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The Qo site of cytochrome b_6f complexes controls the activation of the LHCII kinase

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We created a Qo pocket mutant by site-directed mutagenesis of the chloroplast *petD* gene in *Chlamydomonas reinhardtii*. We mutated the conserved PEWY sequence in the EF loop of subunit IV into PWYE. The *pwye* mutant did not grow in phototrophic conditions although it assembled wild-type levels of cytochrome b_6f complexes. We demonstrated a complete block in electron transfer through the cytochrome b_6f complex and a loss of plastoquinol binding at Qo. The accumulation of cytochrome b_6f complexes lacking affinity for plastoquinol enabled us to investigate the role of plastoquinol binding at Qo in the activation of the light-harvesting complex II (LHCII) kinase during state transitions. We detected no fluorescence quenching at room temperature in state II conditions relative to that in state I. The quantum yield spectrum of photosystem I charge separation in the two state conditions displayed a trough in the absorption region of the major chlorophyll *a/b* proteins, demonstrating that the cells remained locked in state I. $^{33}P_i$ labeling of the phosphoproteins *in vivo* demonstrated that the antenna proteins remained poorly phosphorylated in both state conditions. Thus, the absence of state transitions in the *pwye* mutant demonstrates directly that plastoquinol binding in the Qo pocket is required for LHCII kinase activation.

Keywords: *Chlamydomonas reinhardtii*/plastoquinol/Qo site/site-directed mutagenesis/state transitions

Introduction

Chloroplasts have an as yet undetermined number of protein kinases and phosphatases which catalyze the reversible phosphorylation of several thylakoid membrane proteins (for reviews see Allen, 1992; Gal *et al.*, 1997). Among these are the antenna proteins, light-harvesting complex II (LHCII), which reversibly associate with either photosystem I (PSI) or photosystem II (PSII) depending on their state of phosphorylation. Since the majority of the two photosystems are located in distinct thylakoid membrane regions, i.e. the grana and stroma lamellae domains (Albertsson, 1995), changes in LHCII phosphorylation cause a lateral migration of the antenna proteins along the thylakoid membranes. The displacement of LHCII antenna proteins has provided a molecular clue to the mechanism of short-term chromatic adaptation, known from the late 1960s as state transitions (Bonaventura and Myers, 1969; Murata, 1969). The picture that emerged from extensive studies, pioneered by Bennett and co-workers (Bennett, 1991), is that state I corresponds to a low phosphorylation state for LHCII, which is then functionally connected with PSII, whereas state II corresponds to an increased phosphorylation of LHCII (Allen, 1992), which then serves as a PSI antenna (Delosme *et al.*, 1994, 1996).

In vivo studies with the unicellular green alga *Chlamydomonas reinhardtii* have demonstrated that state transitions are also controlled by the intracellular demand for ATP: in the total absence of illumination, *C.reinhardtii* cells are locked in state II when the intracellular content of ATP is low, whereas they adopt a state I configuration when the ATP pool is restored (Bulte *et al.*, 1990). Since the PSI-containing domains of the thylakoid membranes display an increased content of both LHCII and cytochrome b_6f complexes in state II (Vallon *et al.*, 1991), this state can be regarded as a supramolecular organization of the photosynthetic apparatus favoring cyclic electron flow around PSI, a functional organization well suited to cope with an increased demand for ATP production.

The changes in the state of phosphorylation of antenna proteins result from the combined actions of an LHCII kinase, whose activation is redox dependent (Allen *et al.*, 1981), and a phosphatase that is considered permanently active (Elich *et al.*, 1997), although some recent data suggest that it may be regulated by its interaction with an immunophilin-like protein (Fulgosi *et al.*, 1998). Although far from being elucidated fully, studies on the mechanism of kinase activation have achieved significant progress over the years. Starting with the observation that an increased reduction of the plastoquinol pool correlated with kinase activation (Allen *et al.*, 1981; Horton and Black, 1981), a search for a specific role for known quinone-binding proteins from the thylakoid membranes led us to exclude that PSII was required for kinase activation *in vivo* (Wollman and Lemaire, 1988). In contrast, we observed that *C.reinhardtii* mutants lacking cytochrome b_6f complexes were in a state I configuration, and that they were unable to undergo transitions from state I to state II, even though the redox state of the plastoquinone pool could be poised to go from an oxidized to a fully reduced state (Lemaire *et al.*, 1987; Wollman and Lemaire, 1988). Similar conclusions were reached subsequently with several cytochrome b_6f mutants from higher plants (Coughlan *et al.*, 1988; Gal *et al.*, 1988). That the activation signal was transduced through the cytochrome b_6f complexes was supported further by the

presence of an LHCII kinase activity in partially purified cytochrome b_6f fractions (Gal *et al.*, 1990, 1992). However, the mechanism through which the redox poise is transduced to the kinase for its activation still remains obscure. Some insight on the process came with the recent studies of Vener and colleagues (Vener *et al.*, 1995, 1997) who found that a reversible acid-induced transient reduction of ~20% of the plastoquinone pool was sufficient to activate the kinase *in vitro*. They reported that kinase activation persisted even when the plastoquinone pool was fully reoxidized, provided that one single plastoquinol molecule was retained per cytochrome b_6f complex (Vener *et al.*, 1997). These observations led the authors to propose that kinase activation occurred as soon as one plastoquinol is available to the Qo site of cytochrome b_6f complexes that have a fully reduced high potential chain. However, this proposal conflicts with the absence of kinase activation *in vivo* in aerated cells of *C.reinhardtii*, although the fraction of reduced plastoquinone is sufficiently high to meet the criteria suggested by Vener and co-workers for state transition. Thus we took a different approach to investigate directly, by site-directed mutagenesis, the possible contribution of the Qo site to the activation of the LHCII kinase.

