.

The benzoquinone (27), naphthoquinone (28), and anthraquinone analogs (29) used in this work were synthesized as previously described.

All chemicals used in this work were of analytical grade.

RESULTS

Stability of LHCII Kinase-activated State—The thylakoid-bound LHCII kinase can be reversibly activated by redox reagents such as plastocyanin or duroquinol (15, 30, 31). The resulting active state can be deactivated by oxidizing reagents such as plastocyanin or ferricyanide (31). LHCII kinase can also be irreversibly inactivated by alkylating agents (21). To distinguish between enzyme deactivation (yet preserving the potential for reactivation by reducing agents) and loss of activity due to irreversible inactivation, we shall refer below to the kinase as being in different activation states: deactivated or having a basal activity, an activated state, and an irreversibly inactive state (Scheme 1).

To test the stability of the activated state of the Acetabu-
Effect of Quinone Analogs on Deactivation of LHCII Kinase

Deactivation of thylakoid LHCII kinase during storage in the dark. Isolated thylakoids (20 μg of chlorophyll·ml⁻¹) of Acetabularia and pea kept at −80 °C were thawed in the dark and stored in ice. Pea thylakoid samples were illuminated for 30 s to activate the kinase and further stored in the dark for 2 h. At times as indicated, samples of the dark-incubated Acetabularia and preactivated pea thylakoids were assayed for LHCII phosphorylation in the dark or light for 10 min at 25 °C. The phosphorylation activity was estimated by excising the LHCII polypeptide bands resolved by SDS-PAGE and counting the incorporated radioactivity. The ratio of the dark activity to that measured in the light are plotted, while the light activity was taken as 100%. Samples were assayed for 27-kDa LHCII band for the light-driven reaction. The amount of LHCII was estimated as in Ref. 9.

Measurements of fluorescence induction kinetics using either pea (32) or Acetabularia thylakoids (data not shown) demonstrate that following light-driven reduction, the plastoquinone pool is reoxidized in the dark within less than 2 min. Thus, the persisting activated state of pea thylakoid LHCII kinase was also measured. The LHCII kinase of isolated pea thylakoids was found in the deactivated or basal state. Following an initial exposure to the light (30 s) to activate the enzyme, the thylakoids were further stored in ice, in the dark, and at times as indicated, LHCII phosphorylation was assayed in the dark or light. The results (Fig. 1) show that in the dark-stored pea thylakoids, the LHCII kinase-activated state deactivates rapidly (t½, 15 min) as compared with the kinase of Acetabularia thylakoids (t½, >2 h).

Effect of Quinone Analogs on Deactivation of Acetabularia LHCII Kinase by Quinone Analogs—The nature of the kinase activator, plastoquinol or a reduced component of the cytochrome b₆/f complex, can be disclosed by measuring the redox midpoint potential of the activation process. The midpoint potential for the activation/deactivation of the Acetabularia enzyme was measured. The results of these experiments are shown in Fig. 2. The midpoint potential in the dark is εₘₐₜ, 120 ± 10 mV, at pH 8.0. However, it was not possible to determine unequivocally whether n = 2 or 1. This εₘₐₜ value is closer to the reported midpoint potential of plastiquinol (εₘₜ, 80–100 mV, pH 7.5 (33–35)) than to that of the b₆ and b₄ components of b₆ (εₘₜ, −146 and −50 mV, pH 7.0, respectively) so far reported (36, 37). Thus, a reduced quinol binding site seems to be involved in the Acetabularia LHCII kinase activation as well.

Information on the properties of the binding site involved in the activation/deactivation process could be obtained by assaying the effect of various substituted quinone analogs on the enzyme deactivation in the dark. Under these conditions the quinone analogs may not compete for the binding site with the plastiquinol pool reduced via light-driven photosystem II electron flow. Furthermore, the possible inhibitory effect of the analogs on the reduction of plastoquinone by PSII may not interfere with the effect of the analogs on the binding site.

