In Vivo Analysis of the Effect of Dicyclohexylcarbodiimide on Electron and Proton Transfers in Cytochrome *bf* Complex of *Chlorella sorokiniana*†

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ABSTRACT: The effect of *N*,*N*′-dicyclohexylcarbodiimide (DCCD) on electron and proton transfers within the cytochrome (cyt) *bf* complex has been analyzed in living cells of the green algae *Chlorella sorokiniana* under anaerobic conditions. DCCD induces a partial decoupling of the protomotive Q-cycle, in agreement with the conclusions of Wang and Beattie (1991) *Arch. Biochem. Biophys. 291*, 363−370. In the presence of 20 μM DCCD, we observe the development of a lag phase in the kinetics of the slow electrogenic phase associated with electron and proton transfers within the cyt *bf* complex. In the same conditions, the initial rate of cyt *b* and cyt *f* reduction is decreased by about 30%. We propose that in the absence of DCCD, a transmembrane movement of proton is coupled to the oxidation of plastoquinol at site Q_o. In the presence of 20 μM DCCD, this redox-coupled proton pump is inhibited, and the kinetics of phase b and cyt *b* reduction become close to that predicted on the basis of a pure Q-cycle process. In agreement with this hypothesis, we observe that upon a weak-flash excitation, two charges are translocated through the membrane in addition to the charge translocated at the level of photosystem I. Part of this large electrogenic phase could be associated with the translocation of a proton from the stroma to the lumen. A tentative mechanism is discussed that remains in the frame of the Q-cycle but accounts for an additional proton-pumping process or for the partial decoupling observed in the presence of DCCD, as well.

The cytochrome (cyt)[1] *bf* complex catalyses the electron-transfer reactions between plastoquinol and plastocyanine. The formation of a transmembrane potential and the transfer of proton from the stroma to the lumen are associated with electron-transfer reactions within the cyt *bf* complex. The functional properties of cyt *bf* complex are reviewed in refs *1* and *2*. Four electron carriers are included within the complex. The Rieske FeS protein (FeS) and cyt *f* [E_{m8} + 330 mV, (*3*)] belong to a high-potential chain and are located close to the lumen. The low-potential chain consists of two hemes: cyt b_h and cyt b_l [$E_{m8} \sim -84$ mV, and $E_{m8} \sim -158$ mV, respectively (*3*)]. The properties of a fifth electron carrier G (*4*), a hemoprotein localized on the stromal side of the complex, will not be discussed here. Plastoquinol (PQH_2) is oxidized at a site Q_o close to the lumen, while plastoquinone PQ is reduced at a site Q_i close to the stroma. The processes of electron transfer and proton pumping within the cyt *bf* complex are most generally interpreted according to the mechanism of the Q-cycle, proposed by Mitchell (*5*) and modified by Crofts et al. (*6*). In dark-adapted algae, in anaerobic conditions in which the plastoquinone pool is reduced prior to the flash excitation, the Q-cycle is initiated by the transfer of a positive charge from P700, the primary donor of photosystem (PS)I, to the high-potential chain of the cyt *bf* complex. Then, in complexes with a fully oxidized low-potential chain (b_l^+ b_h^+), the following sequence of reactions occurs:

$$PQH_{2\ (Q_o)} + (\text{FeS cyt } f)^+ + (b_l^+\ b_h^+) \rightarrow PQ_{(\text{membrane})} + (\text{FeS cyt } f) + (b_l\ b_h^+) + 2H^+_{(\text{lumen})} \quad \text{(a)}$$

$$(b_l\ b_h^+) \rightarrow (b_l^+\ b_h) \quad \text{(b)}$$

In complexes including a semireduced low-potential chain (b_l^+ b_h), the following sequence of reactions occurs:

$$PQH_{2\ (Q_o)} + (\text{FeS cyt } f)^+ + (b_l^+\ b_h) \rightarrow PQ_{(\text{membrane})} + (\text{FeS cyt } f) + (b_l\ b_h) + 2H^+_{(\text{lumen})} \quad \text{(a′)}$$

$$(b_l\ b_h) + PQ_{(Q_i)} + 2H^+_{\leftarrow \text{stroma}} \rightarrow (b_l^+\ b_h^+) + PQH_{2\ (\text{membrane})} \quad \text{(b′)}$$

Under repetitive saturating flashes (<1 s apart) or continuous light excitation, about half of the complexes are in the (b_l^+ b_h^+) state and half in the (b_l^+ b_h) state. The fraction of complexes in the (b_l^+ b_h) state increases with the time interval between flashes because of the reduction of cyt b_h^+ in the dark, which occurs in a several-second range of time (*7*).

A flash excitation induces a fast increase of the membrane potential (phase a <100 ns). When PSII is inactive, the amplitude of the phase a is proportional to the number of PSI charge separations (*8*). A second phase (phase b), completed in 50 to 200 ms, is associated with electron and proton-transfer reactions within the cyt *bf* complex (*9*). This

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[1] Abbreviations: cyt, cytochrome; b_h, cytochrome b_h; b_l, cytochrome b_l; PS, photosystem; FeS, the Rieske iron sulfur protein; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone; NQNO, 2-*n*-nonyl-4-hydroxyquinoline oxide.