In cytochrome b_6f complexes, cytochrome b_6, subunit IV and the Rieske protein contribute residues to the formation of the Qo pocket. We have shown previously that the loops between helices C and D of cytochrome b_6 (Finazzi *et al.*, 1997) and helices E and F of subunit IV (Zito *et al.*, 1998) contribute to the formation of the Qo pocket in *C.reinhardtii*. The lumenal EF loop in subunit IV comprises a short sequence of four amino acids, PEWY, which is strictly conserved in all *bc*-type cytochrome complexes (Degli Esposti *et al.*, 1993). From crystallographic data, it has been possible to establish the position of the PEWY sequence with respect to heme b_l at a distance which allows van der Waals contacts. The side chains of the PEWY sequence contribute to the internal folding of the Qo pocket. We have shown previously that this region is indeed deeply involved in the function of the Qo site and plays a critical role in cytochrome b_6f turnover (Zito *et al.*, 1998). In the present work, we demonstrate that the PEWY region, unequivocally involved in Qo pocket formation, is strictly required for a functional binding of plastoquinol at the Qo site. Its alteration to a PWYE sequence abolishes both the binding of plastoquinone/plastoquinol and LHCII kinase activation. The resulting mutant is locked in a state I configuration.

Results

The pwye mutant is a non-phototrophic strain that accumulates cytochrome b_6f complexes

We have demonstrated that the glutamic residue in the PEWY sequence of the EF loop of subunit IV has a critical role in the turnover of the cytochrome b_6f complex (Zito *et al*., 1998) even though it is not strictly required for its function (Crofts *et al*., 1995). We further investigated by site-directed mutagenesis the contribution of the $_{77}$PEWY$_{80}$ sequence to the function of cytochrome b_6f complexes, carrying out permutation of its three last residues, which yields a $_{77}$PWYE$_{80}$ sequence.

Our first attempt to recover phototrophic clones from a transformation of the Δ*petD* strain, deleted for the *petD* gene, with plasmid pdD*pwye* proved unsuccessful. Therefore, the PWYE mutation is detrimental to photosynthesis. We then used the wild-type strain as a recipient for transformation with plasmid pdDK*pwye* which carries, in addition to the PWYE mutation, a selectable marker, the *aadA* cassette, that confers resistance to spectinomycin to the transformants (Goldschmidt-Clermont *et al*., 1991). With this strategy, we recovered several transformants that were non-phototrophic, which confirmed the detrimental character of the mutation.

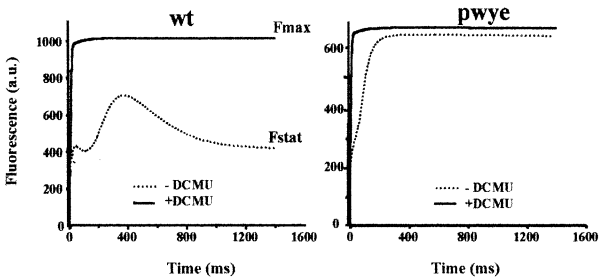

Fig. 1. Fluorescence induction curves of dark-adapted cells from wild-type and *pwye* mutant in the presence or absence of 10 μM DCMU. F_{max}, maximal level of fluorescence; F_{stat}, stationary level of fluorescence.

Photosynthesis mutants of *C.reinhardtii* can be classified easily according to their fluorescence induction pattern upon continuous illumination. Figure 1 (left panel) shows a typical induction curve for the wild-type strain of *C.reinhardtii*. It reaches a steady-state level (F_{stat}) well below the F_{max} level attained in the presence of DCMU, a PSII inhibitor that prevents reoxidation of the primary quinone acceptor by the plastoquinone pool. In contrast, the fluorescence yield of the *pwye* mutant increases continuously upon illumination (Figure 1, right panel), reaching the same level as that attained in the presence of DCMU. However, the fluorescence kinetics were much slower in the absence than in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). This is indicative of a block in electron transfer at the step of reoxidation of the plastoquinol pool (Delepelaire and Bennoun, 1978). In these circumstances, several turnovers of PSII occur before the plastoquinone pool is fully reduced, which eventually prevents reoxidation of the PSII primary quinone acceptor. Thus, the spectinomycin-resistant transformants that contained the modified PWYE sequence showed fluorescence induction kinetics typical of that of mutants lacking cytochrome b_6f (Lemaire *et al*., 1987; Kuras *et al*., 1997; Zito *et al*., 1997).

We then probed the content in three major cytochrome b_6f subunits in the *pwye* mutants. Two transformants are presented together with a wild-type control in Figure 2. Surprisingly, these mutants, although blocked in the reoxidation of the plastoquinol pool, still accumulated the major subunits of the cytochrome b_6f complex at about the wild-type level. In particular, the content of subunit IV, which bears the PWYE mutation, was unaltered in the mutants.