The extent of deactivation of the Acetabularia LHCII kinase in the dark or light by substituted benzoquinones, naphthoquinones, and anthraquinones is shown in Table I. The pKₐ values for this deactivation are given in Table II together with the pKₐ values for the inactivation of the electron flow to photosystem I. For comparison, the pKₐ values for the inactivation of DCIP reduction (PSII) by pea thylakoids are given as well. The concentration-dependent deactivation of LHCII kinase for representatives of these three types of quinone analogs in the light, dark, or dark with addition of duroquinol are given in Figs. 3 and 4. In Tables I and III, only the percent inactivation at a concentration of 0.5 μM was tested for all the analogs, since at this concentration, one could compare the effect of all the compounds tested both in the dark and in the light. Although this approach does not resolve in detail differences between the analogs causing extensive deactivation (≥85%) at the concentration used, it allows a comparison of the effects of those of lower potency.

The kinase was deactivated in the dark (in absence of added activators) by DBBB or DIBB with a pKₐ value of 0.1 μM.
Effect of 1,4-naphthoquinone analogs

The results presented in Fig. 4 and Tables I and II clearly demonstrate that the inhibitory effect of the quinone analogs is drastically reduced with the increase in the ring size. The pIC50 values were 0.05, 1.0, and 3 μM for benzoquinone, naphthoquinone, and anthraquinone, respectively. The lower activity of the trichloroanthraquinone is ascribed to the ring size rather than to the presence of an additional chlorine at position 4 as compared with benzoquinone and naphtoquinone derivatives, since no significant difference was found between the activity of di-, tri- and tetrachloroanthraquinone (Table I). However, even the halogenated-substituted anthraquinone ring is still a potent inhibitor at 0.5 μM concentration.

The kinase deactivation is not related to the quinone binding sites associated with photosystem II (Table II). Furthermore, the pIC50 values for the inactivation of electron flow from duroquinol to methyl viologen are similar to those of the LHCII kinase deactivation, indicating that these inhibitors interact with the cytochrome b6/f complex (Table II).

The high inhibitory activity in the dark of some benzoquinone analogs was significantly diminished when the assay was carried out in the light. This was the case mostly for those compounds that had only a small inhibitory effect on the activity of photosystem II, such as that of unsubstituted benzoquinone or of the tribromomethyl benzoquinone. This could be due to competition for the binding site with plastoquinol generated by electron flow via photosystem II. Compounds such as 2-bromo-5-t-butyl-benzoquinone and DBMIB significantly inhibit the activity of photosystem II but only partially deactivate the kinase in the light (Table I). This may indicate that the relatively low plastoquinol concentration formed in the presence of these compounds in the light compete better with these analogs for the kinase activation site than with other analogs such as 2,3-dichloro-5-t-butylbenzoquinone. The latter deactivates the kinase in the light despite a higher rate of photosystem II activity, which could generate a higher plastoquinol/plastoquinone ratio in the thylakoid membranes.

Substituted quinone analogs may be reduced by light-incubated thylakoids (38, 39). The possibility that the reduced analogs may serve partially as activators could explain their lower inhibitory effect in the light. It is possible that such an activating effect may occur at the lower concentrations of the inhibitors as observed in Figs. 3 and 4.

The nonsubstituted naphthoquinone ring had no effect on the Acetabularia kinase activity in the dark or in the light. However, it partially inhibited DCIP reduction. The effect of the naphthoquinone analogs on kinase deactivation in the dark is drastically enhanced by halogen substitution at position 2- and a further increase in the effect is obtained by addition of a second halogen at position 3- (Table I, compare 2-chloro- with 2,3-dichloro-, 2,3-dibromo, or 2,3-dibromo-naphthoquinones). The effect of all these compounds is significantly lower in the light as expected, since photosystem II is only partially inhibited, and thus, the level of reduced plastoquinol is sufficient to compete with the inhibitors for the binding site.

It is also noteworthy that anthraquinones have a significant inhibitory effect on DCIP reduction and only a limited deactivation of the kinase in the dark (13-32%), yet they exhibit a higher inhibitory activity of the kinase in the light (50-66%, Table I).

