Glu78, from the Conserved PEWY Sequence of Subunit IV, Has a Key Function in Cytochrome b_6f Turnover†

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ABSTRACT: We have investigated the structure to function relationship at the Q_o site in cytochrome b_6f complexes in vivo. To this end, we created site-directed mutants of *Chlamydomonas reinhardtii*, at position 78 in the sequence of subunit IV. The target glutamic acid, present in the highly conserved $_{77}$PEWY$_{80}$ sequence, was changed to residues of different polarities which did not prevent the functional assembly of cytochrome b_6f complexes. Spectroscopic analysis performed in anaerobic conditions in vivo revealed distinct alterations in cytochrome b_6f function, depending on the nature of the substituted residue. The semiconservative E78D substitution, in which only the length of the side chain is reduced, retained the functional features of the wild-type configuration. The E78K and E78L substitutions caused a significant decrease, by factors of 3 and 5, respectively, in the rate of the concerted oxidation process at the Q_o site without a change in the affinity of Q_o for reduced plastoquinones. The E78Q and E78N substitutions modified the characteristics of cytochrome b_6f turnover under repetitive flash illumination. They caused a large increase in the electrogenicity of the electron-transfer reactions through the mutated cytochrome b_6f complex. This increase was specifically sensitive to the electrical component of the proton-motive force. Surprisingly, despite the larger number of charges translocated across the membrane per charge injected in the high potential chain, the reduction phase for cytochrome b_6 became barely detectable in the mutants, unless inhibitors at the Q_i site were present. We show that similar functional characteristics can be observed with the cytochrome b_6f complex in the wild-type in anaerobic conditions, provided a single flash illumination regime is used. These observations suggest that cytochrome b_6f turnover may involve a mechanism implying an extra proton pumping activity.

Quinol-oxidizing complexes—the cytochrome bc_1 complex of mitochondria and bacteria or its chloroplast counterpart, the cytochrome b_6f complex—are the most widespread protein complexes among energy-transducing membranes. In the green alga *Chlamydomonas reinhardtii*, the cytochrome b_6f complex comprises four major subunits (*1*, *2*): the nuclear gene *PetC* encodes the Rieske protein, which binds an Fe_2S_2 cluster; the chloroplast genes *petA*, *petB*, and *petD* encode a *c*-type cytochrome, cytochrome *f* with E_m of +330mV (*2*), a *b*-type cytochrome, cytochrome b_6 which binds the b_h and b_l hemes, with E_ms of −84 and −158 mV, respectively (*2*), and subunit IV. There are three additional small subunits of unknown function, the chloroplast *petG* and *petL* products and the nuclear product *PetM* (*2*, *3*). Sequence comparisons have shown that cytochrome b_6 and subunit IV are, respectively, homologous to the N- and C-terminal parts of mitochondrial and bacterial cytochrome *b* (*4*).

The *bc*-type proteins couple proton translocation across the membrane to electron transfer from a lipophilic two-electron donor (ubiquinol or plastoquinol) to a hydrophilic one-electron acceptor protein (plastocyanin or a *c*-type cytochrome). This electron transfer operates through a high-potential chain formed by the Rieske protein and the *c*-type cytochrome, cytochrome *f* in the case of cytochrome b_6f complexes, both of which are membrane bound. In agreement with the "Q cycle" hypothesis of Mitchell (*5*), it has been documented that oxidation of the substituted quinol also involves a cyclic electron transfer around the *b* heme chain, termed the low-potential chain. This cycle postulates both an oxidation and a reduction of the lipophilic electron carrier at two distinct sites of the protein, the Q_o and Q_i sites, on opposite sides of the membrane. Thus, one electron is transferred across the membrane per electron transferred along the high-potential chain. The Q cycle mechanism results in an extra-proton translocation across the membrane, thereby increasing the net ATP production per electron transferred through the *bc*-type protein.

According to the Q cycle (*5*) as modified by Crofts et al. (*6*), the oxidation of a plastoquinol at the Q_o site is associated with the reduction of both cytochrome *f* and b_l (*5*, *6*) and the release of protons. Oxidation of the b_6 hemes occurs through a two step reduction of a PQ^1 molecule at the Q_i site. In oxidizing conditions, the electron-transfer sequence

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is then a reduction by plastoquinol of cytochrome b_l, which is in turn oxidized by cytochrome b_h. A second cytochrome b_6f turnover places both the b_l and b_h hemes in a reduced state. Consecutive reduction of plastoquinone to a plastoquinol at the Q_i site is coupled to *b* hemes oxidation and to a proton uptake from the stromal space.

The Q cycle hypothesis is widely accepted (*7*) even though Wikstrom and Krab (*8*) have proposed an alternative model, the *b*-cycle in which one semiquinone interacts with the two *b* hemes. In a subsequent version of this model, Joliot and Joliot (*9*) proposed that a semiquinone molecule ($PQ^{\bullet -}$) is translocated through the membrane from the Q_o to the Q_i site where it is reduced by an electron transferred from the b_h heme.

While the semiquinone cycle is rather uninfluenced by the redox state of the *b* hemes, electron transfer through the Q cycle can be initiated only if one of the *b* hemes, at least, is in an oxidized state. In support of the semiquinone cycle hypothesis, cytochrome b_6f-mediated charge translocation across the membrane was reported to still occur under complete reduction of the b_6 cytochromes (*9*, *10*). To take into account this observation, a "reaction chain" mechanism, which keeps with the Q cycle hypothesis but requires multiple turnovers of the cyt b_6f complex, was proposed by Kramer and Crofts (*11*).

Mutagenesis approaches and studies of inhibitor resistance in bacteria and yeast (*6*, *12*−*14*) have provided a number of data on the putative localization of Q_o and Q_i sites in bc_1 complexes. Because of the sequence conservation between bc_1 and b_6f complexes, it is assumed that the cytochrome b_6 and subunit IV both contribute to the formation of the two sites, as well as the Rieske protein in the case of the Q_o site. The scarcity of information pertaining to the structure to function correlations on cytochrome b_6f complexes originates from the fact that most photosynthetic organisms do not tolerate deep alterations in the function of these protein complexes. One exception is *C. reinhardtii* because it shows dispensable photosynthesis. Since it is amenable to chloroplast gene transformation, it is one of the few photosynthetic organisms in which site-directed mutants of cytochrome b_6f complexes can be studied.

