New Insights on the Proton Pump Associated with Cytochrome b_6f Turnovers from the Study of H/D Substitution Effects on the Electrogenicity and Electron Transfer Reactions†

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ABSTRACT: We have studied the effect of protium/deuterium substitution on different kinetics associated with the turnovers of cytochrome b_6f complex in whole cells of *Chlamydomonas reinhardtii*. Both the oxidation of cytochrome *f* and the reduction of hemes *b* were only little affected by the isotopic substitution. Contrasting with this, the initial slope of the electrogenic phase associated with cytochrome b_6f turnover was slowed by a factor of 4 by H_2O/D_2O substitution. Whereas in the presence of H_2O the electrogenic phase developed concomitantly with cytochrome *b* reduction, it lagged for a few hundreds of microseconds after cytochrome *b* reduction in the presence of D_2O. We propose that a proton pump is triggered by the oxidation of plastoquinol at the Q_o site. The proton transfer is specifically delayed upon isotopic substitution, accounting for the lack of significant effect on the electron-transfer reaction as well as for the strong decrease of the initial rate of the electrogenic phase.

Quinol oxidizing enzymes such as the cytochromes b_6f or bc_1 complex are found in most of the energy-transducing membranes. They catalyze the injection of two electrons into two bifurcated electron-transfer chains. Associated with these electron-transfer reactions, the release and uptake of proton at both sides of the membrane participate in the building of the electrochemical potential which drives ATP synthesis. In green photosynthetic membranes, cytochrome b_6f comprises four major subunits (*1*, *2*): the Rieske protein that binds an Fe_2S_2 cluster with an E_m of 320 mV (*3*), the cytochrome *f* with an E_m of 330 mV (*2*), the b_6 cytochromes which bind the b_l and b_h hemes with E_m values of −160 and −87 mV, respectively (*2*), and subunit IV. The Rieske protein and cytochrome *f* belongs to the high potential chain, the two *b* hemes to the low potential chain. While the Rieske protein and cytochrome *f* are located on the same side of the membrane so that the electron transfer between the two redox cofactors does not participate in the building of the transmembrane potential, the electron transfer between the two *b* hemes, located on each side of the membrane, is electrogenic (*4*). Furthermore, proton uptake at the reduction site of quinone (the Q_l[1] site) and proton release at the oxidation site of quinol (the Q_o site) participate in the building of the transmembrane potential.

3D structures of bc_1 complexes have been solved recently (*5*−*8*). Based on the differences between these structures, it has been proposed that the Rieske protein undergoes a conformational change between a "close to the cytochrome c_1" position to a "close to the cytochrome b_l" position (*8*). Considering the strong sequence homology between the mitochondrial bc_1 complex and the cytochrome b_6f complex (*9*), it is most likely that this also applies to the latter. The 3D structures of the soluble domain of cytochrome *f* and Rieske protein have also been solved (*10*, *11*).

In the framework of the Q-cycle (*12*, *13*), both cytochromes *f* and b_l are reduced at the expense of a plastoquinol at the Q_o site (see Scheme 1). Associated with this quinol oxidation, two protons are released in the lumen. A plastoquinone is then reduced at the Q_l site at the expense of the two *b* hemes of cytochrome b_6. Two protons are taken up from the stroma during this reduction. Various authors have disputed the Q-cycle model. Interestingly, it was often suggested that beside the proton-transfer associated with the shuttling of a plastoquinone from one side of the membrane to the other, additional proton pumping would occur according to a mechanism similar to that proposed by Wikström (*14*) for cytochrome oxidase. This extra proton would not be carried through the membrane by an electron carrier, but rather taken up and released by a sequence of pK_a shifts undergone by protonatable residues and triggered by redox or structural changes in their close vicinity. The evidence for invoking this extra proton pumping, although diverse, was mostly indirect. The persistence of an electrogenicity associated with cytochrome b_6f turnover under strongly reducing conditions was first put forward (*15*, *16*). Indeed, in the framework of the Q-cycle, no electrogenicity is expected when the two *b* hemes of cytochrome b_6 are reduced, since the complete oxidation of a plastoquinol into

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DNP-INT, dinitrophenyl ether of iodonitrothymol; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; NQNO, 2-*n*-nonyl-4-hydroxyquinoline oxide; Q_o and Q_l sites, site of quinol oxidation and quinone reduction, respectively.

