Redox-Coupled Proton Pumping Activity in Cytochrome b_6f, As Evidenced by the pH Dependence of Electron Transfer in Whole Cells of *Chlamydomonas reinhardtii*†

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ABSTRACT: The pH dependence of cytochrome b_6f catalytic activity has been measured in whole cells of the green alga *Chlamydomonas reinhardtii* over the 5−8 range. An acid pH slowed the reactions occurring at the lumenal side of the complex (cytochrome b_6 and *f* reduction) and affected also the rate and amplitude of the slow electrogenic reaction (phase b), which is supposed to reflect transmembrane electron flow in the complex. On the other hand, a direct measurement of the transmembrane electron flow from the kinetics of cytochrome b_6 oxidation revealed no pH sensitivity. This suggests that a substantial fraction of the electrogenicity associated with cytochrome b_6f catalysis is not due to electron transfer in the b_6 hemes but to a plastoquinol-oxidation-triggered charge movement, in agreement with previous suggestions that a redox-coupled proton pump operates in cytochrome b_6f complex. The pH dependence of cytochrome b_6f activity has also been measured in two mutant strains, where the glutamic 78 of the conserved PEWY sequence of subunit IV has been substituted for a basic (E78K) and a polar (E78Q) residue [Zito, F., Finazzi, G., Joliot, P., and Wollman, F.-A. (1998) *Biochemistry 37,* 10395−10403]. Their comparison with the wild type revealed that this residue plays an essential role in plastoquinol oxidation at low pH, while it is not required for efficient activity at neutral pH. Its involvement in gating the redox-coupled proton pumping activity is also shown.

The cytochrome b_6f complex is a central component of the photosynthetic chain, which transfers electrons from photosystem II (PSII)[1] to photosystem I (PSI). It comprises four major subunits (*1*, *2*): the Rieske protein, which binds an Fe_2S_2 cluster ($E_m = +290$ mV; *3*), a *c*-type cytochrome, cytochrome *f* ($E_m = +330$ mV; *2*), a *b*-type cytochrome, cytochrome b_6, which binds high- and low-potential hemes, b_h and b_l ($E_m = -84$ and -158 mV, respectively; *2*), and subunit IV, all of which are involved in the catalytic activity. In addition, the complex contains several minor subunits of small molecular weight (*4*), probably involved in complex stabilization.

As a member of the *bc*-type proteins, the cytochrome b_6f complex couples proton- and electron-transfer reactions: it catalyzes the oxidation of a lipophilic two-electron donor (plastoquinol, PQH_2, generated by PSII) and the reduction of a hydrophilic one-electron acceptor protein (plastocyanin, Pc, oxidized by PSI). According to the "Q cycle" model (*5*), the most widely accepted model for *bc* complex catalysis, electron transfer operates through a high-potential chain, formed by the Rieske protein and cytochrome *f*, and through a low-potential chain, composed of the two b_6 hemes (b_l and b_h). This mechanism, as modified by Crofts et al. (*6*), postulates both an oxidation and a reduction of plastoquinol at two distinct sites of the protein, the Qo and Qi sites, on opposite sides of the membrane. The oxidation of plastoquinol at the Qo site is associated with the reduction of both cytochrome *f* and b_l (*7, 8*) and the release of two protons into the lumen. Two in-series turnovers of the complex are required to reduce both the b_l and b_h hemes and trigger the generation of a PQH_2 molecule at the Qi site.

The recent elucidation by X-ray crystallography of the structure of the respiratory analogue of the cytochrome b_6f, the bc_1 complex (*9*−*11*), has suggested a mechanism for plastoquinol oxidation at the Qo site (see ref *12* for a review). It has been reported that the extramembrane domain of the Rieske protein assumes different conformations with respect to the transmembrane part of the complex (*10*−*12*). The same movement has been later observed also in the case of the cytochrome b_6f complex (*13*). In addition, the displacement of the quinone molecule between a proximal and a distal end of the Qo site has also been hypothesized on the basis of the distinct positions of residues responsible for the

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[1] Abbreviations: b_h, high-potential heme of cytochrome b_6; b_l, low-potential heme of cytochrome b_6; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenyl ether of iodonitrothymol; E_m, redox midpoint potential; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HA, hydroxylamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; Pc, plastocyanin; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; Qi, quinol reducing site of the cytochrome b_6f complex; Qo, quinol oxidizing site of the cytochrome b_6f complex.

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resistance to different inhibitors (*12*). This has led to the proposal of a "structural basis" for the Q cycle (*12*), which assumes that the quinol and the oxidized Rieske protein are situated close together at the Qo site upon binding to the cytochrome complex. After the transfer of an electron and a proton to the FeS cluster, they move apart to reduce the b_l and the c_1 (or *f*) heme, respectively. At some point during this reaction, a second proton is released from the semiquinone to a nearby recipient amino acid. The crystallographic structure of the bc_1 complex has suggested that the glutamic residue of the conserved PEWY sequence (E272 of cytochrome *b* in the bc_1, E78 of subunit IV in the b_6f) might be the acceptor of the proton delivered by the semiquinone (*14*).

In a previous analysis of mutants of *Chlamydomonas reinhardtii*, it has been demonstrated that the removal of this residue (*15*) does not affect the rate of PQH_2 oxidation nor its coupling to proton release at neutral pH. On the contrary, its substitution for a polar (strain E78Q) or a basic (strain E78K) residue (*15*) modified the amplitude of the electrogenic reactions associated with cytochrome b_6f activity. This suggested that this amino acid is involved in the regulation of the stoichiometries of charges translocated in the membrane per cytochrome b_6f catalytic cycle (*15*).