We have shown recently that the loop between the C and D helices of cytochrome b_6 is critically involved in the folding of the Q_o site in *C. reinhardtii* (*15*), as is the case of a similar loop of cytochrome *b* in bacterial or mitochondrial cytochrome *bc* complexes. This observation supports the view that the Q_o site involves the same polypeptide regions in cyt *bc* and cyt b_6f complexes (*12*). Among these is a lumenal loop between helices E and F in subunit IV which contains a short sequence of four amino acids, $_{77}$PEWY$_{80}$, strictly conserved in all cytochrome *b* from bc_1 and subunit IV from b_6f complexes (*16*). In the crystal structure of the cytochrome *bc* complex, where one can identify the position of the conserved PEWY sequence with respect to heme b_l, the side chain on the E residue is close to van der Waals contact with the edge of the b_l heme (*17*; atomic coordinates deposited in the Brookhaven Protein database, 1BCC and 3BCC). We turned this charged residue, E78, into a variety of substituting residues, most of which introduced some limited changes in bulkiness and large changes in polarity. In two cases, E78Q and E78N, we observed remarkable changes in the stoichiometry of charges translocated across the membranes during the turnover of cytochrome b_6f complexes.

MATERIAL AND METHODS

Cell Growth Conditions. The wild-type strain (mt +), derived from strain 137C, and transformants were grown on tris-acetate phosphate (TAP) and minimum media, pH 7.2 at 25 °C under 6 μeinstein m^{-2} s^{-1} and 60 μeinstein m^{-2} s^{-1} of continuous illumination, respectively.

Oligonicleotides, Mutagenesis, and Plasmids. Plasmid pdWQ, encompassing the coding region for subunit IV, has been previously described (*18*). Mutations of the glutamate residue (E), from the $_{77}$PEWY$_{80}$ sequence, were introduced in plasmid pdWQ by site-directed mutagenesis according to the method of Kunkel et al. (*19*). PdWQ single-stranded DNA was used for annealing with the following mutagenic oligonucleotides: Wild-type, AGGGTAGAAATACCATTCTGGTAAAATTTCAAGTGGAGTAG; E78D, 5′-AGGGTAGAAATACCA**A**TCTGGTAA**G**AT**C**TCAAGTGGAGTAG-3′; E78L, 5′-GGGTAGAAATACCAT**AA**TGGTAA**G**AT**C**TCAAGTGGAGTAG-3′; E78N, 5′-AGGGTAGAAATACCA**G**T**T**TGGTAA**G**AT**C**TCAAGTGGAGTAG-3′; E78Q, 5′-GGTAGAAATACCATT**G**TGGTAA**G**AT**C**TCAAGTGGAGTAG-3′; E78K, 5′-GGTAGAAATACCATT**T**TGGTAA**G**AT**C**TCAAGTGGAGTAG-3′.

Letters in bold indicate the mutated nucleotides, while a new restriction site *Bgl*II generated by a silent mutation is underlined. This leads to plasmids pdD78D, pdD78L, pdD78N, pdD78Q, and pdD78K. Amplification was performed in *Escherichia coli.* Screening of candidate colonies was carried out using unique restriction site (*Bgl*II) for each mutation.

Chloroplast transformation in C. reinhardtii. Δ*petD* strains (*18*) were transformed by tungsten particle bombardment according to Boynton et al. (*20*) with a particle gun, operating under vacuum, built in the laboratory by P. Bennoun and D. Beal. Phototrophic transformants were selected on minimum medium at 60 μeinstein m^{-2} s^{-1}. The presence of the E78 mutations was checked by PCR amplification of the region of interest with suitable oligonucleotide primers and RFLP analysis (data non shown). Mutations were purified to homoplasmy.

DNA Analysis. Polymerase chain reaction (PCR) was performed directly on *C. reinhardtii* colonies. About 10^6 cell were resuspended in 20 μL of lysis buffer (0.1 M Tris, pH 8, 0.1 M EDTA, 0.25 M NaCl, and 0.1% SDS), then 180 μL of water were added and 1 μL of cell suspension was used for PCR reaction. Total DNA was amplified using the specific oligonucleotides SubIVcod1 (5′-TTTAAGCGATCCAGTTTT-3′) and SubIVrev2 (5′-TCAATAGGGAATGTTGAACC-3′). Routine amplification was performed with 40 cycles, using a cycle of denaturation at 94 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C.

[1] Abbreviations: cyt, cytochrome; DCMU 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DTT 1,4-dithiothreitol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HA, hydroxylamine; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonate); NQNO, 2-*N*-4-hydroxyquinoline-*N*-oxide; *p*BQ, *p*-benzoquinone; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosytem; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SQ, semiquinone.

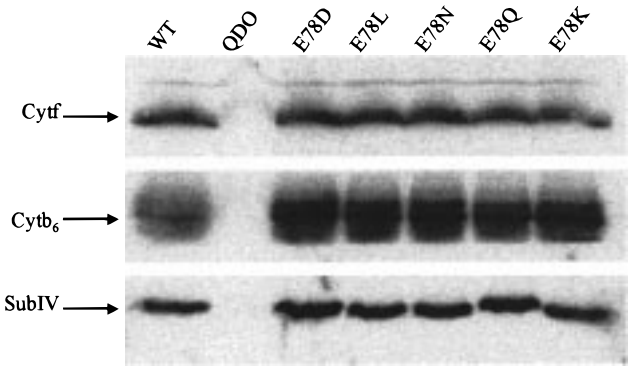

FIGURE 1: Immunoblots of whole cell protein extracts. The protein extracts where probed with specific antibodies against cytochrome *f* and b_6 and subunit IV. A 1/100 dilution was used for detection with protein-A ^{125}I-labeled (Amersham). Loads for each sample correspond to 20 μg of chlorophyll. QDO, strain deleted for the *pet*D gene; E78Q, E78N, E78D, E78K, and E78L, strains mutated in the position 78 in the PEWY sequence.