The cytochrome b_6f complex from the pwye mutant is unable to perform plastoquinol oxidation

In order to gain further insight into the step at which electron transfer through the cytochrome b_6f complex was

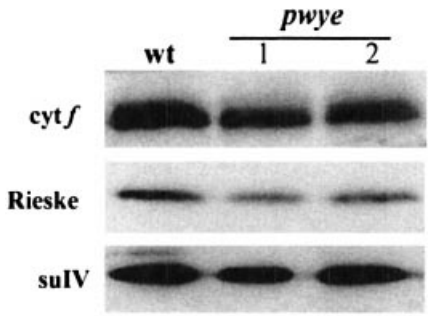

Fig. 2. Immunoblot of whole cell protein extracts probed with specific antibodies against cytochrome *f*, Rieske protein and subunit IV. Loads for each sample correspond to 20 μg of chlorophyll.

blocked in the mutants, we studied, by time-resolved flash spectroscopy, the cytochrome b_6f-related absorbance changes. Electron transfer through the cytochrome b_6f complex is coupled to a charge translocation across the membrane that is detected as the slow phase (phase b) of the 515 nm electrochromic shift (Joliot and Delosme, 1974). As previously reported (Finazzi *et al*., 1997), the $t_{1/2}$ of phase b, measured under anaerobic conditions using non-saturating flashes, is ~2.5 ms in the wild-type (Figure 3A). In the *pwye* mutant, the charge separation in the reaction centers can still be detected as the fast phase of the 515 nm electrochromic shift, which corresponds to the value of 1 on the ordinate scale of Figure 3A. No subsequent signal changes were detected in the millisecond time range where the cytochrome b_6f contribution occurs.

The absence of phase b is indicative of a loss of charge translocation across the membrane, i.e. of a loss of electron transfer in the low potential chain (the redox path comprising the b_l and b_h hemes). The Q cycle mechanism, as proposed by Mitchell (1975) and modified by Crofts *et al.* (1983), predicts that electron transfer into both the *f* and b_6 hemes occurs in a concerted step. Therefore, the lack of phase b could indicate either a specific block in the electron transfer step from the plastosemiquinone to cytochrome b_l or an impairment of the overall, concerted plastoquinol oxidation at the Qo site. To distinguish between these possibilities, we have measured the kinetics of the redox changes of cytochrome *f* (Figure 3B): the fast oxidation step retained similar kinetics in the wild-type and in the *pwye* mutant. However, its amplitude was larger in the mutant, due to the drastic decrease in the rate of cytochrome *f* re-reduction. The latter reaction was slower than in the wild-type by about three orders of magnitude. We previously have observed such a delayed re-reduction of cytochrome *f* in a mutant lacking the cytochrome b_6f complex but retaining a soluble form of cytochrome *f* (Kuras *et al*., 1995b). Therefore, we attribute the loss of cytochrome b_6f function in the *pwye* mutant to a complete block in the concerted electron transfer reaction from plastoquinol to cytochrome *f* and heme b_l.

The pwye mutant lacks plastoquinone/ plastoquinol binding at the Qo site of the cytochrome b_6f complex

In order to distinguish between a block in electron transfer from a bound plastoquinol to the Rieske protein and the absence of plastoquinol binding at the Qo site, the electron paramagnetic resonance (EPR) characteristics of the

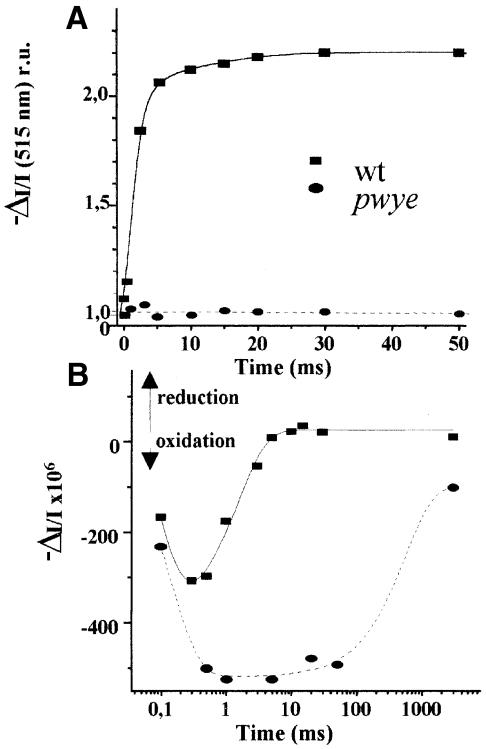

Fig. 3. Slow electrochromic phase (**A**) and time-resolved redox changes (**B**) of cytochrome *f* in wild-type and *pwye* mutant. Algae under anaerobic conditions were illuminated with non-saturating flashes (20% of saturation), given 6.6 s apart. Measurements were performed in the presence of 1 μM carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP).

Rieske center in the *pwye* mutant were examined. The EPR spectrum of the Rieske cluster and more specifically its g_x-trough has been reported to be sensitive to the redox state of the Qo site quinone in the cytochrome b_6f complex from spinach (Riedel *et al*., 1991). A similar effect has been observed previously with cytochrome bc_1 complexes from mitochondria (de Vries *et al*., 1979) and purple bacteria (Matsuura *et al*., 1983) as well as with cytochrome *bc* complexes from Gram-positive bacteria (Liebl *et al*., 1990). Although the exact position of the g_x-trough in the different redox states of the Qo site quinone varies between b_6f, bc_1 and the Gram-positive *bc* complex, the spectral alterations were produced consistently by the interaction of an oxidized quinone with the Rieske center (for a discussion, see Ding *et al*., 1992), whereas only very minor spectral differences were observed between an empty Qo site and a quinol-bound site.