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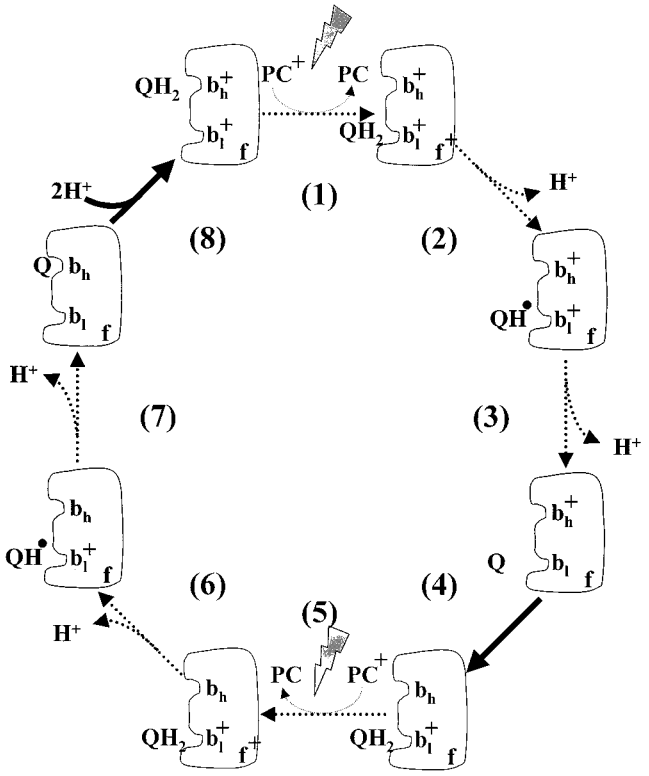
Scheme 1: Scheme Illustrating the Sequence of Reactions in the Framework of the Q-Cycle[a]



[a] (1) Oxidation of cytochrome *f* at the expense of the plastocyanin, (2) reduction of cytochrome *f* via the Rieske protein and proton release, (3) reduction of heme b_l and proton release, (4) reduction of heme b_h at the expense of heme b_l; *this step is electrogenic*; note that only heme b_l is in the oxidized state whereas before step 1 both hemes *b* were oxidized; (5) oxidation of cytochrome *f* at the expense of plastocyanin, (6) reduction of cytochrome *f* and proton release, (7) reduction of heme b_l and proton release, (8) reduction of a plastoquinone at the Q_l site at the expense of the two *b* hemes and proton uptake; *this step is electrogenic*. Note that two plastoquinols need to be oxidized at the Q_o site for a complete cycle. The boldface arrows indicate the electrogenic steps, the dashed ones the nonelectrogenic steps.

a plastoquinone is prevented by the redox state of b_l. As an alternative hypothesis, it was proposed that the plastosemiquinone formed at the Q_o site upon cytochrome *f* reduction could shuttle to the Q_l site and be reduced at the expense of the b_h heme which would in turn be reduced by the b_l heme, thereby accounting for this electrogenic phase (*17, 18*). The amplitude of the electrogenic phase associated with cytochrome b_6f turnovers has also been considered as the signature of additional electrogenic events that would not be described in the Q-cycle model (see ref *19* for a discussion). Furthermore, a large electrogenic phase is observed in the presence of NQNO (*16*), which is known to inhibit the reduction of quinone at the Q_l site (*20*), and should therefore, according to the Q-cycle model, strongly decrease the amplitude of the electrogenic phase. Hope and Rich observed that proton uptake on the stromal side of the thylakoid membrane occurs even in the presence of NQNO (*21*). Thus, the proton uptake process is not necessarily associated with reduction of plastoquinone at the Q_l site. Last, Wang and Beattie reported that DCCD binds to cytochrome b_6 (*22*). Together with the appearance of a marked lag in the electrogenic phase upon DCCD binding (*19*), this was proposed as further support of a proton pumping activity associated with cytochrome b_6f turnovers. Most of these arguments are based upon the analysis of the electrogenic phase associated with cytochrome b_6f turnovers. This phase is a signature of charge movements in the direction of the transmembrane electric field. Comparing its kinetics with those of the electron-transfer reactions proper may allow the identification of transmembrane charge transfers which do not involve solely electron transfer (see, e.g., refs *15, 16, 18*). Unfortunately, this method gives no clue as to the nature of the charge for which the movement is electrogenic. As an example of this vagueness, two (nonexclusive) hypotheses have been proposed to account for the persistence of an electrogenic phase under conditions where heme b_l was reduced: (i) the shuttling of a plastosemiquinone anion from the lumenal side to the stromal side of the membrane (*15, 16*); or (ii) the transfer of a proton from the stromal side to the lumenal side of the membrane (*18*). In an attempt to specifically tune the thermodynamics or kinetic properties of the putative steps involving proton transfer, we have studied the effect of H_2O/D_2O substitution on cytochrome b_6f turnover rates. In the framework of a proton pumping process, one may expect to specifically slow these precise steps by substituting protons for deuterons.