Protein Isolation, Separation and Analysis. Proteins from whole cells, grown to a density of 2×10^6 cells/mL, were resuspended in 100 mM DTT 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% polyacrilamide). Protein analyses were performed by immunoblotting, using specific antibodies against cytochrome b_6f complex subunits as described in Kuras and Wollman (*18*).

Spectroscopic Analysis. Cells were collected during the exponential phase of growth (2×10^6 cells/mL) and resuspended in 20 mM HEPES-NaOH, 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 in Joliot et al. (*21*) and modified as in Joliot and Joliot (*9*). All measurements were performed in anaerobic conditions, to reach steady-state conditions in which inhibition of mitochondrial respiration ensures full reduction of the plastoquinone pool (*22*). 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 RG8). Flashes were of nonsaturating intensity (hitting 20% of the centers) to avoid multiple turnovers of cytochrome b_6f complexes.

The photoinduced transmembrane potential was estimated by the amplitude of the electrochromic shift at 515 nm, which yields a linear response with respect to membrane potential (*23*). In our experimental conditions, the kinetics of the electrochromic shift display two phases (*24*): a fast phase (phase a) completed in less than 1 μs and associated with charge separation within photosystem 1 and photosystem 2 reaction centers, and a slow phase (phase b) in the millisecond time range, associated with cytochrome b_6f turnover. Photosystem 2 absorption changes were prevented by preilluminating the samples in the presence of 10 μM DCMU and 1 mM HA. Under these experimental conditions, phase a is produced by the sole photosystem 1 charge separations.

Kinetic analysis of phase b requires deconvolution of the membrane potential decay. To this end, we assumed that the latter phenomenon has nearly first-order kinetics. We have calculated the rate constant of the membrane potential decay for each experimental curve. The kinetics of phase b were deconvoluted, assuming that the rate of decay of the membrane potential was linearly related to its amplitude. Deconvolution assuming a multiexponential decay of the membrane potential did not alter substantially the kinetic parameters of phase b.

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. For measurements in the presence of NQNO with the E78K and E78L mutants, we noted that a slightly higher concentration of NQNO (8 μM vs 4 μM in wild-type cells) was required to obtain full inhibition of cytochrome b_6 oxidation. We checked that this concentration was still without effect on PQH_2 oxidation kinetics in wild-type cells. NQNO was kindly provided by professor J. Whitmarsh (Urbana University, IL).

p-Benzoquinone Treatments. Algae treated with pBQ before spectroscopic analysis were harvested at a density of 2×10^6 cells/mL, concentrated to 10^7 cells/mL in 50 mM HEPES, pH 7.2, vigorously aerated by shaking in darkness for 1 h to ensure plastoquinol oxidation, and then incubated for 5 min with 500 μM pBQ. They were then centrifuged, washed twice in the same medium without pBQ, and finally resuspended at 5×10^7 cells/mL in 20 mM HEPES-NaOH, pH 7.2, in the presence of 10% Ficoll.

RESULTS

Transformation in the $_{77}$PEWY$_{80}$ Sequence. To investigate the effect of changes in the local charge at the Q_o site, we converted the glutamate GAA codon, in position 78 of the *petD* coding sequence, into GAT, TTA, AAC, CAA, and AAA coding, respectively, for aspartate (D), leucine (L), asparagine (N), glutamine (Q), and lysine (K).

These sequence modifications were introduced by site-directed mutagenesis in plasmid pdWQ yielding plasmids pdD78D, pdD78L, pdD78N, pdD78Q, and pdD78K. To assess easily the efficiency of transformation at the E78 site in the *petD* gene, we generated a screenable RFLP marker, with two additional silent mutations in positions 74 and 75 which created a new *Bgl*II restriction site.

The Δ*petD* strain, deleted for the *petD* gene (*18*), was used as a recipient strain for the recovery of phototrophic transformants. Each substitution E78D, E78L, E78N, E78Q, and E78K yielded phototrophic clones.

The growth rate of the E78 mutants in phototrophic conditions was marginally affected—within a factor of 2—as compared to that of the wild-type. Figure 1 shows an urea/SDS−PAGE of whole cell protein extracts after immunoblotting with specific antibodies directed against the cytochrome b_6f subunits. The main subunits accumulated extensively in each type of mutants, hereafter referred to be the type of substitution at the 78 position.

We then investigated the functional properties of the mutated cytochrome b_6f complexes. All measurements were performed in vivo, after placing the intact cells in anaerobic conditions. The mutants are presented below in two separate sets, which differ widely in their functional alterations.

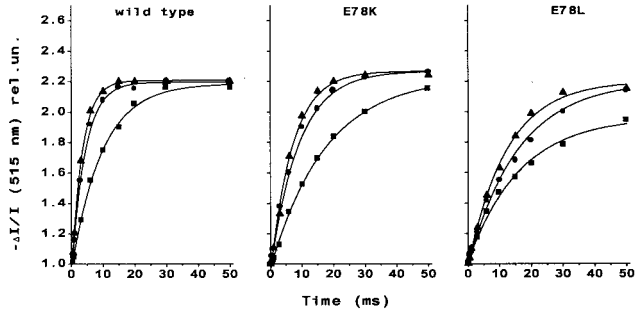

FIGURE 2: Slow electrochromic reaction in wild-type and E78K and E78L mutants. Algae (5×10^7 cells/mL) under anaerobic conditions, were illuminated with nonsaturating red flashes hitting 20% of centers given 6.6 s apart. The incubation medium consisted of HEPES-KOH 20 mM, pH 7.2, Ficoll 10%. DCMU and HA were added at the concentration of 10 μM and 1 mM, respectively, to block PS2 activity. Measurements were performed at 515 nm. Kinetics were corrected for membrane potential decay as described in Material and Methods. Data are normalized to the amplitude of the fast electrochromic signal. Measurements were performed in the absence (squares) of uncouplers, in the presence of nigericin 1 μM (circles) or nigericin plus 1 μM FCCP (triangles).

Functional Analysis of E78K and E78L. These two mutants accommodate an extensive change in the local charge of the residue found in position 78 of suIV, together with a limited increase in bulkiness. The negatively charged E78 residue found in the wild-type was replaced by a positively charged (lysine, E78K) or a neutral (leucine, E78L) residue.