The upper panel of Figure 4 shows EPR spectra of wild-type *C.reinhardtii* thylakoids under conditions where the FeS center is reduced but the plastoquinone pool was either oxidized (continuous line) or reduced (dotted line). The observed shift of the g_x-trough in *C.reinhardtii* corresponds well with what has been observed for spinach b_6f complex (Riedel *et al*., 1991). In addition to this shift, the appearance of a new FeS center with g_y at 1.93 is observed in *C.reinhardtii*. A more detailed characterization of the Rieske center and the g = 1.93 species in wild-type *C.reinhardtii* will be reported elsewhere (F.Baymann and W.Nitschke, in preparation). As can be seen in the lower panel of Figure 4, the g_x-trough of the *pwye* mutant was no longer sensitive to the redox state of the plastoquinone

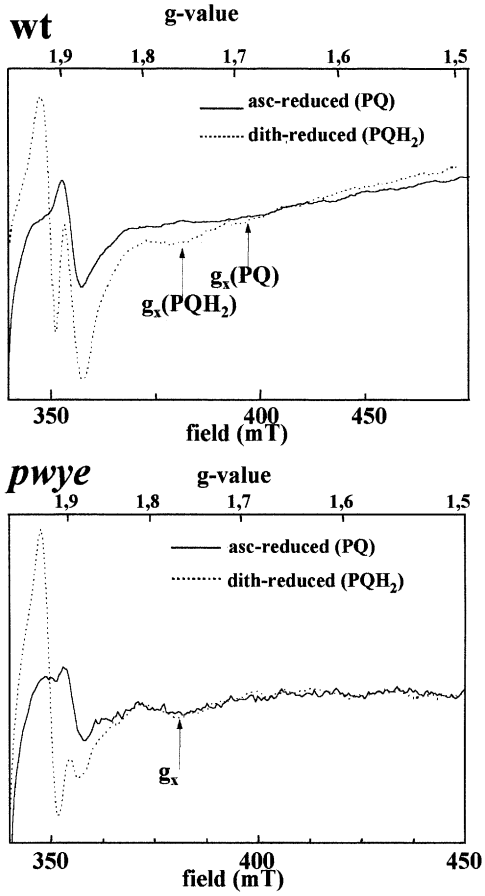

Fig. 4. EPR spectra of thylakoids from *C.reinhardtii* wild-type and *pwye* mutant. Samples were incubated in the presence of either 5 mM ascorbate (continuous lines) or 10 mM dithionite (dotted lines) in order to reduce the Rieske protein while keeping the plastoquinone pool either oxidized or reduced. Instrument settings: microwave frequency, 9.42 GHz; temperature, 15 K; microwave power, 6.3 mW; modulation amplitude, 1.6 mT.

pool. No signal corresponding to an interaction with a plastoquinone was observed. The g_x-trough remained in the position corresponding to an empty or plastoquinol-binding site in the wild-type. From these experiments, we conclude that the *pwye* mutant has lost its ability to bind plastoquinones. Since (i) the affinity of an intact Qo site for quinones and quinols is rather similar (Ding *et al.*, 1992) and (ii) there is no electron transfer from plastoquinol to cytochrome *f* and heme b_l in the mutant, we also conclude that the g_x-trough in the mutant points to an empty Qo site and not to a quinol-binding site. Thus the Qo site of the *pwye* mutant has lost its ability to bind both plastoquinone and plastoquinol molecules.

State transitions are abolished in the pwye mutant

The loss of plastoquinol binding at the Qo site of the cytochrome b_6f complex in the *pwye* mutant offered a unique opportunity to study the specific role of the Qo site in LHCII kinase activation.

We placed the *pwye* mutant in conditions that promote either state I or state II in a wild-type strain. In order not to depend on the photosynthetic electron transfer properties of the strains, the suitable conditions for each state were established in total darkness as previously described

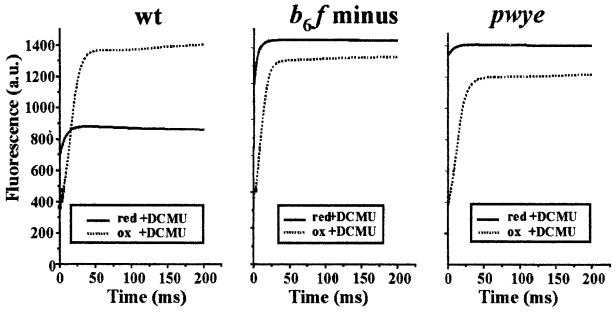

Fig. 5. Fluorescence induction curves, recorded in the presence of 10 μM DCMU, of wild-type, b_6f minus (deleted for the *petD* gene) and *pwye* mutant strains placed either in state I or in state II conditions. Conditions for state I: darkness under strong aeration; for state II: darkness in the presence of 20 mM glucose and 2 mg/ml glucose oxidase.