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phase is fully inhibited by specific inhibitors of the Q_o site as dinitrophenyl ether of iodonitrothymol (*10*). The normalized amplitude of phase b (phase b/phase a) is equal to the ratio between the number of charges translocated from the lumen to the stroma within the cyt *bf* complex and the number of charges translocated at the level of PSI. From experiments in purple bacteria, it is generally assumed that the reduction of cyt b_l^+ [reactions (a) and (a′)] is not electrogenic (*11, 12*). For complexes initially in the (b_l^+ b_h^+) state, phase b is associated with reaction b. The normalized amplitude of phase b associated with the transfer of one electron from cyt b_l to cyt b_h is ~0.6 (*12*). For complexes initially in the (b_l^+ b_h) state, the normalized amplitude of phase b associated with reaction (b′) is $1.4 = 0.6 + (2 \times 0.4)$. Under repetitive-flash excitation, the amplitude of phase b is $1 = (0.6 + 1.4)/2$.

A conventional Q-cycle mechanism does not account for a number of experimental observations in algae and in isolated chloroplasts:

(1) A large electrogenic phase associated with the oxidation of cyt b_l is observed when both *b* cyts are in their reduced form (*13*−*15*). In these conditions, reactions (a) and (a′) cannot operate in a Q-cycle process. Kramer and Crofts (*16*) proposed a cooperative model, in which several positive charges from the plastocyanine pool cooperate at the level of a same cyt *bf* complex. We observe no decrease in the normalized amplitude of phase b or slowdown of its kinetics when the energy of the flash, and therefore the number of positive charges available among PSI secondary donors, tends toward zero (*15*). On the other hand, in reducing conditions, phase b is not inhibited by NQNO (*14*), which is known to bind to site Q_i (*17*). These results make a cooperative mechanism quite unlikely.

(2) According to a Q-cycle mechanism, the complexes in the (b_l^+ b_h) state undergo a nonelectrogenic reaction (a′), followed by an electrogenic reaction (b′). Actually, we observed no lag phase which could be associated with reaction (a′). The initial rate of phase b is independent of the redox state of cyt b_h, and is always larger than that expected from a Q-cycle mechanism (*15*).

(3) Hope and Rich (*18*) reported that the proton uptake on the stromal face is not inhibited by NQNO, suggesting that a proton uptake is not necessarily associated with reactions at site Q_i.

(4) Zito et al. (*35*) observed that, upon repetitive-flash excitation of a mutant strain of C*hlamydomonas reinhardtii* in which a glutamic acid residue in position 78 has been replaced by a glutamine residue, phase b has a larger amplitude (~1.7) than that predicted by a Q-cycle process (<1.4).

(5) In experiments performed with purified cyt *bf* complex reconstituted in liposomes, Wang and Beattie (*19*) reported that DCCD binds to the cyt *bf* complex and induces a large inhibition of the proton-pumping process together with a minor inhibition of the rate of the electron transfer. Beattie and Villalobo (*20*) previously reported similar results in the case of the mitochondrial cyt bc_1 complex. Such behavior is difficult to interpret in terms of a Q-cycle (or semi Q-cycle) process, in which the stoichiometry between electron transfer and proton pumping is invariant.

To answer to points (1) and (2), we proposed (*15*) that the cyt *bf* complex operates according to a semiquinone cycle, as in the model proposed by Wikström and Krab (*21*). In this model, a part of the charged semiquinone PQ^- formed at site Q_o is transferred to the site Q_i; this transfer is responsible for the initial electrogenic phase that is not associated with cyt *b* reduction. In another class of models, a proton pump, involving a translocation of protons, is coupled to the redox changes of electron carriers (*22*). In the original form of the *b*-cycle, Wikström and Krab (*23*) proposed that the proton pump is coupled to the redox changes of cyt *b*. Girvin and Cramer (*13*) assumed that the proton pump is coupled to the redox changes of the FeS protein. We proposed (*14*) a model in which phase b, measured in highly reducing conditions, is associated with the transfer of a proton from the stroma to site Q_o, via a transmembrane proton channel.

In this paper, we analyze the effect of DCCD, an inhibitor of proton channels, on the kinetics of phase b and cyt *b* redox changes in living algae under anaerobic conditions, aiming at a choice between these different hypotheses.

MATERIALS AND METHODS

Experiments were performed under anaerobic conditions with the S8 mutant strain of *Chlorella sorokiniana* lacking PSII, isolated by Bennoun and characterized by Lacambra et al. (*24*). Spectrophotometric measurements were performed with an apparatus similar to that described in refs *25* and *26*. Actinic excitation is provided by xenon flashes (3 μs half-duration) filtered through red RG8 Schott filters. The variation of the membrane potential is measured by the electrochromic shift of membrane pigments at 515 nm. We have checked that the relative amplitude and kinetics of phase b are similar when measured at 515 nm or by the difference (515 nm − 545 nm). The absorption changes measured at 515 nm have been normalized to phase a, which is proportional to the number of PSI charge separations.

In the presence of FCCP, the decay of the membrane potential is close to an exponential function. At each experiment, the rate constant of the membrane potential decay is measured after completion of phase b and the kinetics of phase b is corrected for this decay. In the presence of a low concentration of FCCP (2−3 μM), the decay of the membrane potential ($t_{1/2}$ is 300−600 ms) is a much slower process than phase b, which is completed in ~25 ms. The large difference in the time-range of these two processes makes the correction procedure accurate.