Deactivation of Pea Thylakoids LHCII Kinase by Quinone Analogs—The activated state of pea LHCII kinase is less stable than that of Acetabularia (cf. Fig. 1), and thus, measurements of kinase activity in the dark in the presence of the various quinone analogs is technically more difficult. Nevertheless, it was possible to demonstrate a similar behavior toward substituted quinone analogs for the pea LHCII kinase in the dark (Table III). The effect of the substituted benzoquinone and especially naphthoquinone halogenated at position 2,3- is higher as compared with the nonhalogenated

| TABLE I Deactivation of LHCII Kinase in dark-incubated Acetabularia thylakoids by various quinone analogs |
|---------------------------------------------------------------|
| LHCII kinase activity was assayed under the same experimental |
| conditions in the light or dark as in Fig. 3. DCIP reduction  |
| in the light was assayed as described under "Materials and Methods." The |
| concentration of the quinone analogs was in all cases 0.5 μM. The |
| 100% values measured as in Figs. 3 and 4 were 0.4 and 0.55 μmol "P- |
| mg" incorporated into the 27-kDa LHCII polypeptide in the dark |
| and light, respectively. |

| Effect of 1,4-naphthoquinone analogs |
|-------------------------------------|
| Analogs          | Dark | Light |
|------------------|------|-------|
| 17. 2-Hydroxy    | 0    | 0     |
| 18. 2-Methyl     | 0    | 0     |
| 19. 2-Methoxy    | 0    | 0     |
| 20. 2-Isoproxy   | 0    | 0     |
| 21. 2-Chloro     | 0    | 0     |
| 22. Hydroxy-3-methyl | 0 | 0     |
| 23. Hydroxy-3-isobutyl | 0 | 0     |
| 24. Acetoxy-3-isobutyl | 0 | 0     |
| 25. Methyl-3-(methylthio) | 0 | 0     |
| 26. Isopropylthio-3-methyl | 0 | 0     |
| 27. Amino-3-chloro | 0 | 0     |
| 28. Chlo-isethoxy | 0 | 0     |
| 29. Chloro-3-morpholinol | 0 | 0     |
| 30. Bromo-3-n-propyl | 0 | 0     |
| 31. Bromo-3-n-heptyl | 0 | 0     |
| 32. 2-Dihydroxy   | 0    | 0     |
| 33. 2,3-Dimethyl  | 0    | 0     |
| 34. 2,3-Dichloro  | 0    | 0     |
| 35. 2,3-Dibromo   | 0    | 0     |
| 36. 2,3-Diiodo    | 0    | 0     |
| 37. 2-Bromo-1,2-naphthoquinone | 0 | 0     |
| 38. 1,2-Naphthoquinone | 0 | 0     |

| Effect of 9,10-anthraquinone analogs |
|-------------------------------------|
| Analogs          | Dark | Light |
|------------------|------|-------|
| 39. 1,6-Dinitro   | 0    | 0     |
| 40. 1,7-Dinitro   | 0    | 0     |
| 41. 1,3-Dichloro-2-hydroxy | 0 | 0     |
| 42. 2,3,4-Trichloro-1-hydroxy | 0 | 0     |

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Table II
Comparison of pI50 values for the deactivation of LHCII kinase in the dark with the inhibition of light-driven electron flow by various quinone analogs

The pI50 values for the deactivation of the LHCII kinase and electron flow from duroquinol to methyl viologen are given for Acetabularia thylakoids, while those for the reduction of DCIP were obtained for pea thylakoids.