To further understand the role of glutamic 78 in the electron- and proton-transfer coupling in cytochrome b_6f, the pH dependence of the b_6f complex activity has been measured in the wild type and in the two mutants mentioned above (*15*). The comparative analysis of the three strains indicates the role of E78 in the pH sensitivity of PQH_2 oxidation and its participation in a likely redox-coupled proton pumping activity within the complex.

MATERIALS AND METHODS

Cell Growth Conditions. The wild-type strain (mt +), derived from strain 137C, and transformants were kindly provided by Francesca Zito (UMR 7099, CNRS, France). They were grown on Tris−acetate phosphate (TAP) medium (*16*) at 25 °C under 60 μEinsteins m^{-2} s^{-1} of continuous illumination. Cells were collected during the exponential phase of growth ($\sim 2 \times 10^6$ cells mL^{-1}).

Spectroscopic Analysis. Spectroscopic measurements were performed at room temperature with a home-built spectrophotometer, as described in Joliot et al. (*17*). All measurements were performed in anaerobic conditions to reach the full reduction of the plastoquinone pool in the dark (*18*). To this end, algae were kept under an argon flux in a large reservoir, connected to the measuring cuvette. Actinic flashes were provided by a xenon lamp (3 μs duration at half-height) filtered through a red filter (Schott RG 695). Flashes were of nonsaturating intensity (hitting 20% of the centers) to avoid multiple turnovers of cytochrome b_6f complexes and the generation of a substantial light-induced ΔpH.

The photoinduced transmembrane potential was estimated by the amplitude of the electrochromic shift, as the difference between 515 and 545 nm, which yields a linear response with respect to membrane potential (*19*). In green algae, the kinetics of this signal display four phases (*20*): The first is a fast rising signal (phase a), completed in less than 1 μs and associated with charge separation within PSI and PSII reaction centers. This is followed by a slower rising phase (phase b) in the millisecond time range, which reflects cytochrome b_6f activity. In addition, two exponential decay phases (c and d) are also observed (*20*), which are due to the ion flux though the $CFo-F_1$ ATP synthase complex and to the passive leak through thylakoid membranes, respectively.

Kinetic analysis of phase b requires deconvolution of the membrane potential decay. To this end, the latter phenomenon was assumed to be first-order kinetics. This is justified by the fact that most of the ATPase−ATP synthase complexes are inactive under the conditions employed here, and passive charge leak through the membrane (phase d) largely prevailed over channel-mediated ion flux. After having calculated the rate constant of membrane potential decay for each experimental curve, the kinetics of phase b were deconvoluted assuming that the rate of the membrane potential decay was linearly related to its amplitude. The utilization of a multiexponential decay did not improve the deconvolution procedure, in agreement with the lack of any ATPase-mediated conductance activity, as stated above.

Cytochrome b_6 redox changes were evaluated as the difference between absorption at 564 nm and a baseline drawn between 545 and 573 nm. Cytochrome *f* redox changes were measured as the difference between absorption at 554 nm and the same baseline. A small correction for the contribution of the electrochromic shift (5% of the signal at 515 nm) was applied (*15*).

pH Equilibration. When the pH dependence of the cytochrome b_6f turnover rate was measured, algae were incubated for 30 min with permeant buffers (sodium acetate, 30 mM, in the pH 4.5−6 range or sodium imidazole, 30 mM, in the pH 6−7.5 range). Small amounts of the protonophore FCCP (1 μM) were added to facilitate pH equilibration between the cellular compartments. Ficoll (10% w/v) was also added to avoid cell sedimentation. This procedure is essentially the same as that already employed in the case of *Chlorella sorokiniana* (*21*), with the exception that imidazole was preferred to phosphate in the pH 6−7.5 range, as the former proved to be more effective in the case of *C. reinhardtii*.

RESULTS

To better understand the role of the glutamic 78 of subunit IV in the coupling between electron and proton transfer at the Qo site of the cytochrome b_6f complex, the pH dependence of the single reaction steps catalyzed by this complex was measured in whole cells of *C. reinhardtii.* Three strains were studied: the wild type and two mutants, where the residue has been changed to a glutamine (E78Q) or a lysine (E78K) (*15*).

Wild Type. The results obtained in wild-type cells are reported in Figure 1. There, signals relative to phase b (A) and to the light-induced redox changes of cytochrome *f* (B) and of cytochrome b_6 (C and D) are shown as a function of pH over the 5−8 range. Incubation in more acid or more basic pHs was detrimental for *C. reinhardtii* cells (not shown). The traces are representatives of reactions occurring at different sites of the complex: phase b and the oxidation of cytochrome b_6 are indicative of reduction of a plastoquinone molecule at the Qi site (see below, however). Cytochrome *f* and b_6 reduction are indicative of the rate of

