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Contrasted Effects of Inhibitors of Cytochrome b_6f Complex on State Transitions in *Chlamydomonas reinhardtii*

THE ROLE OF Q_o SITE OCCUPANCY IN LHCII KINASE ACTIVATION*

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We have investigated the relationship between the occupancy of the Q_o site in the cytochrome b_6f complex and the activation of the LHCII protein kinase that controls state transitions. To this aim, fluorescence emission and LHCII phosphorylation patterns were studied in whole cells of *Chlamydomonas reinhardtii* treated with different plastoquinone analogues. The analysis of fluorescence induction at room temperature indicates that stigmatellin consistently prevented transition to State 2, whereas 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone behaved as an inhibitor of state transitions only after the cells were preilluminated. The same effects were observed on the phosphorylation patterns of the LHCII proteins, while subunit V of the cytochrome b_6f complex showed a different behavior. These findings are discussed on the basis of a dynamic structural model of cytochrome b_6f that relates the activation of the LHCII kinase to the occupancy of the Q_o site and the movement of the Rieske protein.

Protein phosphorylation is a general mechanism for signal transduction that is present both in eucaryotes and procaryotes. It is usually triggered by the binding of an external signal molecule to a membrane located receptor, as in the case, for example, of the hormone-induced signal transduction pathway (1). In other instances, however, membrane-bound receptors are not involved in the reception of external signals. This is the case of the short time chromatic adaptation phenomena, known as state transitions (2, 3), that occur in plants and in algae. In these organisms, changes in the quality of the absorbed light energy induce the phosphorylation and reversible migration of a fraction of the light harvesting proteins (LHCII) between the grana and the stroma domains of the thylakoids (4). Following an illumination with light absorbed preferentially by photosystem II (PSII),[1] LHCII is phosphorylated and becomes part of PSI antenna (State 1 to State 2 transition) (5, 6). The illumination with PSI-absorbed light has the opposite effect: a dephosphorylation triggers the re-association of LHCII to PSII (State 2 to State 1 transition (5, 6)). *In vivo* studies with the unicellular green alga *Chlamydomonas reinhardtii* have also demonstrated that state transitions are controlled by the intracellular demand for ATP: dark-adapted cells are locked in State 2 when the intracellular content in ATP is low, whereas they shift to a State 1 configuration when the ATP pool is restored (7).

The changes in the phosphorylation state of antenna proteins result from the combined actions of an LHCII kinase, the activation of which is redox-dependent (8), and a phosphatase that is considered permanently active (9), although recent data have suggested the possibility of a regulation via its interaction with an immunophilin-like protein (10). The mechanism for kinase activation involves the reduction of the plastoquinone pool (3, 11) and requires the presence of cytochrome b_6f complexes (12, 13). The nature of the kinase is still obscure, even though its presence has been reported in partially purified preparations of cytochrome b_6f complexes (14). Although the molecular mechanism through which the redox state of the plastoquinone (PQ) pool is transduced to the kinase is not known, the implication of the quinol binding site, Q_o, of the cytochrome b_6f complex has been demonstrated both *in vivo* with *C. reinhardtii* (13) and *in vitro* with thylakoid preparations from spinach (15, 16). In the latter case, Vener and colleagues (15, 16), have reported that the activation of the kinase *in vitro* could be obtained by a reversible acidification of the thylakoids that induces the reduction of ~20% of the PQ pool. The activation was maintained even after reoxidation of the PQ pool, provided that a Q_o-bound plastoquinol was retained per cytochrome b_6f complex (16).

The same authors have explained the activation in terms of conformational changes of the Rieske subunit, whose flexibility has been recently demonstrated in cytochrome bc_1 (17, 18) and b_6f (19) complexes. The Rieske protein was shown to adopt at least two different positions: one close to the membrane surface, next to heme b_1 (the so called proximal position, Ref. 20), another extending more in the lumen, next to heme c_1 (respiratory, *f*) (the distal one, Ref. 20). The existence of a third position, intermediate between the two, has also been suggested by Iwata and co-workers (17). According to the model proposed by Vener *et al.* (21), the Rieske subunit would be kinase-activating in its distal position, and inhibiting in the proximal one, due to some interaction with a putative transmembrane segment of the kinase. We have recently questioned this hypothesis and suggested that activation of the kinase was produced when the Rieske was in its proximal position (13).