(a) Kinetic Analysis of the Electrochromic Shift. Electron transfer through the cytochrome b_6f complex is accompanied by a charge translocation across the membranes which is detected as a slow rise phase of the 515 nm electrochromic signal, termed phase b (*24*). This process is highly sensitive to the large transmembrane electrochemical proton gradient which develops in darkness when the algae are placed in anaerobic conditions (*9*); consequently, they display a marked decrease in the turnover rate of the cytochrome b_6f complex upon illumination, mainly due to the pH dependence of PQH_2 oxidation reaction (reviewed in ref *25*).

Therefore, we compared the kinetics of phase b, in the wild-type and in the E78L and E78K mutants, in the absence and presence of nigericin, which suppresses the ΔpH component of the permanent electrochemical gradient but increases its electric component (see ref *26* for a discussion on the action mechanism of nigericin), as well as in the presence of both nigericin and FCCP, a treatment which totally collapses the transmembrane electrochemical gradient built up in darkness. The comparison is shown in Figure 2, where we analyzed phase b after a preillumination in the presence of DCMU and HA. This procedure inactivates photosystem 2. The kinetics of phase b are presented after normalization to the amplitude of the PSI-driven phase a, which has been measured 100 μs after the actinic flash. Phase a is set to a relative value of 1 on the ordinate axis, at the origin of the graph. Thus, only the changes in phase b are presented on Figure 2. The amplitude of phase b in the wild-type and the two mutants, reached $\sim$1.2 times that of phase a under repetitive flash illumination, given 6.6 s apart, in agreement with previous findings (*9*, *15*, *27*). It was, however, greater than what has been reported from in vitro measurements (*28*, *29*). The seemingly lower amplitude of phase b in the E78L mutant, when untreated, is the mere consequence of its much slower kinetics (see below) which have not reached their maximum amplitude after 50 ms.

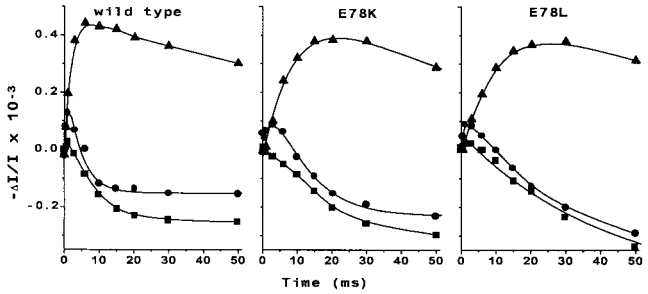

FIGURE 3: Cytochrome b_6 redox changes in wild-type and E78K and E78L cells. Same conditions as in Figure 2. Measurements were performed in the absence (squares) or presence (circles) of FCCP. NQNO (triangles) was added at the concentration of 4 μM (wild-type) or 8 μM (E78K and E78L mutants) to slow cytochrome b_6 oxidation through the Q_i site.

Table 1: Cytochrome *f* Reduction in Wild-type and E78 Mutant Strains[a]

strain	cytochrome *f* reduction (ms)	amplitude
wild-type	2.2	0.20
wild-type (+ *p*BQ)	15.0	
E78K	6.0	0.22
E78K (+ *p*BQ)	20.0	
E78L	10.2	0.26
E78L (+ *p*BQ)	26.3	
E78D	2.5	0.24
E78Q	4.2	0.25
E78N	4.0	0.22

[a] Figures refer to half-time of cytochrome *f* reduction after two saturating actinic flashes, measured in the absence of DCMU and HA, obtained after deconvolution of the signal from oxidation kinetics. The amplitudes are normalized to the amplitude of the phase a signal. For other conditions, see Material and Methods.

The sensitivity of the turnover of the cytochrome b_6f complex to the presence of the resting transmembrane potential in the wild-type is illustrated by the increase in the half-time of phase b from 7 ms, in the absence of uncouplers (squares on Figure 2), to 2.5−3 ms in their presence (triangles and circles). When a lysine (E78K) or a leucine (E78L) replaced the glutamic residue in position 78, the kinetics of phase b were slower in the three conditions we tested, with half-times of, respectively, 12 and 16 ms in the absence of uncouplers, 6 and 11 ms in the presence of nigericin, and 5 and 10 ms in the presence of both nigericin and FCCP. We note that the decrease in phase b kinetics in the mutants, was larger when the negatively charged glutamic residue was replaced by a neutral (leucine) than a positively charged (lysine) residue.

(b) Cytochrome b_6 Redox Changes. In Figure 3 are shown the spectral changes attributed to cytochrome b_6, with reduction phases drawn as an upward change whereas oxidation phases are shown as downward changes. The experimental conditions are the same as for the above detection of phase b of the electrochromic shift. The reduction phase is sensitive to FCCP addition in the three strains (compare circles and squares on Figure 3), in agreement with the increased rate of the concerted oxidation of plastoquinol at the Q_o site in uncoupled conditions (see above and Figure 2). The oxidation kinetics were slowed

to the same extent as those of phase b in E78K and E78L. Since cytochrome b_6 oxidation takes place only after reduction of the b_l heme, the decreased rates could reflect changes at various steps in the sequence of electron-transfer reactions, either from Q_o to b_l, from b_l to b_h, or from b_h to Q_i. Therefore, we measured the kinetics of cytochrome b_6 reduction in the presence of NQNO, in uncoupled conditions (triangles on Figure 3). This compound drastically slows down cytochrome b_h oxidation at the Q_i site (*28*) and therefore allows a better evaluation of the rate of reduction of cytochrome b_l. After correcting for the slow oxidation kinetics which still develop in the presence of NQNO (with $t_{1/2}$ in the time range of hundreds of milliseconds), we obtained half-times for cytochrome b_l reduction of ~5.5 and ~8.5 ms in E78K and E78L, respectively. These values are larger than that in the wild-type—half-time ≈ 2.5 ms—and are in agreement with the respective kinetics of phase b in the three strains.