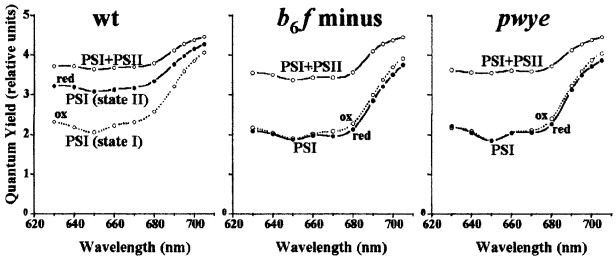

Fig. 6. Spectral dependence of the quantum yield of photochemistry measured by photoacoustic spectrometry in wild-type and mutant strains. PSI + PSII: untreated cells. PSI: quantum yield in the presence of 40 μM DCMU and 4 mM hydroxylamine. Ox and red refer to the redox state of the plastoquinone pool

(Wollman and Delepelaire, 1984): cells were placed either in oxidizing conditions by a strong aeration under vigourous stirring (state I conditions) or in reducing conditions by an incubation in the absence of oxygen (state II conditions). Figure 5 shows the fluorescence induction kinetics recorded in the presence of DCMU for three strains placed in state I or state II conditions. The *pwye* mutant was compared with the wild-type, here used as a positive control, and a cytochrome b_6f minus strain, the Δ*petD* strain, used as a negative control since it cannot undergo state transitions (Lemaire *et al.*, 1987; Wollman and Lemaire, 1988). The maximal fluorescence yield from the wild-type strain dropped by ~40% in state II as compared with state I, as expected from the transfer of a major fraction of LHCII from PSII to PSI, that acts as a strong fluorescence quencher (Bonaventura and Myers, 1969). In contrast, neither the cytochrome b_6f minus mutant nor the *pwye* mutant displayed a fluorescence quenching in state II conditions as compared with state I. Rather, the fluorescence yield increased in state II conditions, a phenomenon previously observed in strains locked in a state I configuration when the plastoquinone pool is fully reduced (Bulte and Wollman, 1990).

We then used a photoacoustic approach (Delosme *et al.*, 1994, 1996) as an independent tool to determine the distribution of the antenna pigments between the two photosystems in state I and state II. Figure 6 shows a quantum yield spectrum in the red region for the same three strains used in Figure 5. Differences in the efficiency of excitation transfer from the various pigment holochromes to the reaction centers appear as spectral variations of

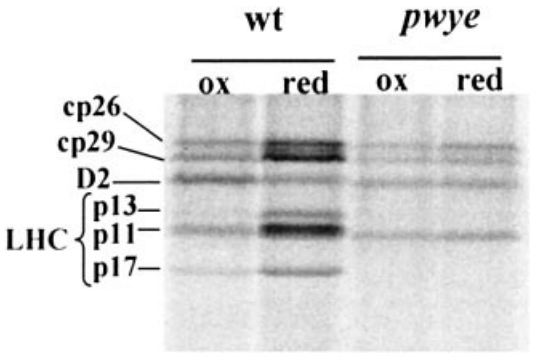

Fig. 7. Autoradiogram of ^{33}P-radiolabeled antenna polypeptides in the 25–35 kDa region. Cells were placed in state I (ox) or state II (red) conditions as in Figure 5.

the quantum yield of charge separation. Thus, an even connection to the reaction centers of all chlorophyll holochromes, which corresponds to a constant quantum yield, would produce a flat spectrum. The typical PSI + PSII spectrum provides a reference spectrum showing the state of connection of the light-harvesting antenna when the two types of reaction centers are active. In state I, the PSI spectrum from the wild-type, obtained by blocking PSII photochemistry by a pre-illumination in the presence of hydroxylamine and DCMU, displays a large drop in quantum yield in the absorbance region of LHCII (from 680 nm and below) which is consistent with the chlorophyll *a/b*-containing antenna being connected primarily to PSII centers. In state II, the PSI spectrum shows a much higher sensitization by the LHCII-associated pigments. It is close to the PSI + PSII spectrum, indicating that most of the antenna is now connected to PSI. In contrast to the wild-type situation, the quantum yield spectrum of PSI hardly changes between state I and state II conditions in both the cytochrome b_6f minus mutant and the *pwye* mutant. The four PSI spectra display a similar trough, peaking at 650 nm, which indicates a disconnection of LHCII from PSI, typical of a state I configuration.

The pwye mutant lacks LHC II kinase activation in state II conditions

The fluorescence and photoacoustic experiments with the *pwye* mutant both agree with the conclusion that LHCII is not transferred from PSII to PSI in state II conditions. In order to assess whether the block in a state I configuration originates from a lack of kinase activation, we performed an *in vivo* protein phosphorylation experiment. Thylakoid membranes were purified from cells that were pre-incubated for 90 min with $^{33}P_i$ and placed for 20 min in state I and state II conditions in a $^{33}P_i$-free medium as previously described (Wollman and Delepelaire, 1984).

Figure 7 shows an autoradiograph of an electrophoretogram from the *pwye* mutant and the wild-type that displays the labeling pattern of the thylakoid membrane polypeptides in the 25–40 kDa region. In the wild-type, the phosphorylation of all phosphopolypeptides that correspond to antenna proteins, CP26, CP29 and LHCII, increases drastically in state II as compared with state I, whereas the PSII phosphoprotein D2 shows an opposite behavior as we reported previously (Delepelaire and Wollman, 1985). In contrast, the *pwye* mutant displays a low and constant level of phosphorylation on CP26, CP29, D2 and LHC-P11, whatever the state conditions. We also noted the absence of phosphorylation of LHC-P13 and LHC-P17 in *pwye*, which is typical of a mutant locked in state I (Wollman and Lemaire, 1988). Thus, the LHCII kinase cannot be activated in reducing conditions in the *pwye* mutant.