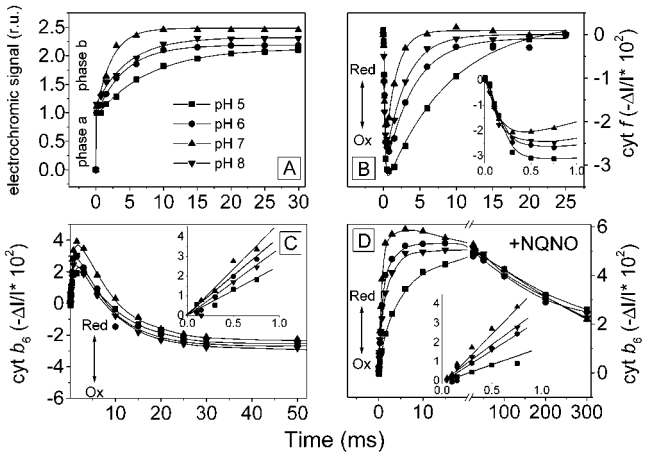

FIGURE 1: Kinetics of the electrochromic signal (A) of cytochrome *f* (B) and b_6 (C, D) redox changes as a function of the absolute pH value in *Chlamydomonas*. Cells were collected in exponential phase and incubated for 30 min with permeant buffers (sodium acetate, 30 mM, in the pH 4.5−6 range or sodium imidazole, 30 mM, in the pH 6−7.5 range). Small amounts of the protonophore FCCP (1 μM) were also added to facilitate pH equilibration between the cellular compartments. Ficoll (10% w/v) was present to avoid cell sedimentation. Actinic flashes were of nonsaturating intensity (20% of saturation). Phase a and phase b represent charge separation within PSI and cytochrome b_6f activity, respectively. Traces in panel D refer to measurements performed in the presence of the Qi site inhibitor NQNO. The inset in panel B shows the oxidation kinetics of cytochrome *f* on a smaller time scale. Those of panels C and D show a linear fit of the reduction kinetics of cytochrome b_6 on a smaller time scale. Traces represent the best fit to data points.

PQH_2 oxidation at the Qo site, while cytochrome *f* oxidation reflects the dynamics of electron flow between this subunit and Pc.

PSII absorption changes were prevented by a preillumination of the samples in the presence of DCMU, 10 μM, and HA, 1 mM (*22*). Under these conditions, phase a was generated by the sole PSI charge separations, and the amplitude of phase b normalized to phase a (panel A) became indicative of the amount of charges translocated across the membrane as a result of a single catalytic cycle of the cytochrome b_6f complex (*18*).

The oxidation and the reduction phases (decreasing and increasing signals, respectively) were reasonably resolved in the case of cytochrome *f* redox changes (B), owing to their relevant kinetic difference (~1 order of magnitude). On the contrary, they were much more superimposed in the case of cytochrome b_6 (C), where the reduction phase was largely masked by the oxidation one. To improve its evaluation, cells were treated with NQNO (D), an inhibitor of the Qi site (*23*). This compound slows down selectively cytochrome b_6 oxidation (compare the initial rate of the positive signal in panels C and D, insert) and allows a more reliable estimation of its reduction (panel D).

Figure 1 reveals that pH deeply affects the electron-transfer reactions triggered by PQH_2 oxidation, as shown by its modulation of cytochrome *f* (B) and b_6 (D) reduction rates. Both decreased at acid pH, in agreement with previous findings in vitro (*24*−*26*) and in vivo (*21*). pH affected also the rate and the amplitude of phase b (A) but was ineffective in the case of cytochrome b_6 oxidation (C). This result is surprising, as the two reactions are supposed to reflect the same phenomenon (*5*). The rate of cytochrome *f* oxidation

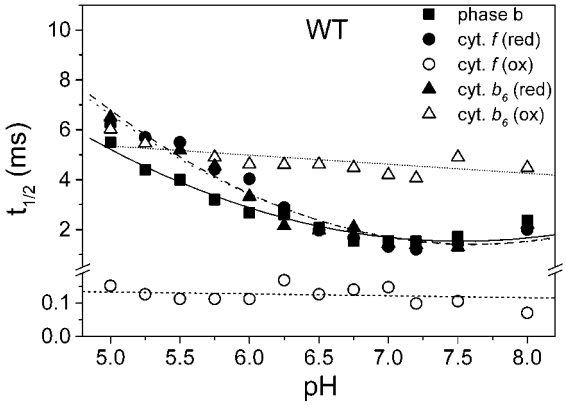

FIGURE 2: Absolute pH dependence of the partial reactions of the cytochrome b_6f catalytic cycle in *C. reinhardtii* wild-type cells. The $t_{1/2}$ values were calculated from fitting of traces as in Figure 1 using a sum of exponentials. See text for further explanation.

did not change appreciably when the pH was modified (panel B, insert).

To quantify the effects of pH on cytochrome b_6f catalytic efficiency, the rates of the partial reactions mentioned above were calculated. To this aim, the reduction and oxidation phases of cytochrome *f* and b_6 redox changes were deconvoluted using a procedure similar to that employed in the case of phase b. The recorded traces were fitted using a sum of two exponentials, which gave the $t_{1/2}$ of the oxidation and reduction phases. Translation parameters were introduced to take into account the existence of a "lag" in the kinetics of phase b (*18*) and of cytochrome *f* (*27*). In the case of the cytochrome b_6 kinetics (panel C), the deconvolution was improved by imposing the $t_{1/2}$ of cytochrome reduction calculated in the presence of NQNO (panel D). In the case of cytochrome *f*, this operation was not necessary to obtain reliable fits. Nevertheless, it was checked that the $t_{1/2}$ of oxidation calculated from the fit was the same in the absence and presence of the Qo site inhibitor DNP-INT (not shown). By competing with PQH_2 for the Qo site (*28*), this compound prevents cytochrome *f* reduction and allows the estimation of the true oxidation rate (see, e.g., ref *27*).