(c) Cytochrome f Redox Changes. Because the kinetics of phase b and of cytochrome b_6 reduction were similarly affected in the two mutants, we analyzed the kinetics of cytochrome *f* rereduction after an actinic flash. As shown in Table 1, the half-times of this reaction correlated well with the above set of measurements, the rates in E78K and E78L being, respectively, 3 and 5 times slower than in the wild-type. These kinetic correlations indicated that the major effect of the mutations was at the PQH_2 oxidation step at the Q_o site, rather than on the interheme electron transfer in the low-potential chain.

To distinguish between an actual decrease in the rate of electron transfer from the plastoquinol bound at the Q_o site and a drop in the affinity for plastoquinol at this site, we looked at the kinetics of cytochrome *f* rereduction after a *p*BQ treatment of E78K and E78L. *p*BQ fully oxidizes the plastoquinone pool in vivo (*30*). Thus, the only PQH_2 available for the reduction of cytochrome b_6f after two saturating flashes, is the one formed at the acceptor side of PS2. The results shown in Table 1 indicate that the rates of cytochrome *f* reduction were less sensitive to a drop in plastoquinol concentration in the two mutants than in the wild-type. Therefore, a decreased affinity at the Q_o site is unlikely to occur in the two mutant strains. The present experiments rather support a slower rate of electron transfer at the Q_o site, which is now limited by the change in polarity introduced by the modified residue in position 78. We conclude that the mutation affects primarily the rate of the concerted electron-transfer reaction from bound plastoquinol to the cytochromes.

Functional Analysis of E78D, E78Q, and E78N. *(a) Kinetic Analysis of the Electrochromic Shift*. Substitution of the glutamic acid by its aspartic analogue caused no detectable change either in the rise or in the amplitude of phase b (compare E78D in Figure 4 with WT in Figure 2). The half-time was ~7 ms in the absence of uncouplers, but became much shorter upon addition of nigericin ($t_{1/2}$ ≈ 3 ms). Addition of FCCP had hardly any further effect in accelerating phase b ($t_{1/2}$ ≈ 2.5 ms), indicating that the kinetics are mainly dependent on the ΔpH component of the electrochemical gradient in this mutant, as is the case in the wild-type.

When a glutamine (E78Q) or an asparagine (E78N) were substituted for the glutamic residue, there was only a moderate effect—within a factor of 2—on the half-time of phase b (Figures 4 and 5). However, the two mutants were deeply altered in two other distinctive traits. First, not only the rate but also the amplitude of phase b became sensitive to the presence of uncouplers. At variance with the wild-type and the E78K, E78L, (Figure 2) or E78D mutants (Figure 4), the E78Q and E78N mutants displayed an amplitude of phase b about two times larger in fully uncoupled conditions than in untreated cells. Nigericin alone caused only a limited increase in the amplitude of phase b, although its half-time was similarly shortened as in wild-type cells. Second, the amplitude of phase b reached in the presence of FCCP was by far larger than that in all other strains compared in this study, with a mean value of about 1.6 instead of the usual 1.2. These values were obtained after normalization to the same amplitude of phase a taken as 1 (compare Figures 2 and 4) and after carefully checking that they were not the result of our procedure of deconvolution from the membrane potential decay (data not shown). It should be emphasized that the same increase in the amplitude of the b phase was observed in the presence of

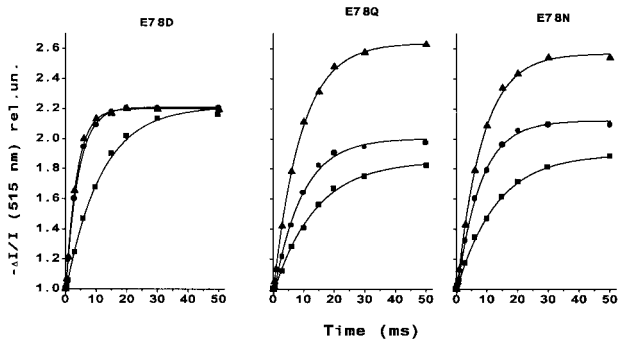

FIGURE 4: Slow electrochromic reaction in wild-type and E78D, E78Q, and E78N mutants. Same conditions as in Figure 2. Measurements were performed in the absence (squares) of uncouplers, in the presence of nigericin 1 μM (circles) or nigericin plus 1 μM FCCP (triangles).

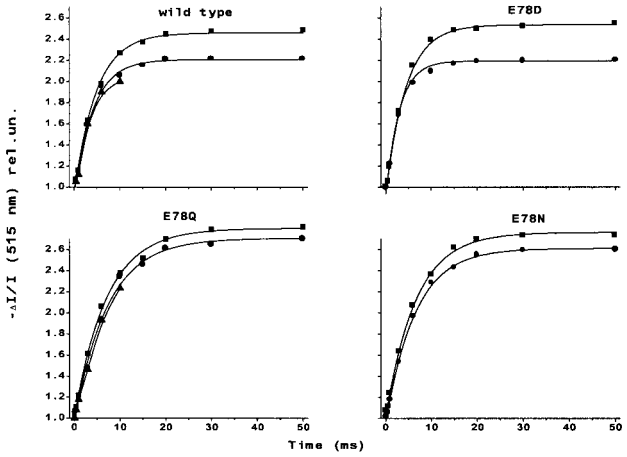

FIGURE 5: Effect of the illumination frequency on the slow electrochromic reaction in wild-type, E78D, E78Q, and E78N cells. (Squares) Single flash illumination. (Circles) Repetitive flashes given 6.6 s apart. (Triangles) Repetitive flashes given 1.25 s apart. FCCP 1 μM was present in all samples to collapse permanent membrane potential. Only the first data points of the kinetics measured under repetitive flash illumination given 1.25 s apart are shown, because this regime did not allow complete decay of the membrane potential, and therefore, the curves could not be deconvoluted correctly.

other ionophores, such as nonactin (data not shown), which excludes any indirect effect of FCCP per se.

The above observations indicate that a change in the stoichiometry of charges crossing the thylakoids membranes per cytochrome b_6f turnover has likely occurred in the E78Q and E78N mutants, through a mechanism highly sensitive to the electrical component of the transmembrane potential, since nigericin allows relaxation of the sole pH component of the electrochemical proton gradient. We checked that the amplitude of phase a, on a cell concentration basis, remained the same in the various strains (data not shown). It should be noted that we observed, in the cases of the E78Q and E78N mutants only, significant variations in phase b/phase a ratios from one batch of mutant to another, with values ranging from 0.7 to 1 in the absence of uncouplers and from 1.5 to 1.8 in their presence. These variations thus appear as being a genuine part of the mutants' phenotype.