To further test the relationship between the movements of the Rieske protein and the activation of the LHCII kinase, we

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[1] The abbreviations used are: PS, photosystem; PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

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have studied the effects on state transitions of two Q_o site inhibitors of electron transfer in the cytochrome b_6f complex. We have used stigmatellin, which blocks electron transfer in both the bc_1 and b_6f cytochrome complex (22) by fixing the iron sulfur protein in its proximal conformation (17–20). We have also used DBMIB, which inhibits cytochrome b_6f but not bc_1 complexes (22), and develops contrasted interactions with the Rieske protein depending on its redox state (23). Remarkable differences were observed between the effects of the two inhibitors, indicative of the existence of a rather complex relationship between the occupancy of the Q_o site and the activation of the LHCII kinase. We present here a structural hypothesis that could account for these observations.

EXPERIMENTAL PROCEDURES

Strains and Culture Growth Conditions—Wild type (mt^+) derived from strain 137C and FUD7 mutant lacking PSII were grown on Tris acetate-phosphate (TAP) at 25 °C under and 60 $\mu E\ m^{-2}\ s^{-1}$ of continuous illumination. They were harvested during exponential growth phase and resuspended in a minimal medium (24). Cells were placed in State 1 and State 2 conditions in darkness, either by vigorous stirring to ensure a strong aeration (State 1) or by an incubation in anaerobic conditions, upon addition of glucose and glucose-oxidase (State 2) (25). 13-Tridecyl-stigmatellin was a kind gift of Paulette Hervé from the UPR 9052 of CNRS. DBMIB was purchased from Sigma.

Optical and Fluorescence Measurements—Fluorescence measurements were performed at room temperature on a home-built fluorimeter: samples were excited using a light source at 590 nm. The fluorescence response was detected in the far red region of the spectrum.

Spectroscopic measurements were performed at room temperature, using a "Joliot-type spectrophotometer" as described in Ref. 13. Samples were illuminated with red light provided by a light-emitting diode array placed on both sides of the measuring cuvette. Heat-absorbing filters were placed between the light-emitting diode arrays and the cuvette. Cytochrome *f* redox changes were evaluated as the difference between absorption at 554 nm and a base line drawn between 545 and 573 nm.

Protein Phosphorylation Assays—Cells, grown to a density of 3×10^6 cells ml^{-1}, were harvested and resuspended in a phosphate-depleted medium containing 1 $\mu Ci\ ml^{-1}$ $^{33}P_i$. Then they were treated as described in Wollman and Delepelaire (25). Polypeptides were separated by denaturing SDS-polyacrylamide gel electrophoresis (8 M urea, 12–18% acrylamide).

RESULTS

Effects of Plastoquinone Analogues on State Transition in Dark-adapted Algae—To study the relationship between the occupancy of the Q_o site of cytochrome b_6f and the activation the LHCII kinase, we have tested the effects of two quinone analogues on whole cells of *C. reinhardtii*: stigmatellin, which is effective on both bc_1 and b_6f cytochrome complexes; and DBMIB, which inhibits only cytochrome b_6f complexes (22).

The occurrence of state transitions was studied by measurement of the fluorescence yield at room temperature of intact algae. It is indeed known that under these conditions fluorescence emission is inversely proportional to the yield of PSII photochemistry and proportional to the size of its light harvesting antenna (26). Therefore it is possible to follow directly changes in the antenna size if PSII photochemistry is inhibited by addition of DCMU. Fig. 1 shows the effects of stigmatellin on the fluorescence yield of intact Chlamydomonas cells: this Q_o site inhibitor completely prevented the quenching of fluorescence otherwise induced under conditions that promote State 2 (*A* and *B*, *dashed lines*) without affecting fluorescence emission in State 1 (compare *A* and *B*, *continuous lines*). Its addition also promoted the restoration of a high fluorescence yield, typical of State 1, in algae that were previously adapted to State 2 (Fig. 1*C*).