Discussion

PEWY and PWYE structures in the Qo site

The PEWY to PWYE conversion in the EF loop of subunit IV, which is positioned on the lumenal side of the thylakoid membrane, led to a full inactivation of the electron transfer through the cytochrome b_6f complex, without altering the assembly of its constitutive subunits. Thus a fully inactive protein could accumulate to wild-type amounts in the thylakoid membrane from *C.reinhardtii*. This is an unprecedented phenotype since the other cytochrome b_6f mutants isolated thus far were either defective in the assembly of this oligomeric protein or only partially altered in their electron transfer properties (Wollman and Lemaire, 1988; Finazzi *et al.*, 1997; Zito *et al.*, 1997, 1998). The experiments we describe here show that the loss of function is caused by a loss of the ability of plastoquinol to bind to the Qo pocket of the protein complex.

The PEWY motif, as well as most of the other residues that are close to the Qo site of cytochrome bc_1, is conserved in all cytochrome bc_1/b_6f complexes (Degli Esposti *et al.*, 1993). Since the homology extends well beyond this region, it is possible to resort to the X-ray structure of cytochrome bc_1. Indeed, the structure of chicken and bovine mitochondrial cytochrome bc_1 have been determined independently in the presence of various inhibitors by three different groups (Xia *et al.*, 1997; Iwata *et al.*, 1998; Zhang *et al.*, 1998). Comparison of this region shows a similar conformation of the Qo pocket for the different structures [Protein Data Bank accession Nos 1bcc, 3bcc (Zhang *et al*., 1998); 1bgy (Iwata *et al.*, 1998); 1qcr (Abola *et al.*, 1997; Xia *et al.*, 1997)]. Figure 8 shows a view of the PEWY region with respect to the b_l heme. The proline, glutamate and tyrosine residues are lining the bottom of the Qo pocket, whereas the tryptophan is facing toward the exterior of the protein. The proline occupies a key position which splits the bottom of the Qo pocket into two parts which are directed toward either the high or the low potential chain: it is able to interact with the inhibitor stigmatellin in the vicinity of the Rieske protein in its proximal position (see 3bcc) and with the inhibitor myxothiazol, which is directed toward the heme and also interacting with the glutamate (Iwata *et al.*, 1998). The tyrosine also lies in the vicinity of the b_l heme. Therefore, the residues from the PEWY sequence are likely to provide the steric constraints for a proper positioning of the plastoquinol at the bottom of the Qo pocket, and the permutation of the (P)EWY residues to (P)WYE should induce severe perturbations in this region. If we assume that the polypeptide chain is not undergoing a drastic reorganization, we can infer that the bulky side chain of the tryptophan should hinder proper interactions between the plastoquinol and the b_l heme. On the other hand, the glutamate residue, whose carboxylic group was facing

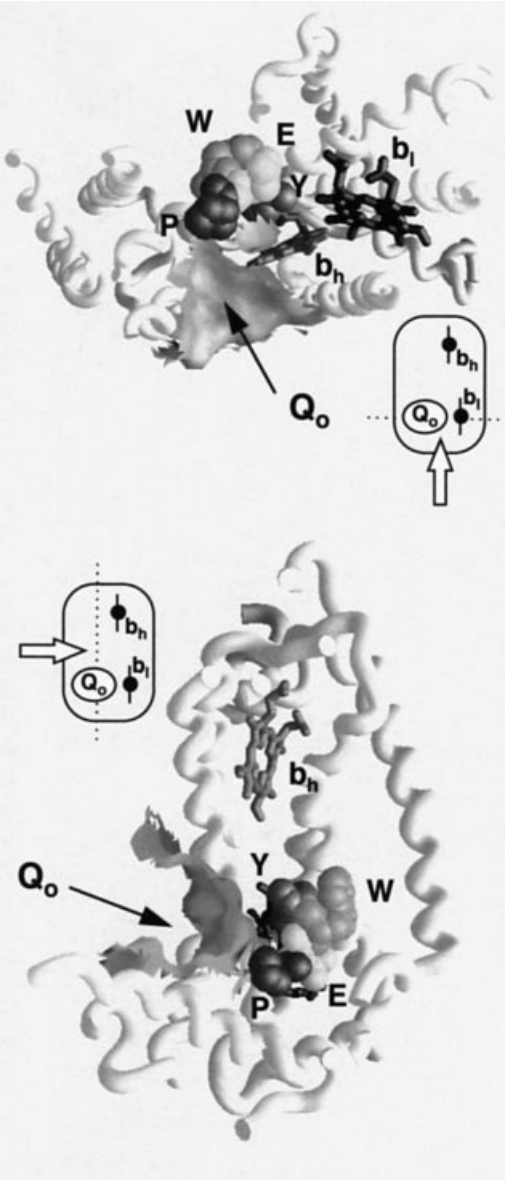

Fig. 8. Localization of the PEWY residues (shown by van der Waals spheres) relative to the Qo pocket in the cytochrome *b* subunit. The figure is drawn with grasp (Nicholls *et al.*, 1991), with the coordinates 1bcc from the PDB (Zhang *et al.*, 1998). The top figure represents a view from the lumenal site (depicted by the hollow arrow) with a cut at the level of the Qo pocket (dotted line). The proline (in black) splits the bottom of the pocket into: (i) on its left, a small opening toward the Rieske protein (not shown) in its proximal orientation and (ii) on its right, a narrow, less defined, side pocket (formed in part by the glutamate and the tyrosine, respectively in white and light gray) which is directed toward heme b_l. This pocket will be targeted primarily by the PWYE mutation. The bottom figure is viewed from the membrane (depicted by the hollow arrow) with a cut roughly normal to the membrane plane (dotted line). The tryptophan residue and the carboxylic group of the glutamate are localized at the surface of the cytochrome *b* on the face opposite to the entrance of the Qo pocket. The heme b_l is hidden behind PEWY.

outside the protein not far from the heme, is now directed toward the pocket and should induce there steric and electrostatic perturbations. This configuration accounts fairly well for the loss of plastoquinol binding in the *pwye* mutant, as documented in the present study by our EPR and visible spectroscopy analysis.