The stock solution of DCCD was prepared in dimethyl sulfoxide. Two procedures were used for the addition of DCCD. (1) In the experiment of Figure 2, DCCD was added at room temperature in the reservoir connected to the measuring cuvette. In these conditions, we observed a large variation of the inhibitory effect of DCCD from one algae culture to another. (2) DCCD was added for 25 min at 35 °C to the algae in the growth medium. Then, after centrifugation, the sample is suspended in the buffer with the same DCCD concentration. This procedure, used in the experiments of Figures 1, 3, and 4, leads to reproducible results.

RESULTS

Figure 1 shows the kinetics of phase b under subsaturating-flash excitation in order to decrease the probability of double

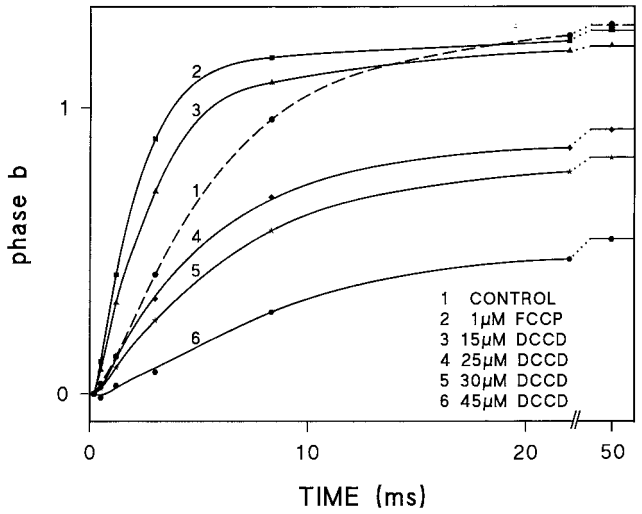


FIGURE 1: Effect of DCCD or FCCP on the kinetics of phase b. 50 mM phosphate buffer, pH 7.2, 7% ficoll; subsaturating-flash excitation, hitting ~25% of PSI centers; time interval between flashes, 5.4 s. The kinetics of the flash-induced increase of the membrane potential has been measured at 515 nm and normalized to the amplitude of phase a, measured at 100 μs. Curve 1, no addition; 2, 1 μM FCCP; 3, algae were incubated in the growth medium for 25 min at 35 °C in the presence of 15 μM DCCD. After centrifugation, algae were resuspended in phosphate buffer *plus* 15 μM DCCD; 4, 5, and 6, same procedure as 3 but with 25 μM, 30 μM, 45 μM DCCD, respectively.

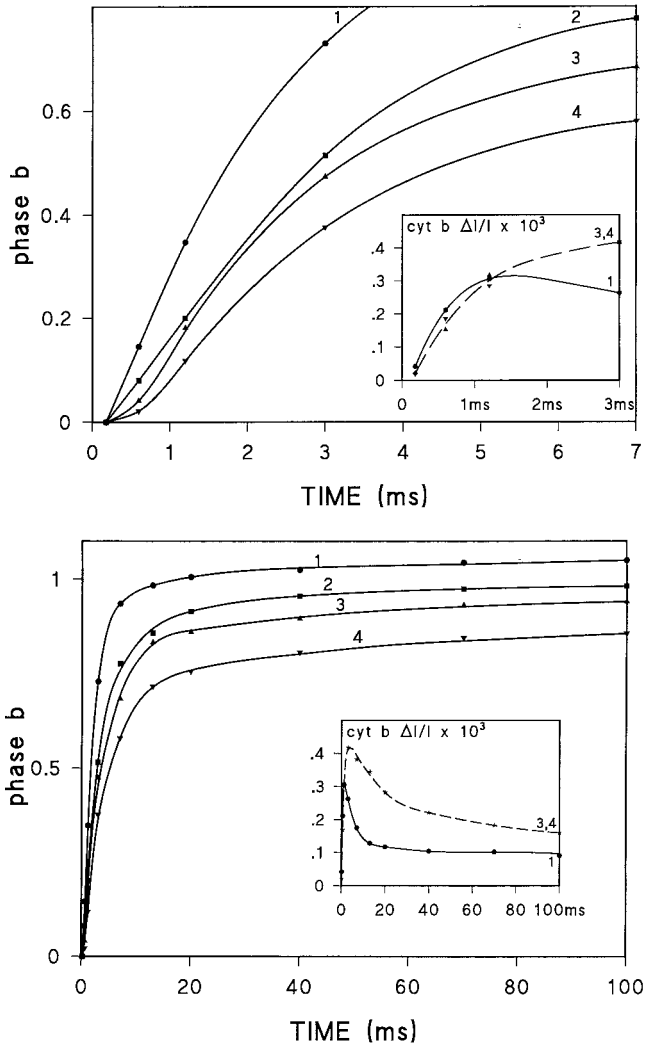


FIGURE 2: (A and B). Effect of DCCD on the kinetics of phase b and cyt *b* redox changes (insert). 50 mM phosphate buffer, pH 7.2, 7% ficoll, 2 μM FCCP; subsaturating-flash excitation hitting 43% of PSI centers; time interval between flashes, 5.4 s; Curve 1, control; 2, 20 μM DCCD added at room temperature, ~60 min incubation; 3, 75 min incubation; 4, 97 min incubation. Insert: kinetics of cyt *b* redox changes $\Delta I/I$ (564 nm − 573 nm); solid line, 1: control. Dashed line, 3, 4: 20 μM DCCD; average of curves 3 and 4 (75 and 97 min incubation, respectively). The signal is not corrected for the small absorption changes associated with plastocyanine and the electrochromic shift. The absorption change $\Delta I/I$ (564 nm − 573 nm) = 1×10^{-3} corresponds to the transfer of 0.6 electron to cyt *b* per positive charge transferred to the high-potential chain.