Fig. 2 shows the fluorescence behavior of Chlamydomonas in the presence of DBMIB. DBMIB addition slightly lowered the fluorescence yield of the nontreated control due to the fact that

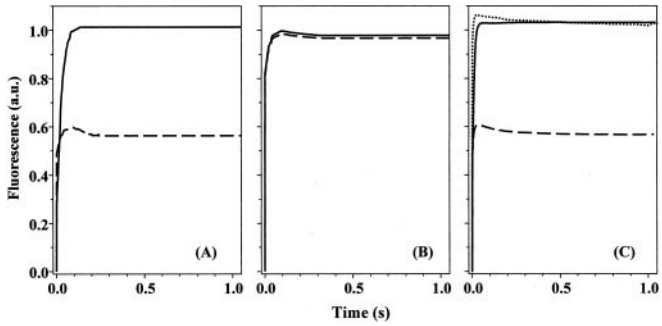


FIG. 1. **Effect of stigmatellin on state transitions in whole cells of *C. reinhardtii*.** *A*, untreated cells; *B*, stigmatellin-treated cells. Cells were harvested during exponential phase of growth and resuspended in minimal medium (24). *Continuous line*, State 1 conditions; *dashed line*, State 2 conditions. State 1 was induced through vigorous agitation in the dark in air. State 2 was obtained through addition of glucose and glucose oxidase (13). DCMU was added at a concentration of 20 μM, stigmatellin was 5 μM. *C*, time course of State 2 to State 1 transition after stigmatellin addition. *Continuous line*, State 1 conditions; *dashed line*, State 2 conditions 1 min after stigmatellin addition; *dotted line*, State 2, 35 min after stigmatellin addition.

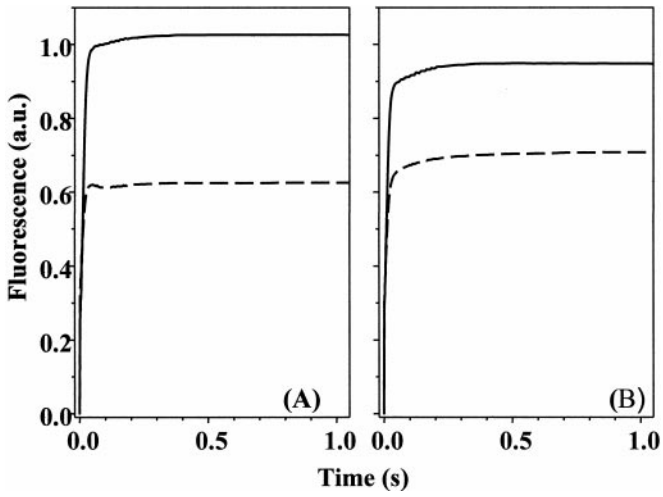


FIG. 2. **Effect of DBMIB on state transitions in whole cells of *C. reinhardtii*.** *A*, untreated cells; *B*, DBMIB-treated cells. DBMIB concentration was 2 μM. Other conditions were the same as in Fig. 1. *Continuous line*, State 1 conditions; *dashed line*, State 2 conditions.

it is a fluorescence quencher (27). However, it did not prevent State 1-State 2 transition in darkness (Fig. 2*B, dashed line*), even at concentration that completely inhibit reduction of cytochrome *f* under continuous illumination (data not shown).

Effects of Plastoquinone Analogues on State Transition in Preilluminated Algae—In a previous study on the effects of DBMIB on electron transfer in the cytochrome b_6f complex (28), we found that the inhibitory efficiency of this compound increased upon a preillumination. We have therefore repeated the measurements performed in Fig. 2 on preilluminated cells, to understand if DBMIB prevented the activation of the LHCII kinase when added in the light. The results are reported in Fig. 3. In these experiments the light intensity was kept low enough not to inhibit a State 1-State 2 transition in the absence of the inhibitor (Fig. 3*A*). While no difference was observed in stigmatellin-treated samples between dark and illuminated cells (compare Fig. 3*B* with Fig. 1*B*), DBMIB addition completely abolished kinase activation under these latter conditions (Fig. 3*C*). Its effect was reversible, provided that the light was switched off (Fig. 3*D*).