We could also observe a larger phase b/phase a ratio in the wild-type and E78D mutants, provided we used a single flash illumination regime instead of actinic flashes given 6.6 s apart (Figure 5). The latter condition was achieved by incubating the algae for at least 1 h in darkness under an argon flux in the reservoir before giving a single actinic flash. Under these conditions, the phase b/phase a ratio increased to a value of 1.5 without any significant effect on the half-time of the reactions. In the case of the E78Q and E78N mutants, we found that the amplitude of phase b was only marginally affected by this illumination regime, reaching the maximal average value of 1.7 under single flash illumination.

(b) Cytochrome b_6 Redox Changes. It has been reported previously that b_h is more rapidly rereduced than b_l in the dark time interval between flashes (*31*). Changes in the dark time interval between flashes thus offered the opportunity to vary specifically the redox state of the latter heme which cannot be otherwise distinguished spectroscopically (*2*). We monitored the kinetics of the redox changes of cytochrome b_6 under single flash illumination and under repetitive flashes given 6.6 or 1.25 s apart. Measurements were performed in the presence of FCCP, to avoid complication related to the proton gradient-dependent decreased rate in cytochrome b_6f turnover (*9*), which are illustrated by the effect of uncouplers on the half-time of the 515 nm changes described above and on cytochrome b_6 turnover (Figure 3).

As shown in Figure 6, the first part of the kinetics of cytochrome b_6 redox changes in the E78D and wild-type strains under single flash illumination corresponds to a small absorption increase, due to cytochrome b_l reduction. It is followed by a large absorption decrease, due to the oxidation of both hemes. Decreasing the dark time interval between flashes to 6.6 then 1.25 s induced a stepwise increase in the extent of cytochrome b_6 reduction, associated with a parallel decrease in the extent of the oxidation signal. These observations are easily explained in terms of less heme b_h being rereduced when the dark time interval between flashes is shorter.

In E78Q and E78N, the amplitude of the reduction signal was much smaller than that in the wild-type for all illumination regimes. This is at variance with the oxidation kinetics which remained of similar amplitude as in the wild-type, although they were somewhat slower. It is noteworthy that the oxidation kinetics retained the same dependency on the duration of the dark period between flashes as in the wild-type.

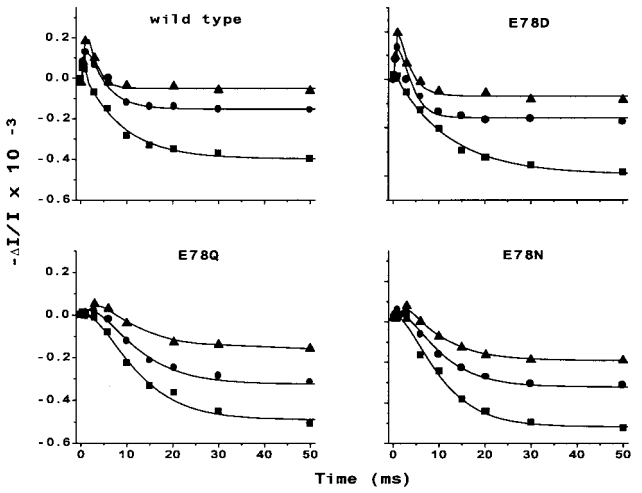

FIGURE 6: Effect of the illumination frequency on cytochrome b_6 reduction in wild-type, E78D, E78Q, and E78N cells. Same conditions and symbols as in Figure 5.

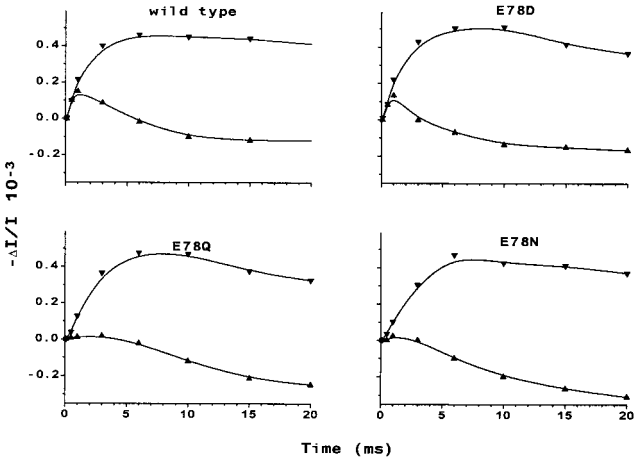

FIGURE 7: Cytochrome b_6 reduction in wild-type, E78D, E78Q, and E78N cells**.** Upward triangles FCCP 1 μM. (Downward triangle) NQNO and FCCP were present at the concentration of 4 and 1 μM, respectively. Others conditions as in Figure 3.

To further investigate the quasi absence of a reduction phase in the kinetics of cytochrome b_6 in E78Q and E78N, we measured cytochrome b_6 kinetics in the presence of NQNO which blocks electron transfer at the Q_i site. We observed that the average values of the amplitude of the reduction phase were now similar in the two mutants and in the wild-type or its E78D counterpart (Figure 7). We noted that the amplitude of the signal with the mutants was somewhat more variable than with the wild-type (data not shown). In the wild-type and E78D mutant, the initial rates of reduction of cytochrome b_6 remained similar whether NQNO was added or not. This is in great contrast to the case of the E78Q and E78N mutants, in which the initial rate of cytochrome b_6 reduction became similar to that in the wild-type only in the presence of NQNO. The overall rates of cytochrome b_6 reduction in the presence of NQNO remained within a factor of 2 between the latter two mutants and the wild-type.