turnovers within cyt *bf* complex. As previously shown (*15*), the addition of 1 μM FCCP induces a ~2.6-fold increase of the rate of phase b (curve 2) compared to the control (curve 1). This concentration of uncoupler is high enough to induce a leak through the membrane, which progressively (in ~10 min) exhausts the ATP reserves and subsequently collapses the dark electrochemical proton gradient. At this low concentration, FCCP induces only a minor acceleration of the decay of the light-induced membrane potential. In the absence of uncoupler, the addition of 15 μM DCCD (curve 3) also induces an acceleration of phase b, although of a lesser extent (~1.9-fold). This acceleration is due to the inhibition of the membrane ATPase, which prevents the formation of a permanent proton gradient. At concentrations higher than 15 μM (curves 4 to 6), DCCD induces the development of a lag phase in the millisecond range and a decrease of the amplitude of phase b. We have obtained similar results with whole cells of *C. reinhardtii* (not shown). The addition of 30 μM DCCD induces a well-defined lag phase (~2 ms duration) and a decrease in the amplitude of phase b of about 40%.

The effect of DCCD on the kinetics of phase b (Figure 2) and of cyt *b* redox changes (Figure 2, inserts) are shown on two time-scales (2A and 2B). Curves 2 to 4, obtained for different times of incubation of 20 μM DCCD added at room temperature, show that the initial rate of phase b tends toward zero for increasing time of incubation in the presence of DCCD. In the same conditions, the initial rate of cyt *b* reduction is decreased by only ~25%. In Figure 2B (insert), one observes that DCCD induces an inhibition of the rate of cyt *b* oxidation.

The effects of DCCD and NQNO on the kinetics of phase b (Figure 3) and of cyt *b* redox changes (Figure 4) have been measured at pH 9 in the presence of FCCP (see also Table 1). As already shown in Figure 2, the initial rate of phase b in the presence of DCCD is close to zero, while the initial rate of cyt *b* reduction is decreased by only 30%. DCCD similarly slows down the rate of cyt *f* reduction ($t_{1/2}$ 0.8 ms and 1.1 ms in the absence or presence of DCCD, respectively). NQNO induces a marked increase of the amplitude of cyt *b* reduction associated with a slight stimulation of the initial rate of both cyt *b* reduction and phase b. The kinetics of phase b displays two phases ($t_{1/2}$ ~ 0.8 and ~10 ms, respectively). The addition of NQNO in the presence of FCCP *plus* DCCD induces a lag phase in phase b and a decrease of ~20% in the initial rate of cyt *b* reduction. We conclude that during the first millisecond following the flash excitation, DCCD acts in a very similar way in the presence or absence of NQNO.

Similar effects of DCCD are observed at pH 6.2 (not shown) and pH 9 with somewhat larger inhibitory effects

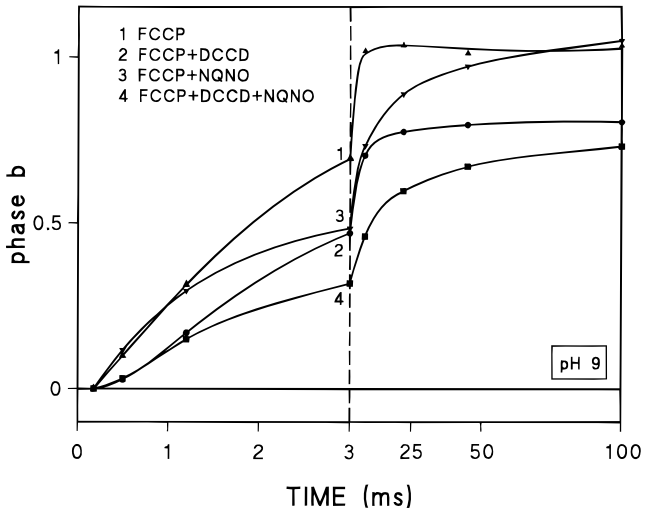


FIGURE 3: Effect of DCCD or NQNO on the kinetics of phase b. 20 mM Tris buffer, pH 9, 7% ficoll, subsaturating-flash excitation hitting 30% of PSI centers; time interval between flashes, 3.4 s. Curve 1, 1.5 μM FCCP; 2, algae were incubated in the growth medium for 30 min at 35 °C in the presence of 30 μM DCCD and then resuspended in Tris buffer in the presence of 30 μM DCCD *plus* 0.25 μM FCCP; 3, same procedure as in 2, but in the presence of 3 μM NQNO instead of DCCD; 4, same procedure as in 2, but *plus* 3 μM NQNO.