It is known that DBMIB and stigmatellin interact not only with cytochrome b_6f complex, but also with PSII: in particular, the former reacts with the light-harvesting subunits of PSII,

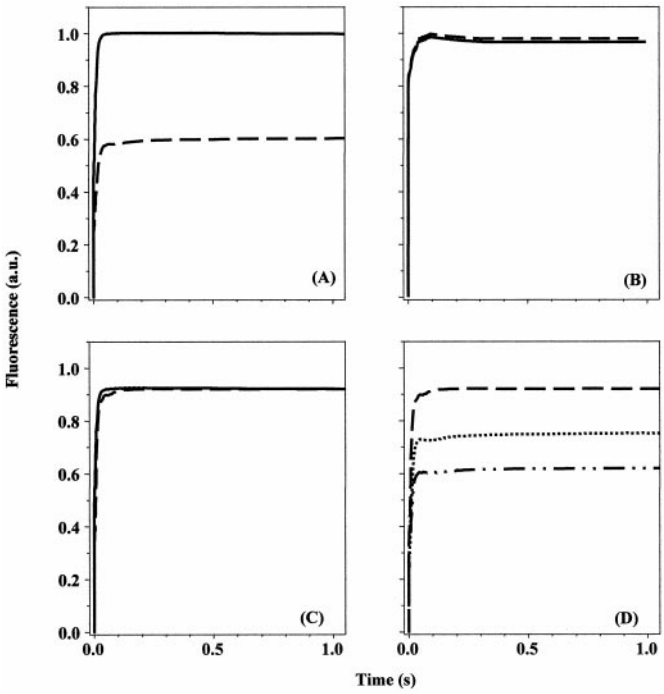


FIG. 3. **State transitions in preilluminated cells of *C. reinhardtii*.** *A*, untreated sample; *B*, stigmatellin-treated samples; *C*, DBMIB-treated sample. *Continuous line*, State 1 conditions; *dashed line*, State 2 conditions. Cells were illuminated with ~60 μE m^{-2} s^{-1} for a few minutes, then fluorescence emission was recorded. *D*, kinetics of State 2 recovery in the presence of DBMIB. Traces were recorded immediately (*dashed line*), 15 min (*dotted line*), or 30 min (*dotted* and *dashed line*) after the light was switched off.

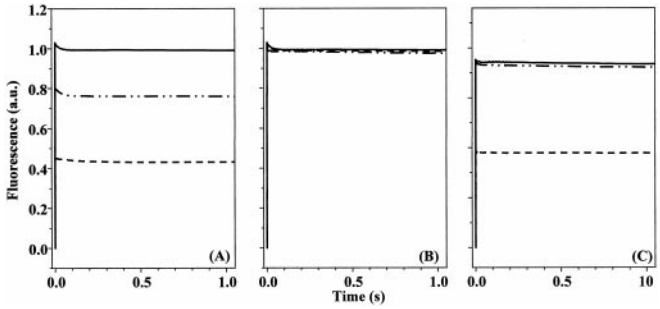


FIG. 4. **Effects of Q_o site inhibitors on state transitions in FUD7 mutant cells.** Same conditions as in Fig. 1. *A*, untreated sample; *B*, stigmatellin-treated samples; *C*, DBMIB-treated sample. *Continuous line*, State 1 conditions; *dashed line*, State 2 conditions (dark); *dotted* and *dashed line*, State 2 conditions (light).

where it acts as a Stern-Volmer quencher of fluorescence (27), while both inhibitors bind at the Q_b site of PSII, where they act as a DCMU-type inhibitor (29). To rule out the possibility that the observed effects of the inhibitors were due to their interaction with PSII, rather than to a deactivation of the LHCII kinase, we have repeated the same experiments with the PSII mutant, FUD7 (30). The results are shown in Fig. 4. In the mutant, both stigmatellin and DBMIB behaved as in the wild type: the former inhibitor blocked the transition to State 2 in both dark-adapted and illuminated cells (Fig. 4*B*), while the latter was effective only on preilluminated samples (Fig. 4*C*). In FUD7 cells, however, the transition to State 2 in light-treated cells was less pronounced than in dark adapted ones (Fig. 4*A*), in agreement with previous reports (25, 30).