(c) Cytochrome f Redox Changes. The amplitudes and half-times of cytochrome *f* reduction after a flash were

compared in the different strains (Table 1). Cells were treated with FCCP and subjected to a repetitive flash illumination regime, with dark time intervals of 6.6 s. The amplitude and half-time of cytochrome *f* rereduction were very similar in E78D and wild-type cells. In E78Q and E78N, the rate of the reduction signal, whose amplitude was similar to that in the wild-type, were about two times slower and within the range we calculated for phase b. These observations indicate that the efficiency of electron injection in the high-potential chain from the Q_o site in the mutants remained close to that in the wild-type. In addition the two reductive pathways—involving the low- and high-potential chain—could not be distinguished kinetically in the E78Q and E78N mutants, a situation similar to that in the other mutants presented in the previous section and in the wild-type.

DISCUSSION

Many mutations conferring resistance to Q_o site inhibitors in cytochrome *bc* complexes have been mapped in the loop between helices E and F of cytochrome *b* (*12*, *32*). The conserved $_{77}$PEWY$_{80}$ sequence from this loop is located in subunit IV in cytochrome b_6f complexes (*16*). In *Rhodobacter sphaeroides*, substitutions within the PEWY sequence resulted in mutants which still assembled cytochrome bc_1 properly but showed some alteration in electron transfer (*33*). These experiments suggested that, although the exact PEWY sequence was not critical for assembly of the protein complex, the constitutive residues were good candidates to contribute in some way to the structure and function of the Q_o site in cytochrome b_6f complexes. Recent progress in the knowledge of the 3D structure of mitochondrial cytochrome bc_1 complexes (*17*, *34*) has provided a molecular basis for the critical function of the PEWY sequence: it closes the Q_o site at the intermembrane side of the inner mitochondrial membrane and the E residue has its side chain ending at about 4 Å from the edge of the b_l heme plane.

In agreement with the substitutions previously performed on the cytochrome *bc* complex (*33*), the single substitutions we made at the E78 position in the PEWY sequence of subunit IV, most of which primarily altered the local polarity, allowed us to recover phototrophic mutants in which assembled cytochrome b_6f complexes displayed marked functional alterations.

Replacement of the glutamic residue by an aspartic residue caused no kinetic changes. Two substitutions, by a lysine or a leucine, caused a decrease in the kinetics of electron transfer through the cytochrome b_6f complex, as measured by the changes in rates of the electrochromic shift and cytochrome *f* reduction. Two other substitutions, by a glutamine or an asparagine, had limited effects on the kinetics but caused a marked increase in the electrogenicity associated with the electron transfer through the cytochrome b_6f. It should be emphasized that most of the properties we discuss below relate to cytochrome b_6f behavior in vivo, in reducing conditions, i.e., in anaerobic conditions where the plastoquinone pool is fully reduced. This is a physiological state which is frequently experienced by green microalgae in their natural environment.

E78K, E78L, and E78D Mutants. In E78K and E78L, the rates of reduction of cytochrome b_6 and cytochrome *f* were both slowed, which suggested a decrease in the rate of the concerted oxidation of PQH_2 at the Q_o site. Quinol oxidation at the Q_o site requires the presence of a bound quinol, which can then be oxidized by the concerted reaction with the Rieske protein and cytochrome b_6, in a complex mechanism which involves proton release in the thylakoid lumen. Therefore, the rate of concerted oxidation of quinols is a function of the affinity constant for the Q_o site and of the rate constant of the electron and proton transfer reactions. We believe that these two mutants are primarily affected in the rate constant of electron/proton transfer. Indeed, decreasing the stoichiometry of PQH_2 to cytochrome b_6f from 10 to 1 (Table 1) had a limited kinetic effect, in marked contrast with what we had observed previously with the FUD2 mutant which displayed a 100-fold decrease in the affinity of the Q_o site for plastoquinol (*15*). Moreover, the relative kinetic decrease upon *p*BQ treatment was lower in the mutants than in the wild-type, as we would expect if the rate of electron transfer was intrinsically slower in the mutants.

Of course, minor changes in affinity cannot be excluded. The affinity of the Q_o site for PQH_2 in vivo is high enough in the wild-type to prevent any functional effect when the PQH_2/PQ ratio is reduced by about a factor of 2 (*15*). Thus, were the E78D mutant to accommodate a decrease in the affinity constant by about the same amplitude as in the homologous *Rb. sphaeroides* E295D mutant (*33*), it would not result in any functional phenotype.

It should be noted that our study does not support a direct participation of the E78 residue in the electron-transfer mechanism at the Q_o site that would implicate a hydrogen bonding, for instance, with the *b* heme: shortening the length of the side chain, as in the E78D mutation, had no effect on the kinetics at variance with the effect of similar substitutions on electron-transfer reactions in the reaction center of photosynthetic bacteria (*35*). Moreover, replacement of a negatively charged residue in position 78 by a positively charged residue, as in the E78K mutant, had less severe consequences on the kinetics than the replacement by a neutral residue (E78L). Rather, a steric hindrance, due to the increase in bulkiness of the K and L residues as compared to the original glutamic residue (*36*), seems primarily responsible for the slower electron transfer.

E78Q and E78N Mutants: Cytochrome b_6 Redox Changes. A most intriguing aspect of the E78Q and E78N phenotypes is the quasi absence of a reduction phase for cytochrome b_6 following an actinic flash. Several lines of evidence led us to exclude that the lack of a reduction phase would be due either to a much slower rate of electron transfer from PQH_2 to the *b* hemes or to a larger regeneration of fully reduced cytochrome b_6f complexes (where both hemes are reduced before the actinic illumination) in the dark time interval between two consecutive flashes. The first hypothesis is ruled out by the NQNO experiment (Figure 7), showing only a limited decrease in the rate of flash-induced cytochrome b_6 reduction in the two mutants, that should be compared with the much slower kinetics at the Q_o site in the E78L and E78K mutants which still display a reduction phase for cytochrome b_6 (Figure 3). The second hypothesis is excluded because (i) the amount of oxidized cytochrome b_6 before a flash, measured by the amplitude of cytochrome b_6 reduction in the presence of NQNO, is the same in the E78Q, E78N,

and wild-type strains; (ii) the oxidation phases for cytochrome b_6 at a given frequency of flashes are of similar amplitudes in E78Q, E78N, and wild-type (Figure 6), which indicates a same proportion of fully plus semireduced cytochrome b_6f complexes between flashes in the three strains.