One usually distinguishes between three types of isotope effects (see, e.g., ref *23*). First, the primary kinetic isotope effect results from the breaking of the bond to the isotopic atoms. Because of the high ratio of the mass of deuterium with respect to that of protium combined with the relatively low energy of the bonds involving protium (or deuterium), the primary kinetic isotope effects are expected to be substantial (e.g., the breaking of an O−D bond is expected to be ~10 times slower than the breaking of an O−H bond). Second, the secondary isotope effect results from changes in the energy of the bond undergoing cleavage induced by changes in the energy of the H bonds due to isotopic substitution. These are usually small ($k_H/k_D < 2$, where k_H and k_D are the rates of the reaction in H_2O and D_2O, respectively). Finally, the solvent isotope effects result from the isotopic substitution in the solvent of the studied molecule. In this case, which is obviously the case for the present study, many physicochemical parameters are changed. Indeed, upon H_2O/D_2O substitution, the viscosity of the solvent is changed (the relative viscosity of D_2O versus that of H_2O is 1.2), and the pK_a's of weak acids are shifted usually by 0.4 pH unit. However, when a O−H or O−D bond is broken, a primary kinetic isotope effect contributes to the overall solvent effect. It is only when a k_H/k_D ratio larger than 2.5−3 is observed that one may conclude that a rate-limiting step involving H-bond breaking or formation is characterized.

From the results presented here, we conclude that whereas the isotopic substitution has little effect on the electron-transfer rates of the quinol oxidation at the Q_o site, the initial step of the electrogenic phase is significantly slowed. These results add further support to the previous proposals of a proton pumping activity associated with cytochrome b_6f turnovers.

MATERIALS AND METHODS

Experiments were performed under anaerobic conditions with wild-type cells of *Chlamydomonas reinhardtii*. The

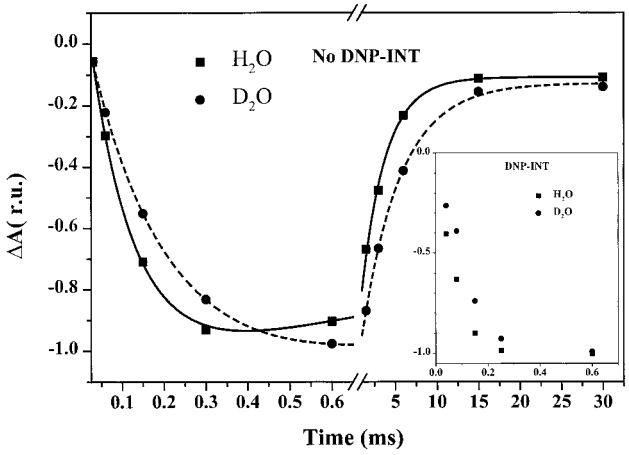


FIGURE 1: Kinetics of absorption changes associated with cytochrome *f* redox changes (measured at 554 nm with the base line drawn between 545 and 573 nm) in the presence of H_2O (squares) and D_2O (circles). The lines are the best fit of the data with two exponentials. The fast phase associated with cytochrome *f* oxidation has half-times of 70 and 110 μs in the presence of H_2O and D_2O, respectively. The slow one, associated with cytochrome *f* reduction, has a half-time of 2.2 ms in the presence of H_2O and 3.5 ms in the presence of D_2O. The inset shows the kinetics of cytochrome *f* oxidation measured in the presence of 5 μM DNP-INT. All the data were normalized to the same amplitude for cytochrome *f* oxidation so that r.u. stands for relative units. For further experimental details, see Materials and Methods.

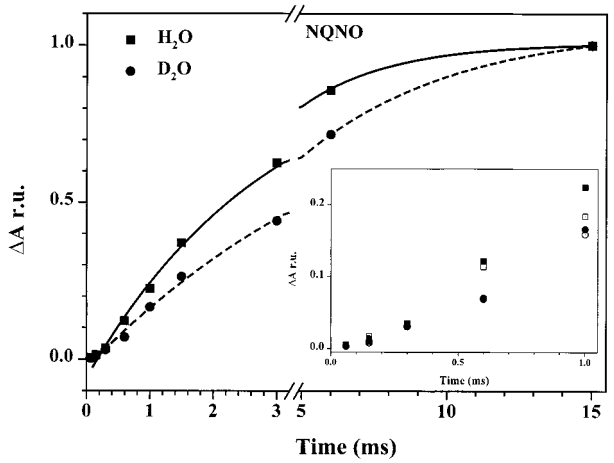


FIGURE 2: Kinetics of absorption changes associated with cytochrome *b* redox changes (measured at 564 nm with a base line drawn between 545 and 573 nm). The reduction of cytochrome *b* was measured in the presence of NQNO (5 μM) and H_2O (squares) or D_2O (circles). The lines are the best fit of the data with a single exponential. The inset shows a comparison of the absorption change kinetics associated with cytochrome *b* reduction in the presence of NQNO (closed symbols) and in the absence of NQNO (open symbols) in both D_2O (circles) and H_2O (squares). The data were normalized to the amplitude of cytochrome *b* reduction measured in the presence of NQNO (r.u. stands for relative units). For further experimental details, see Materials and Methods.