Effects of Plastoquinone Analogues on Protein Phosphorylation Patterns—The results shown in Figs. 1–4 strongly suggest that the activity of the LHCII kinase is modulated by the occupancy of the Q_o site of cytochrome b_6f. Therefore we performed an *in vivo* protein phosphorylation assay. Thylakoid membranes were purified from cells that were preincubated for 90 min with $^{33}P_i$ and placed for 20 min in State 1 and State 2 conditions in a $^{33}P_i$-free medium as described previously (25).

Fig. 5 shows an autoradiography of the 15–40-kDa region of an electrophoretogram that displays the labeling pattern of thylakoid membrane polypeptides. In the absence of inhibitors, the phosphorylation of LHCII polypeptides, LHC-P13 and LHC-P17, increased in State 2 as compared with State 1, whereas the PSII phosphoprotein D2 showed an opposite behavior, as reported previously (25). In the presence of stigmatellin, under conditions suitable to promote State 2, a low level of phosphorylation on LHC-P13 and LHC-P17 was observed, which is typical of State 1 (12) (Fig. 5*A*). Thus, the LHCII kinase was not activated by reducing conditions in the presence of this inhibitor. DBMIB had contrasted effects (Fig. 5*B*): when State 2 conditions were established in darkness, it did not prevent kinase activation, as judged from the high level of phosphorylation on LHC-P13 and LHC-P17. When State 2 conditions were established under illumination, DBMIB prevented most of LHC-P13 and LHC-P17 phosphorylation. Stigmatellin and DBMIB (added to preilluminated samples) blocked dephosphorylation of D2 that normally develops in State 2 conditions.

In contrast, we noted that several minor phosphoproteins in the 15–20-kDa region were detected in State 2 conditions even when LHC-P13 and LHC-P17 showed no significant increase in phosphorylation (Fig. 5). In particular, phosphorylation of the cytochrome b_6f subunit, subunit V (*sub V*) (31), was clearly detectable in the presence of both stigmatellin (Fig. 5*A*) and DBMIB (Fig. 5*B*). None of these polypeptides showed significant phosphorylation in State 1 conditions.

DISCUSSION

Relationship between Q_o Site Occupancy and LHCII Kinase Activation—This work further investigates the modulation of LHCII kinase activity by the interaction between plastoquinone (and its analogues) and the Q_o site of cytochrome b_6f complexes. In particular, it confirms previous observations that substitution of PQH_2 with other quinones resulted in the inhibition of the kinase activity (13, 16, 32), in agreement with the notion that binding of plastoquinol is essential for kinase activation (2, 3).

Since both activation and deactivation can be induced in the absence of light (see Figs. 1 and 2 and Ref. 25), we can conclude that the mere binding of a quinol at the Q_o site and not the function of cytochrome b_6f in electron transfer, is sufficient to activate the LHCII kinase. However, our study also suggests that the signal transducer for LHCII kinase activation is able to discriminate the redox state as well as the nature of the bound quinone. This observation argues for a requirement in a specific binding configuration for signal transduction. As an example of such discrimination, DBMIB allows or prevents kinase activation depending whether it is added in the dark (Fig. 2), *i.e.* bound to cytochrome b_6f complex in a reduced form, or in the light (Fig. 3), *i.e.* bound in a semireduced state (23, 33).