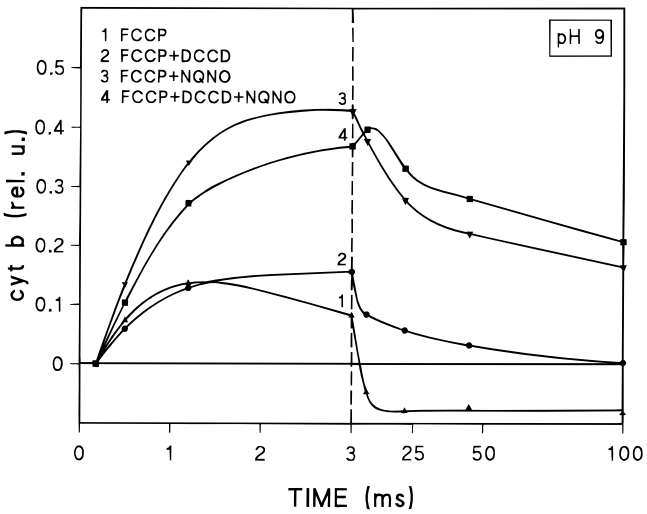


FIGURE 4: Effect of DCCD or NQNO on the kinetics of cyt *b* redox changes. Same conditions as in Figure 3: cyt *b* is measured as the difference between the absorption at 564 nm and a baseline drawn between 545 and 573 nm. This procedure eliminates the absorption changes associated with plastocyanine. The signal is further corrected for the electrochromic absorption changes (i.e., $-0.02 \times (\Delta I/I$ 515 nm). Cyt *b* redox changes are normalized as follows: algae are dark-adapted in anaerobic conditions in the presence of 5 mM dithionite *plus* 1 μM safranine T which induce a full reduction of the two *b* cyts. Upon excitation by a flash weak enough to induce no double turnover within cyt *bf* complex, all the positive charges formed by PSI are transferred to cyt b_l, and the amount of oxidized cyt b_l is proportional to the number of PSI charge separations. The absorption decrease associated with the oxidation of cyt b_l is 0.87 of the absorption increase associated with phase a, measured at 515 nm. Cyt *b* redox changes are divided by 0.87 $\times$ phase a.

on phase b and on cyt *b* reduction at the lower pH. At pH < 7.5, NQNO induces an inhibition of about 50% of the initial rate of phase b with no effect on the initial rate of cyt *b* reduction. Whatever is the pH, NQNO does not inhibit the rate of cyt *f* reduction (not shown).

Table 1: Initial Rate of Phase b and of Cyt *b* Reduction (data from Figures 3 and 4, ms^{-1})[a]

	V_{pb}	V_{cb}	V_{pb}/V_{cb}
1. FCCP	0.33	0.31	1.07
2. FCCP + DCCD	~0.02	0.22	~0.09
3. FCCP + NQNO	0.39	0.42	0.93
4. FCCP + DCCD + NQNO	~0.02	0.34	~0.06

[a] V_{pb}, initial rate of phase b; V_{cb}, initial rate of cyt *b* reduction.

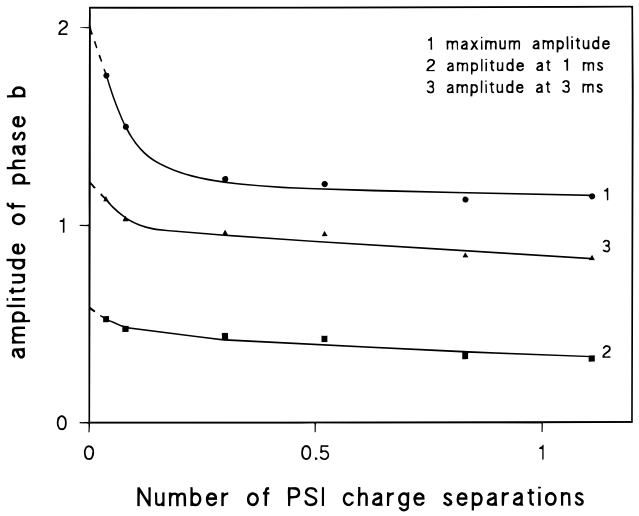


FIGURE 5: Amplitude of phase b as a function of the number of PSI charge separations. 50 mM phosphate buffer, pH 7.2, 7% ficoll, 1.5 μM FCCP; time interval between flashes, 2.6 s; for the weakest flash excitation, average of 200 experiments. Curve 1, maximum amplitude of phase b; 2, amplitude of phase b measured 1 ms after the flash; 3, amplitude of phase b measured 3 ms after the flash. The flash of the highest energy induces ~10% PSI double turnover.