algae were dark-adapted for 20 min before the experiments. Anaerobiosis was reached by keeping the algae under an argon flux in a large reservoir connected to the measuring cuvette. H_2O/D_2O substitution was performed as follows: the algae were centrifuged for 5 min; the pellet was resuspended in a buffer containing H_2O or D_2O (99.8% D atom) and HEPES (20 mM, pH 7.2) and stirred for 1 h. The ratio of the pellet and resuspension buffer volumes was 1/10. After a second centrifugation, the pellet was resuspended in the same buffer with a pellet volume to resuspension buffer volume ratio of 1/20. Then 1 mM hydroxylamine, 10 μM DCMU was added to the samples to inhibit photosystem II activity; 1 μM FCCP was also added to collapse the permanent transmembrane electrochemical difference potential (*18*). No increase in the kinetic isotope effect was observed when the duration of the incubation in D_2O was increased.

The absorption changes were measured with a home-built spectrophotometer (*18, 24*). The absorption changes associated with cytochrome *b* and cytochrome *f* redox changes were measured at 564 and 554 nm, respectively, with a base line drawn between 545 and 573 nm. The electrogenicity was measured by the transmembrane field induced electrochromic shift at 515 nm. This signal yields a linear response with respect to the transmembrane potential (*25*). The experiments were performed under strict anaerobic conditions. The intensity of the actinic flashes was limited to hit about 30% of the photosystem I reaction centers in order to avoid double turnovers at the level of the cytochrome b_6f complex. The absorption changes measurement were performed under repetitive flashes with a time delay between flashes of 7 s.

RESULTS

Figure 1 shows the kinetics of the flash-induced cytochrome f absorption changes in the presence of H_2O or D_2O. The isotopic substitution had only little effect on the rates of both cytochrome *f* oxidation and rereduction. The data could be fitted by two exponentials. The half-times of the oxidation phase were 70 and 110 μs in the presence of H_2O and D_2O, respectively. This latter point could be further evidenced by addition of DNP-INT (an inhibitor of plastoquinol oxidation at the Q_o site) to the samples (inset of Figure 1). This inhibitor slows down cytochrome *f* reduction, thereby allowing an accurate determination of the rate of injection of an oxidizing equivalent into the cytochrome b_6f complex in both conditions. In the presence of H_2O, the half-time of this reaction was 60 μs, in good agreement with the value reported in ref *26*; in D_2O, it was in the 90 μs range. As mentioned in the introduction, such a small decrease in the rate of the reaction most probably results from a nonspecific isotope effect such as, for example, the increased relative viscosity of D_2O which could slightly slow plastocyanin diffusion and thus the injection of an oxidizing equivalent into cytochrome *f*. More importantly, these results show that substitution of H_2O for D_2O should not result in any pronounced lag phase in the kinetics of PQH_2 oxidation at the Q_o site. Moreover, the rate of rereduction of cytochrome *f* was also little slowed upon isotopic substitution. Fitting the data yielded a half-time of 2.2 ms in the presence of H_2O, in good agreement with ref *27*, and 3.5 ms in the presence of D_2O. This finding suggests that the oxidation of plastoquinol at the Q_o site is slightly sensitive to H/D substitution. To further characterize the consequences of this substitution on the electron-transfer reactions catalyzed at the Q_o site, we studied the reduction reaction of cytochrome *b*.

Figure 2 shows the absorption changes associated with cytochrome *b* redox changes in the presence of NQNO (5 μM), an inhibitor of the PQ reduction at the Q_I site, and in the presence of H_2O and D_2O. Again, substitution of H_2O for D_2O resulted in a small decrease of the reaction rate. Fitting the data by single exponential yields half-times of