The results of the deconvolutions are shown in Figure 2. Only three reaction steps (closed symbols) changed as a function of pH: phase b (squares) and the reduction of cytochrome *f* (circles) and of cytochrome b_6 (triangles). Their pH dependence profiles were very similar. On the contrary, the $t_{1/2}$ values of both cytochrome *f* (circles) and b_6 (triangles) oxidation (open symbols) were essentially pH insensitive. The $t_{1/2}$ of cytochrome *f* oxidation was about 1 order of magnitude smaller than the others, in agreement with the notion that its oxidation by Pc is the fastest reaction in cytochrome b_6f catalytic cycle (see, e.g., ref *29*). The $t_{1/2}$ of cytochrome b_6 oxidation was very similar to that of cytochrome b_6 reduction at acid pH. Consequently, the two phases largely overlapped, explaining the decrease of the reduction signal observed at pH 5 (Figure 1C). At higher pH, the $t_{1/2}$ of cytochrome b_6 reduction became smaller. The ratio between these two $t_{1/2}$ values remained, however, below a factor of 3, and consequently, the reduction phase of the signal measured at neutral pH was smaller than that observed in the presence of NQNO (Figure 1, compare panels C and D). Due to its pH insensitivity, the rate of cytochrome b_6 oxidation became slower than that of phase b at neutral pH,

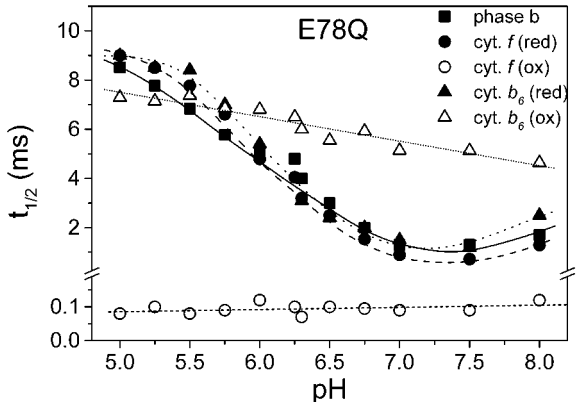

FIGURE 3: Absolute pH dependence of the partial reactions of the cytochrome b_6f catalytic cycle in *C. reinhardtii* E78Q mutant cells. Same conditions as in Figure 2.

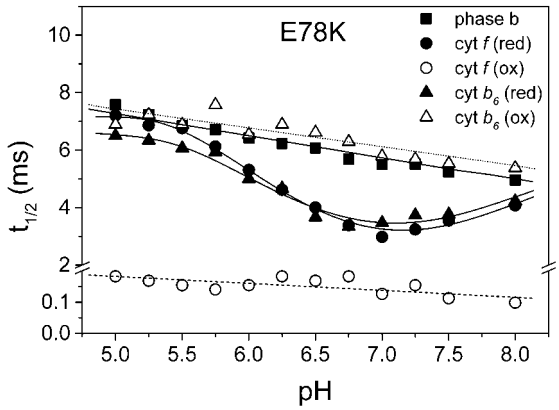

FIGURE 4: Absolute pH dependence of the partial reactions of the cytochrome b_6f catalytic cycle in *C. reinhardtii* E78K mutant cells. Same conditions as in Figure 2.

although both reactions are supposed to reflect the same phenomenon.

E78Q and E78K Mutants. The substitution of the glutamic acid by a glutamine (strain E78Q) did not modify the pH sensitivity of cytochrome b_6f: the kinetics of phase b and of cytochrome *f* and b_6 reduction remained sensitive to pH, while its oxidation did not vary much in the interval tested (Figure 3). The pH profiles of the three sensitive reactions, however, became sharper and shifted toward more basic pH values with respect to the wild type (compare Figures 2 and 3). The mutation did not appreciably affect the activity at acid pH or the maximal rate of cytochrome b_6f turnover measured at neutral pH, in agreement with the findings that the intrinsic catalytic efficiency of the complex is essentially unmodified in this mutant (*15*).

The substitution of the glutamic acid by a lysine (strain E78K) had the same consequences on the pH profile of cytochrome *f* and b_6 reduction as the E78Q mutation (Figure 4, circles and triangles). Two additional phenotypes appeared in this strain: (i) the rate of phase b became much less pH sensitive. Its $t_{1/2}$ was very similar to that of cytochrome b_6 oxidation in all of the pH range and, therefore, larger than that of cytochrome b_6 and *f* reduction at neutral pH. In addition, (ii) the maximum catalytic efficiency of the cytochrome b_6f complex (i.e., the $t_{1/2}$ at neutral pH) was slightly reduced in the E78K mutant, in agreement with previous results (*15*).

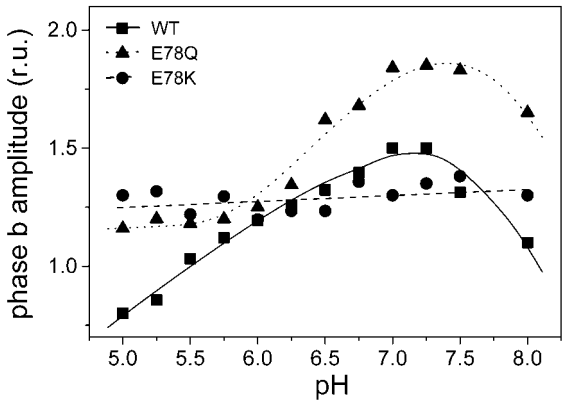

FIGURE 5: Absolute pH dependence of the slow electrochromic shift (phase b) amplitude in *C. reinhardtii*. Data were calculated from traces as in Figure 1A by deconvolution of phase b from membrane potential decay, as explained in Materials and Methods. Then, its amplitude was calculated by subtracting the maximum amplitude of the slow electrochromic shift from that measured at 100 μs. The latter reflects PSI-catalyzed charge separation.