The major effect of DCCD characterized in the experiments of Figures 1−4 is the large inhibition of the initial rate of phase b, which is associated with a minor decrease of the rate of cyt *b* and cyt *f* reduction. A likely hypothesis is that the high initial rate of the electrogenic phase observed in the absence of DCCD, not predicted by a Q-cycle framework, is associated with a transmembrane movement of a proton. This proton-pumping process, inhibited by DCCD, would be superimposed to a classical Q-cycle process. According to this hypothesis, the amplitude of phase b should be larger than that predicted from a pure Q-cycle process. This led us to measure the amplitude of phase b normalized to the number of PSI charge separations (Figure 5). The maximum amplitude of phase b (curve 1) varies from ~1.2 for the flashes of higher energy to ~2 (extrapolation to a flash of null energy). Note that upon repetitive high-energy flash excitation, i.e., when about half of the complexes are in the (b_l^+ b_h^+) and half in the (b_l^+ b_h) states, the amplitude of phase b can vary from ~1 to ~1.4, depending upon the algal culture. Its value is most generally larger than that expected from a Q-cycle process (~1). When the flash energy is decreased, the probability for a given cyt *bf* complex to undergo a turnover decreases and thus, the time interval between turnovers of this complex increases. Taking into account that the reduction of cyt b_h which is highly multiphasic (*7*) is completed in ~30 s, one expects that upon the weakest flash excitation, cyt b_h is fully reduced at the time each flash is fired**.** On the other hand, the initial rate of phase b, measured 1 ms after the flash (Figure 5,

Table 2: Amplitude of Phase b under Weak-flash Excitation Hitting 2.6% of PSI Centers[a]

	amplitude of phase b	
	2 ms	maximum[b]
no Addition, flashes 5.4 s apart	0.38	1.65
2 μM FCCP, flashes 5.4 s apart	0.87	1.89
2 μM FCCP, dark-adapted algae	0.58	1.76

[a] Average of 240 experiments. 50 mM phosphate buffer, pH 7.2, 7% ficoll. [b] The maximum amplitude is measured after completion of phase b ($\geq$30 ms).

curve 2), slightly increases when the flash energy decreases. This excludes the presence of complexes including a reduced cyt b_l, as we have shown (*15*) that the rate of phase b, measured in complexes including both *b*-hemes in their reduced state, is largely slowed compared to complexes in the semireduced state. We therefore conclude that the fraction of complexes in the (b_l^+ b_h) state varies between $\sim$50% for the flashes of higher energy and $\sim$100% for the flashes of lower energy and that the amplitude of phase b is maximum for complexes in the (b_l^+ b_h) state. It is worth pointing out that the amplitude of the slowest part of phase b (difference between curves 1 and 3) presents a larger dependence upon the energy of the flash than its initial rate (curve 2).

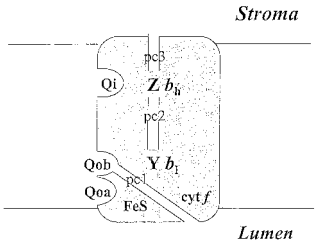
Table 2 gives the amplitude of phase b under weak-flash excitation hitting about 3% of PSI centers. The amplitude and the rate of phase b are maximum in the presence of FCCP, i.e., in the absence of electrochemical proton gradient. A slower phase b of smaller amplitude is observed when measured in dark-adapted algae in which a fraction of the complexes are in the fully reduced (b_l b_h) state.

DISCUSSION

As stated above, the normalized amplitude of phase b computed on the basis of a Q-cycle (or a semi Q-cycle) process varies between $\sim$0.6 and $\sim$1.4, depending upon the initial redox state of cyt b_h. We report here larger variations of the amplitude of phase b than that compatible with a Q-cycle process. The large amplitude ($\sim$2) and the large initial rate of phase b measured in conditions that most of the complexes are in the (b_l^+ b_h) state, suggest that the oxidation of plastoquinol at site Q_o is associated with the pumping of a proton from the stroma to the lumen. Such a redox-coupled proton pump necessarily involves a transmembrane proton channel. Interestingly enough, Brandt and Trumpower (*27*) have previously proposed a mechanism involving a transmembrane proton channel to interpret the decoupling of the protomotive Q-cycle induced by DCCD (*19*, *20*). In this model, a proton movement from the lumen to the stroma (proton slip), i.e., in the opposite direction to a proton pump, occurs in the presence of DCCD. Bruel et al. (*28*) interpret in the same way the partial decoupling between proton pumping and electron-transfer induced by a cyt *b* point mutation (mutant G137E).

We propose a tentative mechanism that remains in the frame of the Q-cycle process, but accounts for an additional proton-pumping process or for the partial decoupling induced by the addition of DCCD, as well. This mechanism is based on a structural model (Scheme 1) close to that proposed by Brandt and Trumpower (*27*). In this model, site Q_o includes

Scheme 1



two subsites called here Q_{oa} and Q_{ob}, close to the FeS protein and to cyt b_l, respectively. Site Q_{ob} is connected to the lumen by a proton channel pc1. A transmembrane proton channel (pc2) includes, at least, two amino acids able to bind protons, one (Y^-) in electrostatic interaction with site Q_{ob} and cyt b_l, and the other (Z^-) in interaction with cyt b_h. A third proton channel (pc3) connects site Q_i to the stroma. As in a conventional Q-cycle process, initiated by the transfer of a positive charge to the high-potential chain of the cyt *bf* complex, reaction 1 leads to the formation of a neutral semiquinone (PQH) at site Q_{oa}.

$$(\text{FeS cyt } f)^+ + \text{PQH}_{2\ (Q_{oa})} \rightarrow (\text{FeS cyt } f) + \text{PQH}_{(Q_{oa})} + \text{H}^+_{(\text{lumen})} \quad (1)$$

PQH is transferred to site Q_{ob} and a second proton is released in the lumen via proton channel pc1.

$$\text{PQH}_{(Q_{ob})} \rightarrow \text{PQ}^-_{(Q_{ob})} + \text{H}^+_{(\text{lumen})} \quad (2)$$

Steps 1 and 2 are supposed to be not electrogenic.