2.1 and 3.5 ms in the presence of H_2O and D_2O, respectively, in good agreement with the results obtained for cytochrome *f* reduction. The similarity between the half-times obtained for cytochrome *f* and cytochrome *b* reduction is noteworthy. Indeed, the former was obtained in the absence of any inhibitor of the b_6f complex whereas the latter was measured in the presence of NQNO. This finding indicates that at least at the concentration of the present study (5 μM) addition of NQNO did not slow the rate of plastoquinol oxidation at the Q_o site (see, however, ref *16* for a discussion of the effect of NQNO concentration on the kinetics of b_6f complex turnover). This point is further illustrated in the inset of Figure 2. Here, we compare the initial slope of the absorption changes associated with cytochrome *b* redox changes in the presence or absence of NQNO (solid and open symbols, respectively) and in the presence of H_2O or D_2O (squares and circles, respectively). In the absence of NQNO, the reduction phase is followed by an oxidation phase indicative of plastoquinone reduction at the Q_I site. These two phases are not well separated in time. This precludes the accurate determination of the rates of cytochrome *b* reduction in the absence of an inhibitor of the Q_I site such as NQNO. However, two conclusions may be drawn from the comparison shown here: (i) the initial slope of the absorption changes associated with cytochrome redox changes is slightly sensitive to the addition of NQNO; (ii) the kinetic consequence of isotopic substitution is similarly small irrespective of the presence of NQNO. Such a small isotope effect may be found surprising since obviously the oxidation of plastoquinol (or reduction of cytochrome *b*) is associated with proton release. Consequently, both the thermodynamic and kinetic parameters of this reaction may be expected to vary upon isotopic substitution. However, a k_H/k_D ratio of about 1.7 is too small to be considered as indicative of a kinetic limitation involving proton transfer. The present finding only shows that the deprotonation of quinol or semiquinone is not a limiting step in the overall mechanism of electron-transfer reactions at the Q_o site. Although discussing the interplay of proton transfer and plastoquinol oxidation at the Q_o site is beyond the scope of the present paper, we would like to briefly address this point. Indeed, it has been reported that the cytochrome b_6f turnover rates are dependent on pH (for a titration of the electrogenic phase, see ref *28*; for the study of pH effects on the kinetics of redox reactions, see refs *29*−*31*). However, the maximal rates are observed in the 6.5−7.5 pH region, which is precisely the pH range of the present study (see Materials and Methods and ref *28*). It follows that if any kinetic limitation involving proton transfer is to be observed for the redox reaction catalyzed by the b_6f complex, it is not expected in the 6.5−7.5 pH range but rather in the more acidic pH domain. This is in line with the present results, which evidence the absence of significant kinetic isotope effect for both the oxidation or rereduction of cytochrome *f* and the reduction of heme *b*.

Since the reduction kinetics of cytochrome *b* have been measured in the presence of NQNO, we have first studied the effect of isotope exchange on the kinetics of the electrogenic phase in the presence of NQNO (Figure 3). For comparison, we have replotted, in the inset, the first 2 ms of the kinetics of cytochrome *b* reduction in the presence of NQNO. Clearly the effects of H/D substitution were larger on the electrogenic phase than on the cytochrome *b* reduction.

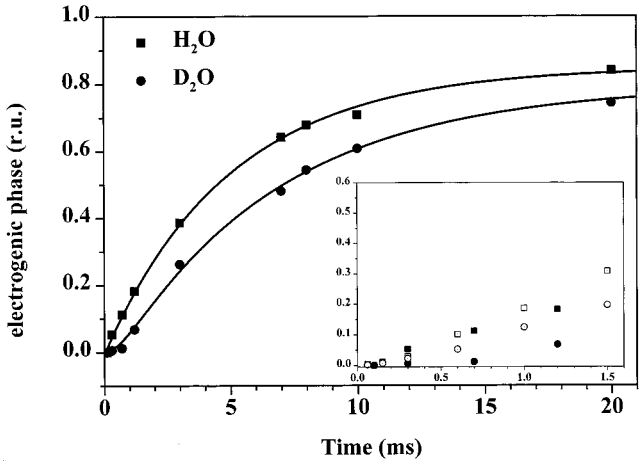


FIGURE 3: Electrogenic phase associated with cytochrome b_6f turnovers in the presence of H_2O (solid squares) or D_2O (solid circles) in the presence of NQNO (5 μM). The electrogenic phase was normalized to the amplitude of the signal measured 100 μs after a flash resulting from the photoinduced transmembrane electric field (phase a). The inset shows a zoom on the first milliseconds of the kinetics. For comparison, the kinetics of cytochrome *b* reduction were replotted from Figure 2 [H_2O (open squares), D_2O (open circles)] after normalization to the amplitudes of the electrogenic phases. For further experimental details, see Materials and Methods.