pH Dependence of the Amplitude of Phase b. In the wild type and the E78Q mutant, not only the rate but also the amplitude of phase b was sensitive to pH changes (Figure 5): in both strains, an increase of ~90% was observed when the pH increased from 5 to 7.25. In the wild type, the number of charges transferred across the membrane by the cytochrome b_6f complex (indicated by amplitude of phase b minus that of PSI-catalyzed phase a, normalized to phase a) varied from ~0.8 to ~1.5. In the E78Q mutant, it varied from ~1.25 to ~1.9. As in the case of the $t_{1/2}$, the pH profile of phase b amplitude calculated in the case of E78Q was sharper and shifted to more basic values than in the wild type. In the E78K strain, the insensitivity of phase b to pH changes was confirmed also by the stability of its amplitude, which was of ~1.3 charges per charge translocated in PSI in the whole pH interval. We note that, in both the wild type and the E78Q mutant, the increase in the rate and amplitude of phase b inversely correlates to the presence of a lag in the kinetics (not shown), which varied from 0.5 ms at acid pH to ~0.1 ms at neutral pH. The lag was also observed in the E78K strain. Its value was pH independent and similar to that measured in the other two strains at acid pH (~0.5 ms).

DISCUSSION

pH Dependence of the Cytochrome b_6f Catalytic Cycle. This work analyzes the pH dependence of the electron-transfer reactions catalyzed by the cytochrome b_6f complex. While some of these reactions, the ones related to the oxidation of PQH_2 at the Qo site, are pH dependent (Figure 1B,D and Figure 2), the reduction of PQ at the Qi site (indicated by the kinetics of cytochrome b_6 oxidation, Figure 1C and Figure 2) is poorly affected by changes in the proton concentration. This suggests that the rate of PQ reduction at the stromal site of cytochrome b_6f complex is not limited by the protonation reaction steps, at least under the conditions employed here.

Furthermore, the cytochrome *f*−Pc interaction (evidenced by the oxidation of cytochrome *f*, Figure 1B and Figure 2) is essentially pH independent, i.e., not influenced by the protonation state of the charged domains located on both

partners. This finding is consistent with previous observations that have ruled out the implication of electrostatic interaction in the formation the Pc−cytochrome *f* reaction complex in vivo (*30*), at variance with the in vitro system (*31*−*34*), unless the cell integrity is disrupted before the measurement (*35*). It has been previously reported, however, that the addition of protonophores to intact cells of green algae (*27*) results in a substantial increase of the oxidation rate of cytochrome *f*. To conciliate these two apparently opposite results, it can be proposed that the protonophore's effect is related to their dissipation of the ΔpH, rather than to the consequent changes in the thylakoid lumenal pH. The presence of a ΔpH might decrease the rate of cytochrome *f* oxidation, for instance, by inducing the swelling of thylakoid membranes (*36*), thereby diluting the Pc concentration in the lumen.

pH Dependence of Plastoquinol Oxidation (First Electron and Proton Transfer). Figures 1 and 2 indicate that the reactions occurring at the Qo site of cytochrome b_6f are significantly influenced by the changes in the absolute pH, their rate being slower at higher H^+ concentration. This result is consistent with previous reports (*37*−*41*) and with the notion that PQH_2 oxidation is the main target of the kinetic inhibition exerted by pH on the cytochrome b_6f complex (see, e.g., refs *25* and *42*−*44*). It has been proposed (*12*) that this reflects the pH modulation of the formation of hydrogen bonds between the PQH_2 and protonable residues in the Qo site. One is H161 of the Rieske protein in *Rhodobacter sphaeroides* (*12*; H126 in the case of *C. reinhardtii*), and the second is E78. The involvement of H161 in the modulation of the pH effects on the quinol oxidation rate has been already asserted (reviewed in ref *12*). Its involvement is confirmed by the pH profile observed in the E78Q and E78K mutants, where a single p*K* is observed, the value of which (6.25−6.5, Figures 3 and 4) is very close to the one previously attributed to histidine 161 (*12*) of the Rieske protein.

The contribution of H126 can be inferred also in the case of the wild type, where the pH profile is, however, more complex: the rate of PQH_2 oxidation (indicated by both cytochrome *f* and b_6 reduction) is faster in the pH 5.25−5.75 range, if compared to the E78Q and E78K mutants, while being nearly identical at more neutral pH (at least in the case of the E78Q strain; compare Figures 3 and 4 with Figure 2). This suggests that another residue, with an acid p*K*, is involved in the kinetic modulation of PQH_2 oxidation. Because of the selective removal of this residue in the mutants, it is reasonable to attribute this p*K* to E78. Its deprotonation in the pH 5.25−5.75 region would help the formation of an H-bond to one −OH group of the quinol and, consequently, increase the p*K* of the opposite (para) −OH group, i.e., the H^+ donor during the first deprotonation.

In principle, the effect of the E78Q and E78K mutations on the pH profile might be also interpreted on the assumption that the absence of E78 simply shifts the curve toward more basic values by removing an electrostatic effect. This hypothesis is less likely than the involvement of two p*K*'s (E78 plus H126) in the wild type for at least two reasons: (i) It is difficult to explain the smoother profile observed in the case of the wild type (Figure 2) in the frame of a single p*K*, unless it is assumed that the amplitude of the curve is by far larger in the wild type, and therefore, the slope observed in the pH 5−7 interval only represents its inflection point. In addition, (ii) the acid shift induced by the glutamic acid is not consistent with a simple electrostatic effect, which would be to raise the overall p*K* of the curve and not to decrease it.