| Quinone analogs                      | LHCII kinase | Duroquinol-MeV | H2O-DCIP |
|--------------------------------------|--------------|----------------|----------|
| 1,4-Benzoquinone-                    |              |                |          |
| 1. 2-Bromo-5-butyyl                  | 7.60         | 7.92           | 4.30     |
| 2. 2,3-Dichloro-5-t-butyyl           | 8.47         | 7.47           | 4.39     |
| 3. 2,3-Dibromo-5-t-butyyl            | 7.69         | 7.69           | 5.28     |
| 4. 2,3-Diido-5,6-t-butyyl            | 7.00         | 7.46           | 6.29     |
| 5. 2-Iodo-3-methyl-6-isopropyl       | 7.69         | 6.23           | 4.12     |
| 6. 2,5-Dibromo-3-methyl-6-isopropyl  | 7.69         | 7.11           | 4.61     |
| 7. Tribromomethyl                    | 7.30         | 7.69           | 4.40     |
| 8. Tetrachloro                       | 6.23         | 4.79           |          |
| 9. Tribromobromo                     | 6.00         | 7.92           | 5.89     |
| 10. Tetraiodo                       | 7.17         | 6.44           | 7.06     |
| 1,4-naphthoquinone-                  |              |                |          |
| 14. 2,3-Dichloro                     | 6.23         |                | 5.48     |
| 15. 2,3-Dibromo                      | 7.77         |                | 5.89     |
| 16. 2,3-Diido                        | 7.69         |                | 6.16     |

Fig. 3. Inhibition of Acetabularia thylakoid LHCII phosphorylation by the benzoquinone analogs DBBB and DIBB. Thylakoid membranes stored in the dark in ice were incubated at a final concentration of 20 μg of chlorophyll·mL⁻¹ in the phosphorylation assay conditions for 15 min in the presence of various concentrations of DBBB (A) or DIBB (B). The reactions were carried out in the light (○), dark (●), or in the dark with addition of 1 mM duroquinol (□). The degree of LHCII phosphorylation was estimated as in Fig. 1. The 100% phosphorylation was 0.7, 0.4, and 0.5 nmol of ³²P·mg⁻¹ incorporated into the 27-kDa LHCII polypeptide, respectively.

Fig. 4. Effect of the quinone analog size on the deactivation of Acetabularia thylakoid LHCII kinase in the dark. Thylakoid membranes were incubated in the presence or absence of various concentrations of 2,3-dichloro-5-t-butylnaphthoquinone (O), naphthoquinone (O), or anthraquinone (Δ). The phosphorylation assay was carried out in the dark for 10 min. The experimental conditions are the same as in Fig. 3. The 100% value was 0.4 nmol ³²P·mg⁻¹ incorporated into the 27-kDa LHCII polypeptide.

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**Table III**

| Pea thylakoids (20 µg of chlorophyll·ml⁻¹) were incubated in the absence or presence of various quinone analogs at a final concentration of 0.5 µM. The experimental conditions are the same as in Table I. The pea-isolated thylakoids were preactivated by 30-s illumination before assay in the dark as described in Fig. 1, and the respective 100% phosphorylation values were as in Fig. 1.

| LHCII kinase | H₂O-DCTP | % inhibition |
|--------------|----------|-------------|
| Effect of 1,4-benzoquinone analogs | | |
| 1. 1,4-Benzquinone | 75 | 7 | 0 |
| 2. 2,3-Dichloro-5-6-butyI | 95 | 73 | 70 |
| 3. 2,3-Dibromo-5-t-butyI | 96 | 85 | 77 |
| 4. 2,3-Diiodo-5-t-butyI | 95 | 96 | 93 |
| 5. 2,3-Diiodo-5,6-dimethyl | 74 | 5 | 0 |
| 6. 2,3-Diiodo-5,6-dimethyl | 83 | 40 | 0 |
| 7. Tetracloro | 83 | 3 | |
| 8. Tetrabromo | 65 | 15 | 37 |
| 9. Tetraiodo | 95 | 84 | 70 |
| Effect of 1,4-naphthoquinone analogs | | |
| 10. 1,4-Naphthoquinone | 30 | 9 | 26 |
| 11. 2-Amino-3-chloro | 46 | 45 | 9 |
| 12. 2,3-Dimethyl | 2 | 18 | 9 |
| 13. 2,3-Dichloro | 88 | 59 | 33 |
| 14. 2,3-Dibromo | 88 | 35 | 22 |
| 15. 2,3-Diiodo | 84 | 80 | 57 |
| Effect of 1,10-anthraquinone analogs | | |
| 16. 9,10-Anthraquinone | 86 | 98 | 86 |
| 17. 1,6-Dinlro | 86 | 88 | 79 |
| 18. 1,3-Dichloro-2-hydroxy | 79 | 69 | 79 |
| 19. 2,2,4-Trichloro-1-hydroxy | 79 | 69 | 79 |