As shown in the inset, in the presence of H_2O, the electrogenic phase starts to develop concomitantly with cytochrome *b* reduction. Contrasting with this, it significantly lags after cytochrome *b* reduction in the presence of D_2O. This suggests the existence of an early electrogenic proton transfer which would account for the initial slope of the electrogenic phase. The mere persistence of a large electrogenic phase in the presence of NQNO has been put forward to hypothesize the occurrence of an electrogenic event not accounted for by a conventional Q-cycle (*16*). To further investigate this point, we then turned to the study of the sensitivity of the electrogenic phases to isotopic substitution in the absence of NQNO. Figure 4 shows the kinetics of the electrogenic phase in the presence of H_2O or D_2O in the absence of any inhibitor. In the presence of H_2O (squares), the electrogenic phase could be satisfyingly fitted by a single exponential with a half-time of 2.3 ms (solid line). This finding is consistent with a previous report on the absence of any significant lag phase under experimental conditions similar to ours (*18*). This is somewhat unexpected as discussed in ref *18* since, according to the Q-cycle model, the electrogenic phase should only develop concomitantly with cytochrome *b* oxidation so that it should be preceded by the reduction of the cytochrome b_l^{ox} (see ref *13*). This discrepancy between data and model-based expectation is further illustrated by the comparison of the rate of cytochrome *b* reduction and of the electrogenic phase. In the inset, we have replotted from Figure 2 (after normalization to the amplitude of the electrogenic phases) the initial slopes of the kinetics of cytochrome *b* reduction obtained in the presence of NQNO (open symbols, squares: H_2O, circles: D_2O). At variance with the expectation drawn from the Q-cycle model, the electrogenic phase develops concomitantly with heme b_l reduction although the latter reaction has been reported to be nonelectrogenic (*4*, *32*). Although these different sets of data were obtained under different

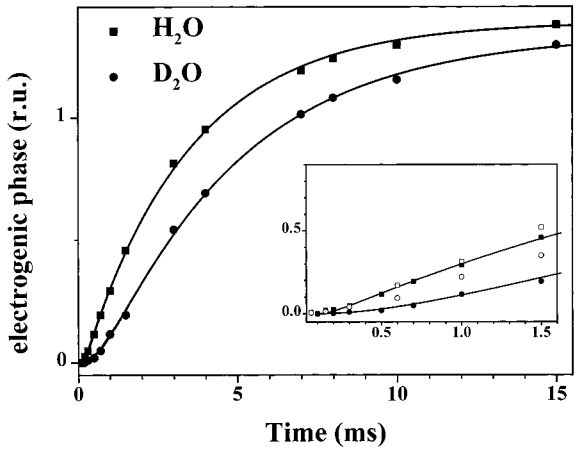


FIGURE 4: Electrogenic phase associated with cytochrome b_6f turnovers in the presence of H_2O (solid squares) or D_2O (solid circles). The electrogenic phase corresponds to the absorption changes measured at 515 nm normalized to the amplitude of the signal measured 100 μs after a flash resulting from the photoinduced transmembrane electric field (phase a). It thus represents phase b. The inset shows a zoom on the first milliseconds of the kinetics. For comparison, the kinetics of cytochrome *b* reduction were replotted from Figure 2 [H_2O (open squares), D_2O (open circles)] after normalization to the amplitudes of the electrogenic phases. For further experimental details, see Materials and Methods.

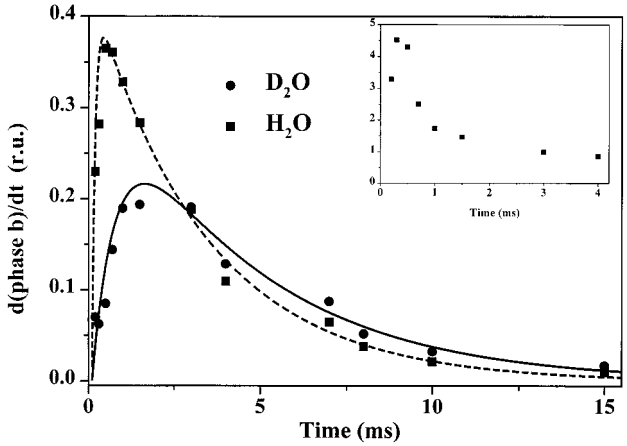


FIGURE 5: First derivatives of the kinetics of the electrogenic phase in the presence of H_2O (squares) and D_2O (circles). The inset shows the ratio of these two derivatives, thereby allowing the quantification of the k_H/k_D ratio.

experimental conditions, we believe this comparison to be meaningful for the purpose of the present discussion which is focused on comparison of the initial slopes of the electrogenic phase and on the kinetics of cytochrome *b* reduction. Indeed, as shown in the inset of Figure 2 the initial slopes (i.e., the initial rates) of cytochrome *b* reduction are not affected by the addition of NQNO. Unfortunately, comparison with the kinetics of cytochrome *b* redox changes measured in the absence of NQNO is not possible without resorting to arbitrary normalization. Indeed, in the absence of inhibitor of the Q_I site, the oxidation of cytochromes *b* which follows their reduction precludes the determination of the amplitude of the reduction phase used here as a normalization factor.