In the E78Q strain, the lack of kinetic effects at neutral pH can be explained either by assuming that a single H-bond (that of H126) is sufficient to stabilize PQH_2 binding or that another residue is able to interact with the quinol in the absence of the E78 residue, but only at neutral pH. This means that the p*K* of this hypothetical residue (hereafter referred as X) should be much closer to that of H126 than to that of E78. The almost complete superposition between the two p*K*'s might explain why the pH dependence measured in the mutants (Figures 3 and 4) is consistent with the involvement of a single p*K*.

pH Dependence of Plastoquinol Oxidation (Second Electron and Proton Transfer). Besides its likely involvement in the binding of PQH_2 (*12*), E78 also participates in the deprotonation of the semiquinone (*14*). Thanks to its high mobility in the Qo site, this residue would be able to accept a H^+ and to transfer it to a water chain, connected with the lumenal side (*12*). It has been recently proposed that the water chain present in cytochrome *f* (*45*) constitutes an essential component of this channel in the cytochrome b_6f complex (*46*). The unmodified efficiency of electron transfer measured in the E78Q mutant at neutral pH (Figure 3) suggests that another residue is able to replace E78, when the latter is removed. As stated before, either H126 or X might perform such a role. While it is not possible to discriminate between the two possibilities in the case of PQH_2 binding, the involvement of H126 in the case of the deprotonation of the semiquinone can be ruled out. Indeed, the occurrence of efficient proton transfer between this species and the Rieske protein should also be accompanied by electron transfer between them, because of the very favorable E_m difference (*29*). As a consequence, double electron transfer to cytochrome *f* would take place, resulting in a diminished efficiency of cytochrome b_6 reduction in the E78Q and E78K mutants, with respect to the wild type. This phenotype has been already observed in cytochrome *f* mutants of the cytochrome b_6f complex (*47*). The data of Figures 3 and 4 clearly indicate that this is not the case in the E78Q and E78K mutants, where cytochrome b_6 is reduced with the same efficiency and pH dependence as cytochrome *f*. The PQH_2 oxidation via the low- and high-potential chains of the b_6f complex is therefore still concerted in E78Q and E78K mutant strains.

The situation where a proton acceptor might be efficiently substituted by other residues is not peculiar to the cytochrome b_6f complex. For instance, in the bacterial reaction center, a system that is better characterized, it has been shown that quinone protonation occurs via a network of ionizable residues and that the removal of one of them is easily compensated by the activity of the whole network itself (reviewed in ref *48*). As a consequence, it is difficult to attribute a measured p*K* to a single residue in the case of this complex, because the removal of a single group alters the ionization properties of the whole network, due to electrostatic interactions (J. Lavergne, personal communication). The situation is apparently less complicated in the case of the cytochrome b_6f complex, where apparently only two

p*K*'s are involved. Nevertheless, the strict attribution of p*K* made above probably represents an oversimplification.

pH Dependence of Phase b. In the wild type (Figure 2) and in the E78Q strain (Figure 3), the rate of phase b is modulated by pH, and its dependence is similar to that of cytochrome *f* and b_6 reduction. On the contrary, phase b is not sensitive to pH changes in the E78K strain, and its rate is slowed to values comparable to those of cytochrome b_6 oxidation (Figure 4). This behavior is predicted by the Q cycle, where cytochrome b_6 oxidation (i.e., the sum of the b_l-b_h electron transfer plus PQ reduction and protonation at the Qi site) is the only electrogenic reaction taking place during the catalytic cycle (*5*).

On the contrary, several results reported in this work are less compatible with this model and suggest instead that a reaction, different from cytochrome b_6 oxidation, is an essential component of phase b kinetics. Among them, are (i) the discrepancies between the $t_{1/2}$ of phase b and of cytochrome b_6 oxidation observed in the wild type and the E78Q, (ii) the pH sensitivity of phase b (Figure 5), which is observed only in the wild type and E78Q strain and not observed in the case of cytochrome b_6 oxidation, and (iii) the strict correlation between the pH profiles of the $t_{1/2}$ of phase b (Figures 2 and 3) and its amplitude (Figure 5).

The Q cycle may account for variable amplitudes of phase b assuming changes in the redox state of the cytochromes. This is due to the fact that one of the two electrogenic reactions (the reduction of PQ at the Qi site; see above) occurs only if the b_h heme is reduced prior to illumination. Thus, in complexes where this heme is oxidized, the only electrogenic reaction taking place is the b_l-b_h electron transfer, which contributes ~0.7 to the overall electrogenicity (*23*, *49*). In complexes where b_h is reduced, both reactions occur, and the amplitude expected, after normalization to PSI-driven charge separation, is 1.3 [0.7 + 2(0.3)], due to a double e^- and H^+ transfer to plastoquinone (*7*−*8*, *23*, *49*). Under repetitive flash illumination (as employed here), the amplitude of phase b may vary between 1 [(0.7 + 1.3)/2, due to the binary oscillation between oxidized and semi-reduced complexes] and 1.3. This depends on whether the b_h heme is completely rereduced in the dark or not (see ref *49* for a further discussion).