As a first explanation, one could consider two distinct binding domains within the Q_o site, the occupancy of which would either activate or inhibit the kinase. This possibility is consistent with previous studies on the Q_o site of bacterial cytochrome bc_1 complex where the binding of more than one quinone per Q_o site was proposed (34), and with the structure of the site, where two distinct (even if partially overlapping) quinone binding domains have been observed (17, 18). According to a recent model for cytochrome *bc* complexes activity (20), the occupancy of the two binding domains by quinones is not simultaneous

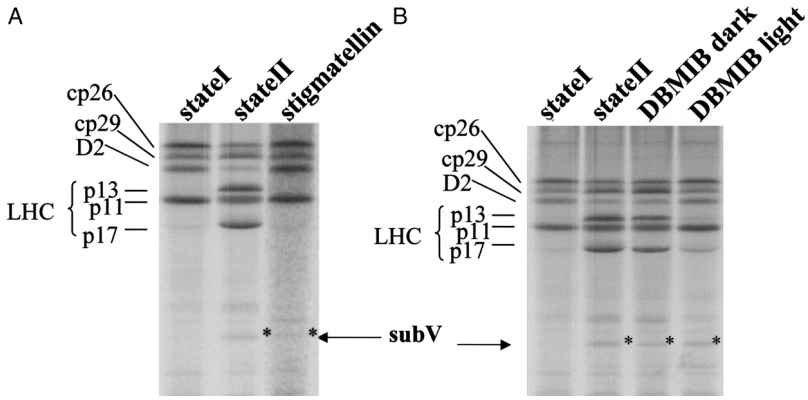


FIG. 5. **Autoradiogram of ^{33}P-radiolabeled antenna polypeptides in the 15–35-kDa region.** Cells were placed in State 1 or State 2 conditions as in Fig. 1. *A*, stigmatellin-treated cells; *B*, DBMIB-treated cells. Other conditions were the same as in Fig. 1.

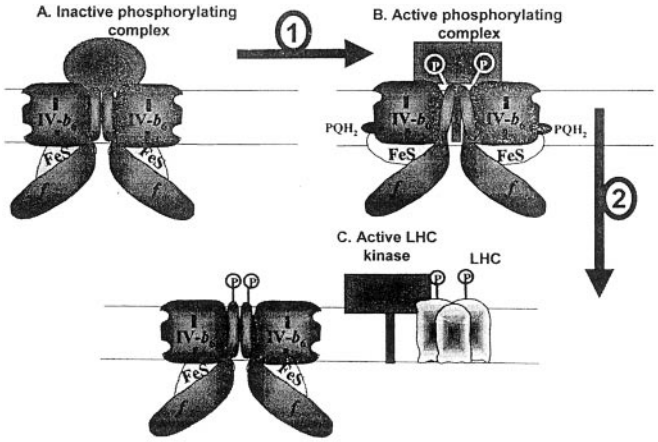
and is regulated by their redox state: they remain in the so called proximal (with respect to the Rieske subunit) domain in the reduced state and move to the distal one upon oxidation by the Rieske protein (17, 20).

The contrasting effects of PQH_2 (State 2 conditions) and PQ (State 1 conditions) on the LHCII kinase would then be explained assuming that only the proximal domain is activating. This hypothesis is also consistent with the differential effects of DBMIB reported here: reduced DBMIB would be activating in dark adapted cells, because it would occupy the proximal binding pocket, whereas it would become inhibitory upon translocation to the distal binding domain when converted to its semiquinone form by a preillumination (33). This hypothesis, however, does not account for the effect of stigmatellin, which inhibits State 2 transition (Fig. 1), although it occupies the proximal pocket of the Q_o site, as does PQH_2 (see *e.g.* Refs. 20 and 35).

We consider then an alternative hypothesis based on the recent discovery that the Rieske protein is a flexible molecule that can move from a distal (close to cytochrome *f*) to a proximal position (close to heme b_l) (17–20). Previous reports have suggested a relationship between quinol binding to the Q_o site and activation of the LHCII kinase in terms of the stabilization of one conformation of the FeS subunit by PQH_2 (13, 16). Unfortunately the data reported here are not consistent with this hypothesis either: on the one hand the requirement of quinol binding for kinase activation (Fig. 1, Refs. 13 and 15) argues against an activating role of the distal position of the FeS protein, which is observed in empty Q_o sites (17–20). On the other hand, the effect of stigmatellin, which blocks kinase activation (Figs. 2 and 3) but locks the Rieske protein in its proximal conformation (17–20), demonstrates that this conformation is also inhibitory. An involvement of the intermediate conformation (17) in LHCII kinase activation could also be considered. We consider this possibility rather unlikely, however, as such a conformation has also been observed in complexes that were devoid of quinone substrate (17).