The results obtained in the presence of D_2O (Figure 3, solid circles) may shed light on the origin of this discrepancy between the data and model-based expectation. Indeed, as shown in Figure 4, the kinetics of the electrogenic phase are markedly affected by the isotopic substitution. The initial slope of the electrogenic phase kinetics is strongly decreased in the presence of D_2O, suggesting that, in this case, the first electrogenic step resulting from cytochrome b_6f turnover is delayed. Furthermore, comparison between the kinetics of the electrogenic phase and those of heme *b* reduction shows that (i) the initial slope of the electrogenic phase is much more decreased than that of cytochrome *b* reduction, and (ii) in the presence of D_2O, the electrogenic phase start to develop later than the reduction of hemes *b*, consistent with both the Q-cycle model and the absence of electrogenicity associated with this electron-transfer reaction (*4*, *32*). This suggests that the electrogenic phase observed in the presence of H_2O reflects, as a matter of fact, a process involving at least two phases, the first one only being significantly affected by isotopic substitution. This point is further illustrated in Figure 5 where the derivatives of the electrogenic kinetics shown in Figure 4 have been plotted. Differentiating the data yields the time course of the apparent rate of the electrogenic phases in both conditions. Figure 5 shows that from 3 ms onward the kinetics of the electrogenic phase are hardly sensitive to the isotopic substitution whereas they were significantly slowed in the submillisecond time range. In the inset, we plotted the ratio of the derivatives of the electrogenic phase (in H_2O versus in D_2O). This computation yields the time course of the k_H/k_D ratio. This ratio is larger than 3 during the first 700 μs and then decays toward 1 in the millisecond time range.

DISCUSSION

The present results show that the electron-transfer reactions catalyzed by the b_6f complex are, at least in the experimental conditions of the present study, not very sensitive to H/D substitution. The k_H/k_D ratio is in the 1.3 range for cytochrome *f* oxidation, that for cytochrome *b* reduction in the range of 1.7, i.e., too small to ascertain a kinetic limitation due to proton transfer associated with these reactions. With respect to electrogenicity, however, a very different picture emerges from the effect of H_2O for D_2O substitution on the kinetics of the electrogenic phase. Indeed we report here a significant sensitivity of the kinetics of the electrogenic phase to H/D substitution. A pronounced lag phase was observed in the presence of D_2O and hardly detectable in the presence of H_2O. Since, as discussed above, the redox reactions catalyzed by the cytochrome b_6f complex are only slightly slowed upon isotopic substitution, this lag phase is not due to a decrease of a putative step that would precede the triggering of the electrogenic phase. Interestingly, in the 3−20 ms time domain, the kinetics of the electrogenic phase are not sensitive to isotopic substitution. We thus propose that this phase consists of a sequence of electrogenic events and that the first step(s) involve(s) pure proton transfer that is (are) revealed by its (their) sensitivity to H_2O/D_2O substitution. The question, which is to be addressed then, is the triggering event of this proton transfer. Extrapolating the results obtained by Glaser and Crofts (*32*) or Roberston and Dutton (*4*) on the *Rhodobacter sphaeroides* bc_1 complex to the b_6f complex yields no electrogenicity associated with the reduction of the Rieske iron−sulfur cluster by a plastoquinol at the Q_o site or with the proton release from the resulting