The amplitudes of phase b measured in the case of the E78K and of the E78Q strains at acid pH (~1.3) are compatible with the occurrence of the Q cycle in semireduced complexes. This suggests that the dark reduction of the b_h heme is almost complete in the dark time between two consecutive illuminations. On the contrary, the amplitudes measured in the wild type at acid and neutral pH (0.8 and 1.5, respectively) and in the E78Q mutant a neutral pH (~1.9) are not consistent with the predictions of this model. This reinforces the notion that an additional charge movement across the thylakoid membranes takes place during the cytochrome b_6f catalytic cycle. Such a phenomenon has been first observed in the case of another energy transducing complex, the cytochrome oxidase (*50*), and has been explained by assuming the existence of a redox-coupled proton pump activity. In the case of *bc* complexes, its occurrence has also been proposed to explain different experimental findings: (i) the inhibitory effect of the carboxyl-binding reagent DCCD on the proton/electron coupling in both cytochrome b_6f and bc_1 complexes (*51*−*53*) and on the amplitude of phase b in *C. sorokiniana* (*49*); (ii) the effect of H/D substitution on the initial rate of phase b (*54*); (iii) the occurrence of proton slip in mutants of the bc_1 complex (*55*); (iv) the lack of correlation between the kinetics of cytochrome *b* and phase b in the bc_1 complex (*56*−*57*), and (v) the existence of an "abnormally" large amplitude of phase b in the E78Q mutant of *C. reinhardtii* (*15*).

A Model for H^+/e^- Coupling in the Cytochrome b_6f Complex. The finding that the pH profile of both the rate (Figures 2 and 3) and the amplitude (Figure 5) of phase b is similar to that of reactions occurring at the Qo site strongly suggests that PQH_2 oxidation triggers the activity of a redox-coupled proton pump. In addition, the differences observed between the wild type (Figure 2) and the E78Q mutant (Figure 3) suggest that E78 is involved in the modulation of this phenomenon. The observation of a pH-dependent lag in the kinetics of phase b in the wild type and the E78Q strain indicates that the redox-coupled proton transfer occurs at the beginning of the cytochrome b_6f catalytic cycle, i.e., before the oxidation of cytochrome b_6 hemes. This is in agreement with the finding of a relevant isotopic effect on the initial rate of phase b (*54*). It is also consistent with the existence and the pH independence of the lag in the E78K strain, where the pump is apparently blocked.

To explain all these results, a mechanism is proposed that combines two existing models. The first (*12*) explains the dynamics of quinol oxidation and deprotonation in the Qo site, while the second (*53*) accounts for proton slip in DCCD-treated complexes (see also ref *49* for a discussion on its application in the case of the cytochrome b_6f complex). The combined scenario presupposes that E78 is involved in quinol binding (Figure 6A,C,E) and in proton transfer from the semiquinone (Figure 6B,D,F, reaction 1) to a H^+ channel (*12*, *46*; Figure 6D,F, reaction 2). This would occur thanks to the rotation of the E78 residue on one C−C bond (*14*). The channel (indicated here as PC1, using the same nomenclature as in ref *53*) would be located close to the b_l heme pocket and in contact with the lumen. Our model also assumes the existence of a second proton channel (PC3; *53*), which connects the Qi site to the stroma, and of a third one (PC2) that provides a proton path between the two quinone binding sites. This latter channel would be gated by two protonable residues (W and Z; see also ref *53*). The model also includes the existence of another protonable residue (X), which is able to accept H^+ from the semiquinone and has a p*K* higher than that of E78, as discussed above.

Our model predicts that, after its deprotonation at the distal end (Figure 6A,C,E), the charged semiquinone would interact electrostatically (Figure 6B,D,F, dashed line) with residue W, increasing its p*K* and inducing its protonation (*49*, *53*). This would occur either via residue Z (Figure 6D,F, reaction 3) or the E78 residue, which would be connected to residue W thanks to its high mobility in the site (*14*; Figure 6B,D, reaction 4). We do not expect the X residue to be connected to W, as the conformational rearrangements in the Qo site appear to be specific of the sole E78 side chain (*12*, *14*). After its deprotonation, the semiquinone would be oxidized by the b_l heme. This would remove the electrostatic effect on the W residue and induce its deprotonation, either in the lumen via the PC1 channel (Figure 6D,F, reaction 5) or back to residue Z (Figure 6B,D, reaction 6). The next step of the