A Dynamic Model for LHCII Kinase Activation—One of the major problems in understanding LHCII kinase activation is the mechanism by which the signal generated in the lumenal side of the cytochrome b_6f complex is transduced to the stromal side of the membrane, where kinase activity develops. Recent structural data on the cytochrome b_6f complex (19) support a mechanism whereby the occupation of the Q_o site by stigmatellin in transduced across the membrane through a conformational change not only in the lumenal-located head of the Rieske protein, but also in some transmembrane domains of the complex, in particular in those that are close to the monomer to monomer interface (19).

Still, neither the stigmatellin-bound state nor the empty state are competent for activation. We are thus led to suggest



SCHEME 1.

that a fixed conformation of the cytochrome b_6f complex is inappropriate for kinase activation. Activation may require a more dynamic situation that can be explained assuming that the activating state includes at least a two step process (Scheme 1): a signal transduction step, step 1 from (*A* to *B*), that involves the movement of the Rieske from the distal (empty site) to the proximal position (PQH_2-bound site). This switch induces changes of the whole cytochrome b_6f conformation that allow activation of the kinase through a change in protein/protein interaction. The next step, step 2 (from *B* to *C*), would be the relaxation of the Rieske at the distal position, that would release the activated kinase from the cytochrome b_6f complex and allow its interaction with its LHC substrates.

We consider the transmembrane subunit of the b_6f complex protein (subunit V) that can be reversibly phosphorylated in a redox-controlled way in *C. reinhardtii* (31), as being likely involved in the transition from step 1 to step 2. This possibility is supported by the significant phosphorylation of subunit V in State 2 conditions, even in the presence of stigmatellin and DBMIB when little if any phosphorylation of LHC-P13 and LHC-P17 is detected.

State Transitions and Binding Properties of Quinones in the Q_o Site—The dynamic model for kinase activation proposed above suggests a mechanism for recognition of quinones, which depends on their binding dynamics.

Binding of stigmatellin is of dead end type (22, 33). Thus, it is characterized by a very small unbinding rate (k_{off}), which is the consequence of its strong interaction (via hydrogen bonds) with the FeS protein in its proximal conformation (20). Its inhibition of LHCII kinase is therefore explainable assuming that it blocks the Rieske in one conformation, after the formation of the pre-active state (Scheme 1*B*).

Plastoquinol binding is apparently different from that of stigmatellin. Despite its interaction with the Rieske protein in the proximal position, with the same hydrogen bonding as stigmatellin (20, 36), it does not prevent kinase activation. Therefore it should not trap the Rieske protein in one conformation. Two possibilities can be proposed to explain this fact: (i) PQH_2 is not tightly bound to the Q_o site. It can be rapidly released (k_{off} higher than stigmatellin) from the cytochrome complex, leaving an empty site where the FeS protein is in its distal position. (ii) PQH_2 is also a tightly bound quinone (low k_{off} as for stigmatellin), but it does not interact firmly with the FeS protein, because it oscillates between the proximal and distal domains of the Q_o site, where it does not make hydrogen bonds with the FeS cluster. We believe that the first hypothesis is more likely, since PQH_2 and stigmatellin equally affect the EPR spectrum of the Rieske protein at low temperature (35), suggesting that they occupy similar positions within the Q_o site.

The binding properties of DBMIB depend on whether it is in its fully reduced state (addition in darkness) or in a semireduced state (addition in the light). In the former case it behaves as PQH_2, and its contribution to kinase activation can be explained following the same lines as above. In the semireduced state it behaves as stigmatellin, being an inhibitor of electron transport and kinase activation, although it presumably occupies the distal Q_o pocket instead of the proximal pocket. This result indicates either that the Rieske protein is also locked in the proximal position as long as a semiquinone resides in the Q_o site, as suggested by Iwata (17) or that $DBMIB^-$ occupies the proximal Qo domain, instead of the distal one, where it acts as a dead-end inhibitor. In both cases, upon its binding the fixed conformation of the Rieske prevents the dynamic activation of the LHCII kinase.

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