**Table IV**

| Acetabularia kinase activity copurifies with the cytochrome bs/f complex |
|-----------------------------|-----------------------------|-------------------|-----------------|
| Purification steps | Total protein | Kinase activity | Cytochrome f / | Cytochrome f / | Cytochrome f / |
| | mg | pmol [32P]/mg protein | nmol | nmol/mg protein | nmol/kinease |
| NaBr-washed membranes | 98 | 615 | 35.0 | 0.36 | 5.8 |
| Solubilized membranes | 5 | 1,823 | 8.4 | 1.68 | 2.2 |
| Ammonium sulfate precipitate | 0.25 | 5,000 | 0.6 | 2.40 | 4.8 |

*The ratio is given as nmol of cytochrome f/kinease activity × 10⁻⁴. The yield of both cytochrome and kinase is in the range of 2% as compared with 20-30% reported for the purification of similar preparations from spinach (10). Due to the low yield, the last step in the purification consisting of sucrose density centrifugation could not be performed.

*The solubilized membrane fraction is the supernatant after membrane solubilization in the detergent mixture and centrifugation (22).
FIG. 5. Western blot and polypeptide pattern of Acetabularia thylakoids and cytochrome b6/f-enriched fraction. Membrane samples (15 μg of protein) and enriched cytochrome b6/f preparation (2–5 μg of protein) were resolved by SDS-PAGE followed by Western blotting with the corresponding antibodies raised against spinach cytochrome b6/f components. A, immunoblot pattern of membrane fraction (m) and enriched cytochrome b6/f preparation (c). The different lanes were each treated with the respective antibodies against Rieske protein (R), cytochrome b6 (b6), subunit IV (IV), cytochrome f (F), and LHCII kinase (kin); arrow indicates the position of the 64-kDa putative LHCII kinase identified in this preparation only by silver staining (data not shown). Note that lanes R and F show some nonspecific reaction of the antibodies with other components of the b6/f complex. B, polypeptide pattern of Acetabularia cytochrome b6/f preparation as resolved by SDS-PAGE and stained with Coomassie Blue.

to form the anthraquinone, significantly diminished the inhibitory effect of the analog. The most effective analogs inhibiting the LHCII kinase are those that bind to the Q, site of the cytochrome b6/f complex such as DBMIB, DIBB, and DBBB (38, 44), suggesting that this complex is one of the components involved in the activation/deactivation processes.

Some of the quinone analogs used in this work were reported to bind to nucleophilic groups (38). Since it was demonstrated that the LHCII kinase can be inactivated by alkylating reagents (21) one could expect that at least part of the inhibitory effects observed in this work may be due to their interaction with the kinase —SH groups. If this were the case, one would expect that the inhibition would be practically irreversible. To test this possibility, the effect of DBMIB and tetrabromo-1,4-benzoquinone (Bromanil) known to interact with —SH groups was tested and found to be reversible (data not shown). Similar results were also found by Coughlan et al. (40) for the inhibitory activity of DBMIB on thylakoid-bound LHCII kinase from spinach.

It was previously reported that reduced DBMIB does not bind to the cytochrome b6/f complex (45). Since DBMIB can be reduced by electron flow from PSII, one may consider that DBMIB may interact with the kinase proper. Assay of the pI50 of the kinase deactivation by DBMIB-H2 was similar to that of oxidized DBMIB (data not shown). This may indicate that the LHCII kinase had a distinct quinone binding site as previously suggested (11). It was reported that the isolated 64-kDa LHCII kinase appears to be a hydrophilic protein despite its strong binding to the thylakoid membrane (40). Thus, the presence of a quinone binding site in the kinase deserves further investigation.