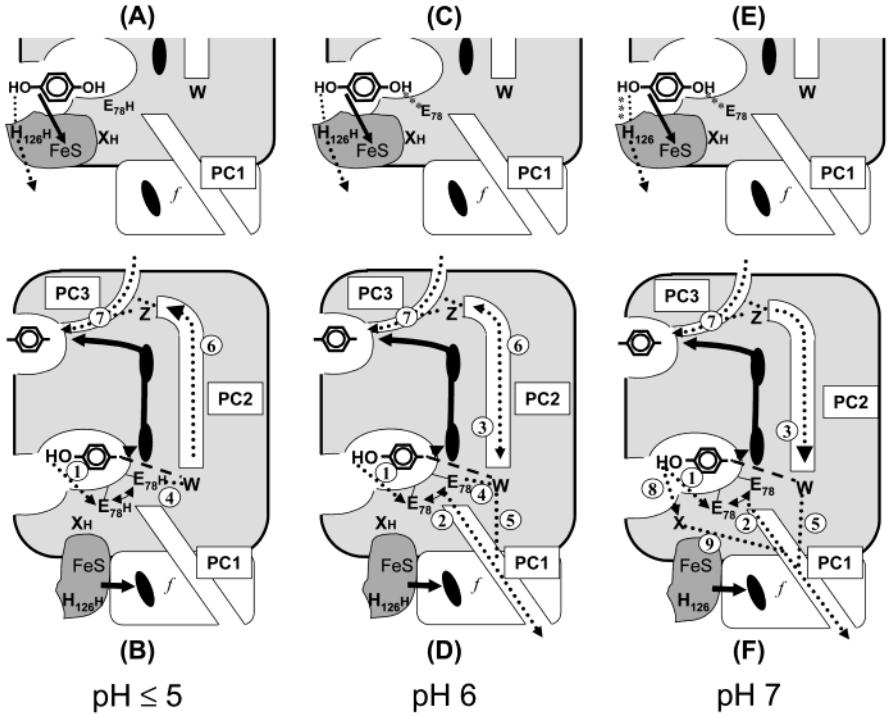

FIGURE 6: Proposed mechanism for H^+/e^- coupling during plastoquinol oxidation in the cytochrome b_6f complex. Plastoquinol binding at the Qo site occurs via H-bonding with H126 and E78 (stars). This results in H^+ and electron transfer to the Rieske protein (A, C, E). The Rieske protein is then oxidized by cytochrome *f*, thanks to a conformational change occurring in its head (B, D, F). In parallel, a movement of the protonated semiquinone allows the transfer of an electron to the b_l heme. Oxidation of the neutral semiquinone is proposed to take place via the E78 or the X residue. Both would be connected to the proton channel PC1. E78 would modulate the activity of a transmembrane H^+ channel (PC2) gated by two protonable residues (W and Z). Their protonation would be regulated by electrostatic interactions with the semiquinone (dashed line) and the b_6 hemes. The activity of this channel would affect the H^+/e^- stoichiometries of plastoquinol oxidation. Dotted lines: proton-transfer reactions. Solid lines: electron-transfer reactions. See Discussion for further explanations.

catalytic cycle would be the reduction (solid lines) and the double protonation of a quinone at the Qi site (Figure 6B,D,F, reaction 7). This reaction occurs specifically in semireduced complexes. However, we assume that it takes place in all of the cytochromes, i.e., that all of them are semireduced in our experimental conditions. This assumption is justified by the finding that the normalized amplitude of phase b is 1.3 (i.e., the value predicted when b_h is reduced in the dark) in the E78K mutant, where the redox-coupled proton pumping activity is apparently inactive (Figure 5).

The possibility of proton transfer via the PC2 channel is likely to modify the overall electrogenicity of the cytochrome b_6f catalytic cycle, which has to be recalculated accordingly: the occurrence of reactions 1 + 2 + 3 would result in a net proton pumping from the stroma to the lumen and the consequent increase of phase b amplitude to 2 (1.3 + 0.7). On the contrary, reactions 1 + 4 + 6 would reduce the amplitude of the electrogenic reactions because of the H^+ transfer from the Qo to the Qi site. In this case a value of 0.6 (1.3 − 0.7) is to be expected. The overall amplitude of phase b remains unchanged with respect to the predictions of a "pure" Q cycle mechanism only in the case where reactions 1 + 4 + 6 + 3 or 1 + 4 + 5 take place.

Given these considerations, the results reported in Figures 2−5 can be explained by assuming that the rate of plastoquinol oxidation is slowed in the wild type at pH ≤5 because H126, E78, and X are protonated (Figure 6A). This would limit the formation of the enzyme−PQH_2 complex and slow all the reactions in a concerted manner. Once the cycle is initiated, a proton would be transferred from the semiquinone to W via E78 (Figure 6B, reactions 1 + 4). Due to the high H^+ concentration the deprotonation of W via PC1 would be precluded, while protonation of Z (reaction 6) could occur. This would favor proton transfer to the plastoquinone present in the Qi site via PC2 and result in a proton slip. As a consequence, the amplitude of phase b would be reduced, as observed in Figure 5. A rough estimation based on the stoichiometries predicted above indicated that nearly 80% of the protons delivered by the semiquinone follow this pathway in the wild type at pH 5.

At pH ∼6 the H^+ concentration in the lumen would decrease and E78 would be largely deprotonated (Figure 6C). This would increase PQH_2 binding (Figure 6C, stars) and deprotonation, resulting in a concerted increase of both cytochrome b_6 and *f* reduction rates. At the same time the probability of semiquinone deprotonation via the E78−PC1 path (reactions 1 + 2) would increase (Figure 6D), reducing the fraction of complexes engaged in proton slip up to ∼50%. At neutral pH (Figure 6E) the deprotonation of H126 would enhance the rate of PQH_2 oxidation (Figure 6E, stars). In parallel, the deprotonation of X would open a new path for semiquinone deprotonation (reactions 8 + 9, Figure 6F). X not being connected to W, the overall electrogenicity associated to the cytochrome b_6f complex would increase (see above), because of the occurrence of reactions 3 + 5 in complexes where the X residue is chosen as the H^+ acceptor.

In the E78Q mutant, removal of the E78 residue would leave X as the only residue involved in semiquinone deprotonation. This might explain the p*K* shift observed in the pH dependence of both phase b kinetics and amplitude (Figures 3 and 5), as well as the absence of proton slip in the whole pH range. The amplitudes of ∼1.3 measured at

acid pH and of ~1.9 measured at neutral pH are consistent with this latter hypothesis. Finally, the E78K phenotype, where phase b is uncoupled from PQH_2 oxidation cycle, can be explained by assuming that the presence of a basic residue might counterbalance the destabilizing effect of the charged semiquinone on the W residue. By increasing its p*K*, K78 would prevent its protonation and, consequently, both proton slip and redox-coupled proton pumping (Figure 5). In addition, its bulkiness, which is larger than that of E78, would slow the movements of the plastoquinol−semiquinone pocket in the Qo site, thus justifying the observed reduced maximal catalytic efficiency (Figure 4).

Interestingly, the major transition from proton slip and proton deposition is observed in the pH 5−6 region, i.e., in the range that includes the value of the lumenal pH measured in dark-adapted living algae (*21*). This suggests that the regulation of the redox-linked proton pumping activity by pH might represent a useful means to help in maintaining the pH homeostasis in the chloroplast lumen (*58*).

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