Complexes Initially in the (b_l^+ b_h) State. The charged semiquinone at site Q_{ob} shifts the p*K* of Y^- toward higher values and induces a transmembrane movement of proton, from ZH to Y^- via channel pc2 (step 3) and from the stroma to Z^- via channel pc3 (step 4).

$$\text{ZH} + \text{Y}^- \rightarrow \text{Z}^- + \text{YH} \quad (3)$$

$$\text{Z}^- + \text{H}^+_{(\text{stroma})} \rightarrow \text{ZH} \quad (4)$$

The amplitude of phase b associated with this proton-pumping process is equal to 1. The next step is the reduction of cyt b_l^+.

$$\text{PQ}^-_{(Q_{ob})} + (b_l^+\ b_h) \rightarrow \text{PQ}_{(\text{membrane})} + (b_l\ b_h) \quad (5)$$

Following the binding of PQ to site Q_i, the oxidation of both *b* cyts leads to the reduction of plastoquinone at site Q_i.

$$(b_l\ b_h) + \text{PQ}_{(Q_i)} + \text{ZH} + \text{H}^+_{(\text{stroma})} \rightarrow (b_l^+\ b_h^+) + \text{PQH}_2 + \text{Z}^- \quad (6)$$

The amplitude of phase b associated with step 6 is 0.6 (transfer inter *b*-hemes) + 0.4 (protonation of PQ) = 1.

The last event is the release of a proton from YH, which might be facilitated by the p*K* shift of Y associated with the oxidation of cyt b_l. The electrostatic interaction between the positively charged cyt b_h^+ and Z^- prevents or slows down a back transfer of proton from YH to Z^-. We thus expect

most of the protons to be released in the lumen at this step.

$$YH \rightarrow Y^- + H^+_{(lumen)} \quad (7)$$

The amplitude of phase b associated with steps 3−7 is equal to 2.

Complexes Initially in the (b_l^+ b_h^+) State. Because of the presence of cyt b_h^+, Z^- is not protonated and the formation of PQ^- at site Q_{ob} is not associated with the pumping of a proton.

$$PQ^-_{(Q_{ob})} + (b_l^+ b_h^+) \rightarrow PQ_{(membrane)} + (b_l b_h^+) \quad (3')$$

Electron transfer from cyt b_l to cyt b_h^+ induces the protonation of Z^-.

$$(b_l b_h^+) + Z^- + H^+_{(stroma)} \rightarrow (b_l^+ b_h) + ZH \quad (4')$$

The amplitude of phase b associated with steps 3′ and 4′ is 0.6 (transfer inter *b*-hemes) + 0.4 (protonation of Z^-) = 1. Upon repetitive flash illumination, i.e., when half of the complexes are in the (b_l^+ b_h) and half in the (b_l^+ b_h^+) state, the amplitude of phase b will be (2 + 1)/2 = 1.5. It is worth pointing out that the amplitudes of phase b computed above represent the maximum theoretical values expected from the mechanism discussed here.

Two processes, not mutually exclusive, can decrease the yield of the electrogenic phase or of the proton-pumping processes: i) At step 2, the proton stored on PQH can be transferred to Y^-, in competition with its release in the lumen, as proposed by Brandt and Trumpower (*27*). This transfer will prevent the redox-coupled proton-pumping process to operate (step 3). (ii) The proton stored in YH can be transferred to Z^- instead of being released in the lumen. This back transfer of proton via the proton channel pc2 (proton slip) will lead to a decrease of the amplitude of phase b and of the yield of the proton-pumping process. If processes i and ii occur sequentially, the amplitude of the electrogenic phase will be lower than that predicted by a Q-cycle mechanism. If process ii is after the pumping of a proton (step 3), the amplitude of phase b will be identical to that of a pure Q-cycle process. Nevertheless, the kinetics of phase b will be faster than that expected from a Q-cycle process with no lag phase.

We will discuss to what extent the mechanistic model proposed here can take into account the kinetics data reported in Results.

Yield of Phase b. Under weak-flash excitation, the maximum amplitude of phase b (∼2) is close to that theoretically predicted by our model. As shown in Table 2, the amplitude of phase b is significantly decreased in the presence of a permanent electrochemical gradient of protons. This suggests that the electric or osmotic component of the electrochemical proton gradient increases the probability of process i or ii.

Under repetitive strong-flash excitation, the amplitude of phase b varies (1−1.4) from one algae culture to another, but is always lower than the maximum amplitude expected (∼1.5). The large membrane potential induced by a strong-flash excitation may increase to a variable extent the probability of a back transfer of proton (process ii). It is worth pointing out that the amplitude of phase b is always lower when measured with broken chloroplasts (*13*, *29*) than in living algae. This suggests that a partial uncoupling of the redox-coupled pump and of the protomotive Q-cycle occurs in broken chloroplasts, according to processes i and ii.

