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 plastocyanin 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 pro-caryotes. 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 plastocyanin 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 plastocyanin (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 plastocyanin 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, plastocyanin; DBMB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3,4'-dichlorophenyl-1,1-dimethyleurea.

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have studied the effects on state transitions of two Qo site inhibitors of electron transfer in the cytochrome b6f complex. We have used stigmatellin, which blocks electron transfer in both the bc1 and b6f cytochrome complex (22) by fixing the iron sulfur protein in its proximal conformation (17–20). We have also used DBMIB, which inhibits cytochrome b6f but not bc1 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 Qo 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 µE 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 Herve 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. 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 cuvet. 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 × 10⁶ cells ml−1, were harvested and resuspended in a phosphate-depleted medium containing 1 µCi ml−1 ³²P. 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 Qo site of cytochrome b6f and the activation of the LHCII kinase, we have tested the effects of two quinone analogues on whole cells of Chlamydomonas reinhardtii: stigmatellin, which is effective on both bc1 and b6f cytochrome complexes; and DBMIB, which inhibits only cytochrome b6f 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 Qo site inhibitor completely prevented the quenching of fluorescence otherwise induced under conditions that promote State 2 (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. 1C).

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 it is a fluorescence quencher (27). However, it did not prevent State 1-State 2 transition in darkness (Fig. 2B, 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 b6f 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. 3A). While no difference was observed in stigmatellin-treated samples between dark and illuminated cells (compare Fig. 3B with Fig. 1B), DBMIB addition completely abolished kinase activation under these latter conditions (Fig. 3C). Its effect was reversible, provided that the light was switched off (Fig. 3D).

It is known that DBMIB and stigmatellin interact not only with cytochrome b6f complex, but also with PSII; in particular, the former reacts with the light-harvesting subunits of PSII,
where it acts as a Stern-Volmer quencher of fluorescence (27), while both inhibitors bind at the Qo site of PSI1, 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 PSI1, rather than to a deactivation of the LHCCI kinase, we have repeated the same experiments with the PSI1 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. 4B), while the latter was effective only on preilluminated samples (Fig. 4C).

In FUD7 cells, however, the transition to State 2 in light-treated cells was less pronounced than in dark adapted ones (Fig. 4A), 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 LHCCI kinase is modulated by the occupancy of the Qo site of cytochrome b6f. Therefore we performed an *in vivo* protein phosphorylation assay. Thylakoid membranes were purified from cells that were precultivated for 90 min with 33P and placed for 20 min in State 1 and State 2 conditions in a 33P-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 LHCCI polypeptides, LHCCI-13 and LHCCI-17, increased in State 2 as compared with State 1, whereas the PSI1 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 LHCCI-13 and LHCCI-17 was observed, which is typical of State 1 (12) (Fig. 5A). Thus, the LHCCI kinase was not activated by reducing conditions in the presence of this inhibitor. DBMIB had contrasted effects (Fig. 5B); when State 2 conditions were established in darkness, it did not prevent kinase activation, as judged from the high level of phosphorylation on LHCCI-13 and LHCCI-17. When State 2 conditions were established under illumination, DBMIB prevented most of LHCCI-13 and LHCCI-17 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 LHCCI-13 and LHCCI-17 showed no significant increase in phosphorylation (Fig. 5). In particular, phosphorylation of the cytochrome b6f subunit V (sub V) (31), was clearly detectable in the presence of both stigmatellin (Fig. 5A) and DBMIB (Fig. 5B). None of these polypeptides showed significant phosphorylation in State 1 conditions.

**DISCUSSION**

**Relationship between Qo Site Occupancy and LHCCI Kinase Activation**—This work further investigates the modulation of LHCCI kinase activity by the interaction between plastoquinone (and its analogues) and the Qo site of cytochrome b6f complexes. In particular, it confirms previous observations that substitution of PQH2 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 Qo site and not the function of cytochrome b6f in electron transfer, is sufficient to activate the LHCCI kinase. However, our study also suggests that the signal transducer for LHCCI 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 b6f 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 Qo site, the occupancy of which would either activate or inhibit the kinase. This possibility is consistent with previous studies on the Qo site of bacterial cytochrome bc complex where the binding of more than one quinone per Qo 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.
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 PQH₂ (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 Q₀ site, as does PQH₂ (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 b₆) (17–20). Previous reports have suggested a relationship between quinol binding to the Q₀ site and activation of the LHCII kinase in terms of the stabilization of one conformation of the FeS subunit by PQH₂ (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 Q₀sites (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 luminal side of the cytochrome b₆f complex is transduced to the stromal side of the membrane, where kinase activity develops. Recent structural data on the cytochrome b₆f complex (19) support a mechanism where the occupation of the Q₀ site by stigmatellin transduced across the membrane through a conformational change not only in the luminal-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 that a fixed conformation of the cytochrome b₆f 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 (PQH₂-bound site). This switch induces changes of the whole cytochrome b₆f 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 b₆f complex and allow its interaction with its LHC substrates.

We consider the transmembrane subunit of the b₆f 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 Q₀ 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 (kᵤₑₗ), 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 1B).
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 ($k_{\text{off}}$ 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 $k_{\text{off}}$ 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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