plastosemiquinone. Consequently, this proton transfer is most likely triggered after reduction of the Rieske iron−sulfur cluster and before reduction of the oxidized b_l heme. This supports the model proposed earlier by Joliot and Joliot (*19*). To account for the specific slowing of the initial slope of the electrogenic phase observed upon addition of DCCD, they put forward the occurrence of a proton pumping step triggered by the formation of a plastosemiquinone in the Q_o site. Somehow, the effects of DCCD or isotopic substitution are very analogous: both specifically decrease the initial slope of the electrogenic phase without significantly affecting the electron-transfer rates. This is a strong indication of electrogenic charge-transfer events, which do not reflect electron transfer but rather proton transfer. This would result in an additional electrogenicity which would add up to the electrogenicity of the electron-transfer reaction between the two *b* hemes of cytochrome b_6 and of the proton uptake associated with quinone reduction at the Q_l site. A redox-induced proton pumping associated with cytochrome b_6f complex turnovers has been hypothesized on the basis of various observations. Among them are the following: (i) the persistence of electrogenicity associated with b_6f turnovers under strongly reducing conditions (*15, 18*); (ii) the evidence for DCCD binding sites taken as indicative of the existence of a proton conducting channel (*22, 33*); (iii) the amplitude of the electrogenic phase which is too large to be accounted for by the electron transfer between the two *b* hemes and the proton uptake at the Q_l site associated with plastoquinone reduction (*18, 19*); and (iv) the effect of DCCD addition of the initial slope of the electrogenic phase (*19*). Interestingly, this proton pumping is unlikely to be constitutive. As a matter of fact, the driving force needed to drive both the PQH_2 oxidation at the Q_o site and the proton pumping simultaneously is likely to be available only when the PQ pool is fully reduced. Indeed, the permanent transmembrane electrochemical potential difference observed in darkness, that can be considered as a lower limit for the transmembrane $\Delta\tilde{\mu}_{H^+}$, is in the 110−140 mV range (*28*). Thus, with respect to the free energy difference of quinol oxidation, an extra driving force of at least 110 mV is needed to pump an extra proton, a figure which is close to the driving force of the oxidation of a plastoquinol molecule when the PQ pool is fully reduced. Altogether, this suggests that the proton pump may produce some work or may not, depending on (at least) the redox condition. Furthermore, it suggests that stoichiometric proton pumping is not required for the electron-transfer reactions to proceed. This conclusion accounts for the variability of the amplitude of the electrogenic phase (see ref *19* for a comprehensive discussion), as well as for the kinetic data reported here. Indeed we show, in the present paper, that the additional electrogenic step may be significantly slowed without decreasing to a similar extent the rates of electron-transfer reactions to cytochromes *f* or b_l. This latter result is particularly interesting since (see discussion above) the proton pumping step, when active, most likely precedes cytochrome b_l^{ox} reduction. It follows that it is branched in parallel with cytochrome b_l^{ox} reduction so that the electron-transfer step may undergo hardly any kinetic limitation when the proton pumping step is dramatically slowed. At this stage, one may notice that the amplitude of the electrogenic phase is only slightly diminished when substituting H_2O for D_2O. We have proposed that the proton pumping is triggered by the formation of the semiquinone at the Q_o site. It follows that it competes with the reduction of the oxidized heme b_l. Thus, one might expect that, at variance with the present results, a decrease in the rate of proton pumping would result in a decrease in the overall electrogenicity. We propose two hypotheses to account for this paradox. The proton pumping could be strongly reversible. It would, then, contribute little to the overall electrogenicity, but, being triggered by the formation of the semiquinone, would still be kinetically detectable. Interestingly, one finds in the b_6f complex, at the position equivalent to glycine 137 of cytochrome *b* of the bc_1 complex, an aspartate residue. It has been reported that substituting G137 for a glutamic residue (building a "b_6f like" bc_1 complex with respect to this position) results in a partial decoupling of the proton pumping (*34*). Alternatively, the equilibrium constant of the reduction of b_l^{ox} by the semiquinone could be low. In this framework, although slower than the rate of the forward electron-transfer reaction, the route via the proton pumping step could still be followed because of the relatively long lifetime of the semiquinone. We note, however, that the formation of semiquinone to a significant level has, until now, escaped EPR detection (*35*−*37*). According to various authors, this result indicates that the oxidation of plastoquinol into plastosemiquinone is the rate-determining step of the overall reaction; so that a small equilibrium constant for the reduction of heme b_l would be unlikely (*37, 38*). However, as proposed by Link, the iron−sulfur cluster/semiquinone could be antiferromagnetically coupled and thus EPR-silent (*39*).

It has been proposed recently that, beside the structural rearrangement associated with the Rieske protein motion (*8*), Glu272 of the highly conserved PEWY sequence of the bc_1 complex (Glu78 of subunit IV in the b_6f complex) may undergo a side-chain rotation upon plastosemiquinone formation (*40*). Both these structural rearrangements of the Q_o site may obviously serve as a switch for the proton pump. Alternatively, the formation of the plastosemiquinone anion may electrostatically trigger the pumping process through the p*K* shift of a nearby protonatable species as proposed in refs *22* and *33*. Glu272 of the bc_1 complex has also been proposed to be involved in the proton release process from the plastosemiquinone upon cytochrome b_l reduction (*40*). This precise proton release is not electrogenic (*4, 32*). Furthermore, no decrease in the amplitude of the electrogenic phase was observed when substituting this glutamate residue in cytochrome b_6f complexes for nonacidic residues (*41*). Consequently, Glu78 is unlikely to be involved in the proton pumping process proper. Concerning the role of this residue, it has been shown that cytochrome *f* reduction is significantly slowed in mutants where this residue has been substituted for nonacidic residues (*41*). It may thus participate to the proton release process as proposed in ref *40* but must also somehow interact with the plastoquinol in the Q_o site so as to modulate the rate of plastoquinol oxidation as proposed in ref *41*. In this framework, one may consider the involvement of this side chain in the ligation of the quinol, in agreement with Crofts et al. (*40*).

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