Kinetics of Phase b. The absence of a lag phase in the kinetics of phase b implies that steps 1 and 2 are not rate-limiting. According to a conventional Q-cycle mechanism, the maximum value of the ratio V_{pb}/V_{cb} is 0.3, when half of the complexes are in the (b_l^+ b_h^+) state. The mechanism proposed here accounts for a much higher value of this ratio (∼1, see Table 1). On the other hand, the tail of phase b is associated with the oxidation of *b* cyts and the reduction of plastoquinone at site Q_i, which occurs exclusively in complexes initially in the (b_l^+ b_h) state. As expected, the normalized amplitude of the slowest part of phase b (Figure 5, curve 1 *minus* curve 3) doubles from high- to low-energy excitation, i.e., when the concentration of complexes in the (b_l^+ b_h) state is doubled.

Effect of DCCD. According to Wang and Beattie (*30*), DCCD binds preferentially to the cyt *b* polypeptide at aspartate 155 or glutamate 166, localized on an amphipathic helix which is a part of the loop which connects helices C and D. According to the structure of cyt bc_1 complex from bovine heart mitochondria, proposed by Xia et al. (*31*), this amphipathic helix is located between site Q_o and the lumen. These amino acids could be involved in the proton channel pc1, which establishes a link between site Q_{ob} and the lumen. Brandt and Trumpower (*27*) proposed that, in the presence of DCCD, the proton movement via channel pc1 is blocked or slowed and the proton stored in PQH is transferred to Y^- rather than released in the lumen (process i). We suggest that the presence of Y^- prevents the electron transfer from PQ^- to cyt b_l^+ (reaction 5). The protonation of Y^- via proton channel pc2 (reaction 3) is a faster process than its protonation in the presence of DCCD (process i). This could explain the 20−30% inhibition of the initial rate of reduction of cyt b_l^+, observed in the presence of DCCD (Figure 2A, Figure 4, and Table 1). In this hypothesis, for complexes initially in the (b_l^+ b_h) state, the proton-pumping process is inhibited, thus accounting for the lag phase in phase b. For complexes initially in the (b_l^+ b_h^+) state, DCCD would sequentially induce processes i and ii. The back transfer of proton decreases the yield of the electrogenic phase associated with the reduction of cyt b_h^+.

The effect of DCCD on the rate of oxidation of *b* cyts suggests that the cyt *bf* complex includes other binding sites, localized in the proton channel pc3, which connects site Q_i to the stroma. Moreover, it is likely that DCCD is able to bind, with different affinities, to many carboxylates in an apolar environment. It is worth pointing out that a refined structural analysis of bacterial reaction centers suggests that several water channels including dicarboxylic acids and working in parallel could be involved in the proton transfer (*32*). The involvement of several DCCD-binding sites associated with several proton channels could explain the progressive inhibition of phase b observed for increasing concentrations of DCCD (Figure 1).

Effect of NQNO. At neutral pH, NQNO does not affect the initial rate of cyt *b* and cyt *f* reduction. This argues against a semiquinone process, which could not operate when NQNO binds to site Q_i. At pH 9, NQNO induces no

inhibition of the initial rate of phase b (Table 1). This agrees with the hypothesis that the initial rate of phase b is associated with steps 3 and 4 (proton-pumping process) or step 4′ (cyt b_h^+ reduction), which do not involve the NQNO-binding site Q_i. The development of a lag phase in phase b in the presence of DCCD *plus* NQNO also suggests the involvement of a proton pump not impaired by NQNO. On the contrary, NQNO induces a large inhibition of the slowest part of phase b associated with the oxidation of the *b* cyts, as previously proposed (*17*). The inhibition of the initial rate of phase b we observe at lower pH suggests that, in these conditions, the binding of NQNO to site Q_i slows down—but does not block—proton movement through channel pc2 or pc3.

Mechanism of Proton Pumping in Highly Reducing Conditions. We proposed (*14*) that the electrogenic phase observed in conditions that both *b* cyts are reduced results from a proton-pumping process at site Q_o. We assume that a sequence of reactions similar to steps 1−4 occurs, leading to the formation of a charged semiquinone at site Q_{ob} and to a transmembrane movement of proton from the stroma to Y^-. Then, one electron is transferred from cyt b_l to PQ^-, as follow:

$$PQ^-_{(Q_{ob})} + \text{cyt } b_l + YH + H^+_{(?)} \rightarrow PQH_2 + \text{cyt } b_l^+ + Y^-$$

As expected, this process is not (*14*), or little (*33*), dependent upon the addition of NQNO.

Possible Involvement of a Movement of the FeS Protein. On the basis of structural data provided by crystallographic analysis, Crofts et al. (*34*) recently proposed that the lumenal part of the FeS protein can occupy two positions in the cyt bc_1 complex, close to the cyt c_1- and cyt *b*-polypeptides, respectively. In the latter position, the FeS protein could contribute to isolate site Q_{ob} from the lumen, making necessary the involvement of the proton channel pc1 for the release of the second proton from plastoquinol (step 2). On the other hand, when the FeS protein is close to the cyt *f*-polypeptide, the release of one proton to the lumen from plastoquinol (step 1) and from YH (step 7) should be facilitated. The movement of the FeS protein could therefore play an important role as a valve in the proton-pumping mechanism.

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