We believe that the most likely interpretation to the quasi absence of a flash-induced reduction of cytochrome b_6 in the E78Q and E78N mutants is that of a shift of the $PQ^{\bullet-}/b_l$ equilibrium in favor of $PQ^{\bullet-}$. The reduction of cytochrome b_6 from the Q_o site is governed indeed by the equilibrium between $PQ^{\bullet-}$, the b_l, and the b_h hemes (see recent reviews in refs *7*, *37*, *38*). When the two *b* hemes are in an oxidized state before the flash, there is little, if any, $PQ^{\bullet-}$ remaining at Q_o after electron donation to the high-potential chain, owing to the large equilibrium constant between b_h- and b_l. In contrast, the proportion of $PQ^{\bullet-}$ increases in semireduced cytochrome b_6f complexes, where b_h is reduced but b_l is oxidized before the flash, because the draining effect of oxidized b_h is lacking. This explains why the extent of cytochrome *b* reduction is sensitive to the illumination regime in wild-type (Figure 6), which is expected to affect the relative proportion of oxidized and semireduced complexes (*31*). Assuming a shift in the $PQ^{\bullet-}/b_l$ equilibrium in the E78Q and E78N mutants, one predicts a decrease in the proportion of electrons residing on b_l after electron donation to the high-potential chain, thus preventing detection of a reduction phase for cytochrome b_6 without affecting the extent of the subsequent oxidation phase.

Phase b/Phase a Ratios. Another striking functional change of the mutated cytochrome b_6f complexes in the E78Q and E78N mutants is the increased number of charges translocated across the membranes per electron injected in the high-potential chain. The steps of electron transfer from b_l to b_h and from b_h to the PQ molecule at the Q_i site contribute, respectively, 60 and 40% to the total electrogenicity of one charge translocated from one side of the thylakoid membrane to the other (*6*, *9*, *39*, *40*, *41*). Given the state of reduction of the cytochrome b_6f complex before the flash, both the Q cycle and semiquinone cycle predict a *fixed* and predictable stoichiometry of charges crossing the membrane during the turnover of cytochrome b_6f. Thus, in the absence of changes in the reduction state of cytochrome b_6f complexes before the flash (see section above), neither the Q cycle nor the semi-Q cycle can account for the increased phase b/phase a ratio observed in the E78Q and E78N mutants (Figure 4). Moreover, the amplitude of phase b becomes larger in the mutants, above 1.7, than the maximal electrogenicity of 1.4 predicted by the Q and semi-Q cycles when all complexes are in a semireduced state before the flash.

The variable amplitude of the phase b can be easily accounted for by a variable contribution of an ionic-pumping activity coupled to cytochrome b_6f turnover. As expected for such a pump, we observed that the extra electrogenicity is sensitive to FCCP and nonactin but not to nigericin. Thus is is collapsed by the $\Delta\Psi$ component of the electrochemical gradient.

Ionic-pumping activities have been identified, or proposed, in several energy transducing complexes, cytochrome *c* oxidase (*42*), bc_1 mitochondrial complex (*43*), and the cytochrome b_6f complex itself (*39*). Also, Mulkidjanian and colleagues (*44*, *45*) have proposed that protons coming from ubiquinol are transferred reversibly toward the Q_i site in *Rb. Spaeroides* bc_1 complex, to compensate electrostatically the electron injection into the *b* heme chain. Brandt and Trumpower (*46*) have recently discussed the effect of DCCD on bacterial and mitochondrial cyt *bc* complexes turnover. They considered the occurrence of a proton slip to account for a partial decoupling of proton translocation in mitochondrial cytochrome *bc*. This concept provides a link between the translocation of protons across the membranes and *b* hemes redox changes. It is fully consistent with the effect of DCCD on the activity of the cytochrome b_6f complex in vivo which behaves as an inhibitor of a redox-coupled proton pump (*47*).

Several residues in the vicinity of the *b* hemes have been identified as proton donors in the case of bc_1 complexes (reviewed in ref *16*). The infrared signal of some of them showed reversible changes during the turnover of the complex (F. Baymann, personal communication). They suggest the existence of several proton wires whose relative contribution may be affected *indirectly* by a substitution at the E78 position. The E78 residue itself should not be a critical component of these proton wires, otherwise dramatic changes in the phase b/phase a ratio would be expected upon an acid−base substitution as in E78K, an effect which was not observed (Figure 2).

Are the Increased Phase b/Phase a Ratio and the Quasiabsence of a Reduction Phase for Cytochrome b_6 Mechanistically Related? These two major changes in cytochrome b_6f function occur together in the E78Q and E78N mutants. Also, they appear together in the wild-type when a single flash illumination regime is used. It is then likely that they are intimately associated.

Correlated changes in proton translocation and charge equilibrium between $PQ^{\bullet-}$ and b_l can be understood equally well in the two following ways: (i) changes in the protonation state of residues located around the Q_o site influence the stability of the semiquinone, thus affecting the $PQ^{\bullet-}/b_l$ equilibrium; (ii) the $PQ^{\bullet-}/b_l$ equilibrium affects the charge distribution in the Q_o site, thus inducing protonation or deprotonation events by means of p*K* shifts of nearby residues. If one assumes that among the alternative proton wires potentially operating one could involve an electrogenic proton transfer from the stromal side of the membrane (as suggested in refs *38* and *46*, for instance), it then turns out that any event affecting the $PQ^{\bullet-}/b_l$ equilibrium should affect the phase b/phase a ratio as well.

The injection of an electron in the low-potential chain represents a critical regulation step in the function of cytochrome b_6f complexes. Some of the mutants presented in this study argue for the contribution of alternative charge transfer pathways, which depend on the redox state of the plastoquinone pool and on the amplitude of the resting electrochemical potential gradient across the membranes. While the experimental conditions we have chosen—a microalga in anaerobic conditions—allowed us to observe the above-described variations in the stoichiometry of charges translocated across the membrane per charge injected in the high-potential chain, we do not know at present if the same properties would apply to other *bc*-type complexes operating in another environment. Further structural data combined with site-directed mutagenesis approaches are now required

to critically assess the presence of a proton pump associated with cytochrome b_6f complexes.

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