Factors Affecting the Rate of Kinase Activation/Deactivation—The slow deactivation process of the kinase in dark-incubated thylakoids contrasts with the relatively fast activation by addition of plastoquinol or by light-driven electron flow. However, even the activation process is slower than that of the plastoquinone pool reduction in light-exposed thylakoids, which is achieved within seconds when isolated thylakoids are illuminated under conditions inducing LHCII kinase activation. The reoxidation of the pool in the dark occurs within 1–2 min. Thus, the kinetics of kinase activation and deactivation do not correlate in a simple way with the changes in the plastoquinol/plastoquinone ratio. The activation time of the kinase in the light was reported to be about 2–5 min as measured by LHCII phosphorylation in pea or Acetabularia (9, 30, 31). ATP-induced fluorescence quenching and state transition resulting from the phosphorylation of LHCII can be detected within 5–10 min (15). However, in this case the measured time includes the process of LHCII dissociation from PSII and its lateral diffusion within the membrane plane. Phosphorylation of LHCII can be activated at 0 °C (47), while the dissociation of LHCII and the associated loss of energy transfer to the reaction center are not affected at this temperature. Since binding of a quinone ligand to a specific binding site occurs on a time scale of seconds (38, 39, 44, 45), one must conclude that the activation of the kinase by plastoquinol or its deactivation by plastoquinone is mediated by an additional slower step(s) besides the quinone binding per se.

Two possible models could be considered to explain this aspect of the kinase activation/deactivation process. In both, the cytochrome-kinase interaction may be the slow step. In both models, cytochrome b6/f is reduced by plastoquinol (fast phase) and binds to the kinase, which becomes activated (slow phase). The cytochrome-kinase complex remains associated and active until the cytochrome is oxidized and dissociates from the kinase (slow phase) or until a quinone binding site on the cytochrome complex, the kinase proper, or both, is occupied by plastoquinone or an appropriate analog causing the kinase deactivation via dissociation of the cytochrome b6/f complex from the kinase (slow phase). Occupancy of the Q, site of the cytochrome complex by quinone analogs preventing its reduction by plastoquinol, or occupancy of the putative quinone binding site on the kinase by such analogs, may prevent this association or accelerate the dissociation process.

The possibility that self-phosphorylation of the kinase and phosphorylation of the cytochrome b6 within the complex may be part of the activation/deactivation processes should also be considered as suggested by recent results demonstrating that both the kinase and the cytochrome b6 are phosphorylated when the isolated cytochrome b6/f-kinase complex is incubated with ATP (48).

An additional factor controlling the rate of these two processes may be the structural organization of the thylakoid. We have demonstrated before that LHCII kinase is specifically localized at the edges of the grana stacks (10). It was reported before that a fraction of the cytochrome b6/f complex is localized at the region between the appressed and nonappressed thylakoid membranes (48). Recent results demonstrate the coexistence of the LHCII kinase in such a fraction possibly associated with some of the cytochrome b6/f complex.

The possibility of the transient existence of "supercomplexes" of LHCII including PSII and cytochrome b6/f complex interconnected by confined plastoquinone molecules has been proposed as a control mechanism of the linear electron flow from PSII (49), while specific diffusion of cytochrome b6/f complex from the granal to the stromal domain during state transition has been implied to be related to the state transition and activation of the photosystem I cyclic electron flow (50). It is thus possible that slow, diffusion-controlled, structural interactions between the cytochrome b6/f complex and the kinase leading to the formation of an active supercomplex may control in a similar way the processes of activation/deactivation of the LHCII kinase.

The observed difference in the stability of the activated

P.-A. Albertson, personal communication.
state between the pea and the *Acetabularia* LHCII kinase could thus be due to differences in the organization or affinity of the kinase/cytochrome b/f complex interactions in different chloroplasts.

Further experimental data concerning the process of kinase deactivation in both isolated thylakoids and cytochrome b/f kinase preparations may contribute to the elucidation of the mechanism of this phenomenon.

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