Consequences of the PEWY to PWYE conversion on LHCII protein kinase activation

A loss of plastoquinol binding at the Qo site offers a unique opportunity to check whether this site is actually part of the kinase activation process that leads to state transitions *in vivo*. Indeed, we observed that the *pwye* mutant showed no increased protein phosphorylation in reducing conditions and was blocked in a state I configuration. The block in state I cannot be ascribed to some undirect effect due to the inability of the cytochrome b_6f complexes to sustain electron flow in the *pwye* mutant since the transitions were performed in darkness, in conditions where cytochrome b_6f complexes do not participate in electron transfer (Bennoun, 1983). Thus our data demonstrate that kinase activation requires quinol binding at the Qo site. The fact that the phosphorylation pattern of the *pwye* mutant was identical to that in strains that lack the cytochrome b_6f complex, with a typical loss of phosphorylation of LHC-P13 and LHC-P17 and a low and constant phosphorylation of LHC-P11, CP29 and CP26, shows that the bands that remain phosphorylated in the mutant originate from a kinase activity that is distinct from that of the regulated LHCII kinase (Wollman and Lemaire, 1988). The loss of inducible phosphorylation of the antenna protein correlated with a lack of fluorescence quenching in state II conditions. Thus no antenna pigments became detached from PSII in state II conditions, as further substantiated by the quantum yield spectrum of PSI, which showed no increased contribution in the absorbance region of LHCII in state II conditions as compared with state I conditions.

LHCII protein kinase activation under physiological conditions

Our study supports the conclusion drawn by Vener and colleagues (Vener *et al.*, 1995, 1997) that was based on *in vitro* experiments performed with spinach thylakoids These authors used an acid shift from pH 7.4 to 4.3 to switch the plastoquinone pool from a fully oxidized state to a partially reduced state. Since the kinase is not active in acidic conditions, they resorted to a reverse pH shift to pH 7.4 to observe kinase activation. In the latter case, the plastoquinone pool was reoxidized rapidly but kinase activity was retained as long as a plastoquinol remained bound to the cytochrome b_6f complex at the Qo site. Flash-induced reoxidation of the bound plastoquinol by PSI deactivated the LHCII kinase. These experiments thus argued for a critical role for a bound plastoquinol at Qo in kinase activation *in vitro*. They also pointed to a much higher affinity of the Qo site for plastoquinol than has been suggested in several other studies (Ding *et al.*, 1992; Kramer *et al.*, 1994; Finazzi *et al.*, 1997). With such a high affinity, living algae such as *C.reinhardtii* would be permanently in state II since the plastoquinone pool is partially reduced even when the cells are kept in aerobic conditions and darkness, owing to the continuing electron flow due to chlororespiration (Bennoun, 1982). This is not observed: *C.reinhardtii* cells are much closer to state I than to state II *in vivo*, under aerobic conditions. An extensive increase in plastoquinone reduction, such as a shift to anaerobic conditions or the use of uncouplers to activate glycolysis, is required to produce kinase activation

and transition to state II (Wollman and Delepelaire, 1984; Bulte *et al.*, 1990).

In cytochrome *bc* complexes, that are highly homologous to cytochrome b_6f complexes, the Rieske protein recently has been demonstrated to undergo a conformational change between at least two positions (Iwata *et al.*, 1998; Zhang *et al*., 1998), one close to the membrane surface next to heme b_1 (hereafter referred to as the position proximal to Qo), the other extending more in the lumen next to heme c_1 (respectively *f*) (hereafter referred to as the position distal to Qo). According to the model recently proposed by Crofts and Berry (1998), the functional turnover of a cytochrome *bc* complex requires the movement of the Rieske protein between the proximal and distal positions. Thus, under physiological conditions, the Rieske protein is expected to oscillate between these two positions.

A model for kinase activation taking into account the movement of the Rieske protein has been proposed recently by Vener *et al.* (1998). It suggests that the Rieske protein, in its proximal position, inhibits the LHC kinase via its interaction with a putative transmembrane segment of the kinase on the lumenal side of the membranes. We favor an alternative view based on the pH titration of the conformation of the Rieske protein performed by EPR in several cytochrome *bc* complexes (M.Brugna and W.Nitschke, in preparation). It was observed consistently that acidic pHs favored the proximal configuration of the Rieske protein. We suggest that the very acidic conditions, pH 4.3, used by Vener *et al*. (1995) in their *in vitro* experiments, with a slow re-equilibration of the lumenal pH when the external pH is raised again, have increased the affinity of the Qo site for plastoquinol because of the stabilization of the Rieske protein in its proximal position, thereby favoring kinase activation. *In vivo*, where the lumenal pH is only ~5.5 (Finazzi and Rappaport, 1998), plastoquinols and plastoquinones would exchange too rapidly for kinase activation to occur.

We conclude that the triggering signal for kinase activation on the stromal face of the thylakoid membranes is the binding of a plastoquinol molecule at Qo. We suggest that the presence of a quinol at the Qo site would favour the positioning of the Rieske protein in its proximal position provided it does not exchange too rapidly with a quinone that would favor relaxation of the Rieske protein to its distal position. Two physiological conditions could lead to this situation, appropriate for LHCII kinase activation: (i) highly reducing conditions, which place most of the plastoquinone pool in a reduced state, or (ii) a large acidification of the lumen compartment, which increases the affinity of the Qo site for plastoquinol by stabilizing the Rieske protein in its proximal position. The N-terminal transmembrane helix of the Rieske protein may then play a critical role in signal transduction for kinase activation. The crystal structure of cytochrome *bc* complexes reveals that the stromal N-terminal segment of the Rieske protein interacts tightly with the core subunits (Xia *et al.*, 1997; Iwata *et al.*, 1998; Zhang *et al.*, 1998). Since there are no such core subunits in cytochrome b_6f complexes, the N-terminus of the Rieske protein may be available for a reversible interaction with other types of proteins, among which the kinase stands as a prominent candidate.

Materials and methods

Cell growth conditions

Wild-type strain (mt+) derived from the strain 137C and transformants were grown on Tris acetate–phosphate (TAP) or minimum media, pH 7.2 at 25°C under 6 and 60 $\mu E/m^2/s$ of continuous illumination, respectively. Wild-type and mutant cells were placed in state I and state II conditions in darkness, either by vigorous stirring to ensure a strong aeration (state I) or by an incubation in anaerobic conditions, upon addition of glucose and glucose oxidase (state II) (Wollman and Delepelaire, 1984). State II conditions could be obtained similarly by adding 5 μM FCCP to aerobic cells in the dark (data not shown).

Mutagenesis and plasmids

Plasmid pdWQ (Kuras and Wollman, 1994), which encompasses the whole *petD*-coding region, was used to perform site-directed mutagenesis according to the method of Kunkel (1985). PdWQ single strand was used to anneal the mutagenic oligonucleotide PWYE 5′-TAATACAGGGTAGAA**TTC**A**T**A**CC**ATGGTAAAATTTCAAG-3′ leading to the plasmid pdD*pwye*. Letters in bold indicate the mutated nucleotides, while a new *Eco*RI restriction site, used for restriction fragment length polymorphism (RFLP) analysis, is underlined. Plasmid pdDK*pwye* was constructed by introducing the 1.9 kb *Sma*I–*Eco*RV fragment of plasmid pUC-atpX-AAD containing the *aadA* cassette (Goldschmidt-Clermont *et al.*, 1991) in the same orientation as the *petD* gene in the *Eco*RV site of plasmid pdD*pwye*.

Chloroplast transformation in C.reinhardtii

The Δ*petD* strain, bearing a deletion of the *petD* gene, and wild-type strains (Kuras *et al*., 1995a) were transformed by tungsten particle bombardment according to Boynton *et al.* (1988) using a device, operating under vacuum, built in the laboratory according to Takahashi *et al.* (1996). At first we used pdD*pwye* to bombard the non-photosynthetic Δ*petD* strain and transformants were selected on minimum medium at 60 $\mu E/m^2/s$. We then used plasmid pdDA*pwye* to bombard the wild-type strain. Transformants were selected on TAP medium for the expression of the *aadA* cassette in the presence of 100 μg/ml of spectinomycin.

Protein isolation, separation and analysis

Whole cells, grown to a density of 3×10^6 cells/ml, were resuspended in 100 mM dithiothreitol and 100 mM Na_2CO_3 and solubilized in the presence of 2% SDS at 100°C for 1 min. Polypeptides were separated by denaturing SDS–PAGE (8 M urea, 12–18% acrylamide). Protein analyses were performed by immunoblotting, using specific antibodies against cytochrome b_6f complex subunits as described in Kuras and Wollman (1994).

In vivo phosphorylation of antenna proteins

Cells grown at 3×10^6 cells/ml were harvested and resuspended in a phosphate-depleted medium containing 1 μCi/ml of $^{33}P_i$. Then they were treated as described in Wollman and Delepelaire (1984).

Fluorescence measurements

Fluorescence measurements were performed at room temperature on a home-built fluorimeter, using a light source at 590 nm. The fluorescence response was detected in the far red region in the near IR region.

Absorption spectroscopy

Cells were collected during the exponential phase of growth (2×10^6 cells/ml) and resuspended in HEPES-NaOH 20 mM pH 7.2 in the presence of 10% Ficoll to avoid cell sedimentation. Spectroscopic measurements were performed at room temperature with a home-built spectrophotometer described by Joliot *et al.* (1980) and modified as in Joliot and Joliot (1984). The slow phase of the electrochromic signal (phase b according to Joliot and Delosme, 1974), which is associated with electron transfer through the cytochrome b_6 hemes, was measured at 515 nm, where a linear response is obtained with respect to the transmembrane potential (Junge and Witt, 1968). Deconvolution of the b phase from the membrane potential decay was performed as described in Zito *et al*. (1998). Cytochrome *f* redox changes were also calculated essentially as described in Zito *et al.* (1998). All measurements were performed in the presence of 1 μM FCCP, to collapse the transmembrane electrochemical proton gradient (Joliot and Joliot, 1994)

Photoacoustic spectroscopy

The quantum yield spectrum of PSI or PSI + PSII was recorded in both state I and state II conditions, as described by Delosme *et al.* (1994, 1996).

EPR measurements

EPR spectra were recorded on broken thylakoids of both the mutant and wild-type strains of *C.reinhardtii* using a Bruker ESP300e X-band spectrometer fitted with an Oxford Instruments He-cryostat and temperature control system. Samples were reduced by 5 mM ascorbate or 20 mM dithionite and incubated in darkness for 2 min prior to freezing.

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