A new catalytic site in the cytochrome b₆ f complex of *Chlamydomonas reinhardtii*

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**Introduction**

The Q-cycle model for the operation of the cytochrome (cyt) b₆ f complex is based on the “oxidant-induced-reduction” phenomenon and proposes that the obligatory sequence of events that, in thylakoids after a flash, leads to the reduction of cyt b is: 1) oxidation of cyt f by plastocyanin, 2) oxidation of Fe₂S₂ center by cyt f (i.e. re-reduction of cyt f), 3) oxidation of PQH₂ to semiquinone by Fe₂S₂ center at the Qₒ site, and 4) reduction of the cyt b chain by semiquinone. Events 3 and 4 constitute a “concerted reaction”. Therefore cyt b(s) should be reduced no faster than cyt f, see Berry et al. (2000). *Chlamydomonas reinhardtii* mutants targeting conserved residues of the heme binding pocket of cyt f, P2V and P2V/R156A, show inhibited rates of cyt f re-reduction (t₁/₂ for wild type (wt) = 3.1 ms, P2V = 60 ms, P2V/R156A = 300 ms), see Fig. 1. These reductions of cyt f are monophasic in both wt and mutants: the stigmatellin-sensitive reductions (difference between the traces without and with stigmatellin) extrapolate monotonically to the origin, with no fast phase. Experiments using very weak flash intensities (6 % P700-saturating) demonstrate that these kinetics in wt, P2V, and P2V/R156A are not affected by multiple turnovers of oxidation. Nevertheless, the photoreduction of cyt b is not correspondingly inhibited (t₁/₂ for wt = 2.8 ms, P2V = 3.4 ms, P2V/R156A = 12 ms) (Fernández-Velasco et al., 2001). Thus, the reduction of cyt b can precede the re-reduction of cyt f, violating the Q-cycle tenets, see also Ponamarev and Cramer (1998).

Two mutually exclusive hypotheses- The fast cyt b reduction that occurs in spite of the slow cyt f reduction could be explained in one of two ways (Fernández-Velasco et al., 2001). The first hypothesis considers a “cyt f-heme-dependent mechanism” where cyt f is the exclusive plastocyanin (PC) reductant and, upon flash activation, the first process at Qₒ is the reduction of the low potential (LP) chain (cyt b). This mechanism would require a conformational change at the Qₒ site, induced by cyt f (photo)oxidation and without any role for cyt f reduction, to change the reductant’s Eₘ at Qₒ. The second hypothesis considers a “cyt f-heme-independent mechanism” where PC can draw electrons not only from cyt f but also directly from the Fe₂S₂ center. This mechanism respects the order proposed for the “concerted reaction”: the high potential (HP) chain is the one reduced first with PC being reduced without the involvement of the cyt f heme. Those two hypotheses predict opposite consequences. If the “cyt f-heme-dependent mechanism” hypothesis is correct two predictions are derived: i) A strong inhibition of cyt f reduction must become the rate limiting step in the cyt b₆ f complex and must propagate to (have limiting kinetic consequences in) the Qₒ-inhibitor-sensitive P700 and PC reductions and to the steady-state electron transfer (Fernández-Velasco et al., 2001). ii) In PQH₂-saturating conditions, the amplitude of cyt b
reduction must be identical, or at least directly proportional, to the amplitude of cyt \( f \) oxidation. Indeed, the putative conformational change in the \( Q_o \) site triggered by the oxidation of cyt \( f \) in a given cyt \( b_6f \) complex will generate the reduction of a cyt \( b \) in the same cyt \( b_6f \) complex. If, instead, the “cyt \( f \)-heme-independent mechanism” hypothesis is correct, then i’') the inhibition in cyt \( f \) reduction must not propagate and ii’’) there should be no obligatory ratio between cyt \( b \) reduction and cyt \( f \) oxidation because these two reactions can be independent.

We demonstrate that cyt \( f \) is not an obligatory path for electrons flowing through the cyt \( b_6f \) complex from PQH\(_2\) to Photosystem I (PSI). The oxidizing equivalents from PSI can be delivered to the HP chain of the cyt \( b_6f \) complex both at the level of cyt \( f \) and, independently, at another site that is connected to the \( Q_o \) site, possibly the Fe\(_2\)S\(_2\) center (Fernández-Velasco et al., 2001).

**Materials and methods**

All materials and methods as in Zhou et al. (1996) and Fernández-Velasco et al. (2001).

**Results and Discussion**

*Kinetic resolution of a cyt \( f \)-heme-independent and \( Q_o \)-site-inhibitor-sensitive electron transfer to P700 and PC*- After a train of flashes, when the reduced PC and cyt \( f \) pools present before the first flash have been exhausted by the repetitive flash-induced P700 oxidations, any P700 reduction requires electrons from the Fe\(_2\)S\(_2\) center and from its reductant PQH\(_2\) at the \( Q_o \) site. That reduction is inhibited by \( Q_o \)-site inhibitors (e.g. stigmatellin or DBMIB). Both inhibitors displace PQH\(_2\) from \( Q_o \). Besides, stigmatellin raises the \( E_m \) of the Fe\(_2\)S\(_2\) from \(~300\) mV to 460 mV (Nitschke et al., 1989). The stoichiometry cyt \( b_6f \) complex:PC:P700 in wt and P2V is 0.32:1.8:1 and 0.32:2.5:1, respectively. Fig. 1 shows the redox kinetics of P700 with a train of flashes. In wt and P2V, without inhibitors, P700 is reduced with a very similar fast (mainly submillisecond) kinetics, which is sustained at every flash in the train. Stigmatellin inhibits P700 reduction (40 fold) and oxidized P700 accumulates with a very similar pattern in both strains; DBMIB also blocks. Thus, for wt and P2V, the stigmatellin inhibition demonstrates that in the absence of inhibitors and after a few flashes following the first, redox equivalents delivered from the cyt \( b_6f \) complex (Fe\(_2\)S\(_2\) center and \( Q_o \) site) are needed for a fast P700 reduction after each flash. In P2V/R156A, the reduction of P700 in the absence of inhibitors shows at least two phases, also inhibited by stigmatellin. As in this mutant, the cyt \( b_6f \) complex is in a lower stoichiometry with P700 (1:6) than in wt and P2V (1:3), that multiphasicity is interpreted as the need of multiple turnovers of the cyt \( b_6f \) complexes for P700 reduction. \( \Delta pet A \) (a cyt \( b_6f \)-less mutant) has a stigmatellin-insensitive blocked P700 re-reduction. The very slow re-reductions of cyt \( f \) in P2V and P2V/R156A (also seen after a train of flashes, not shown) are not consistent with the fast P700 reduction that is sustained during the full train of flashes in both mutants. Indeed, a completely oxidized cyt \( f \) pool that, after a train of flashes, is reduced very slowly cannot be the center responsible for a fast electron donation to P700.
To improve the time resolution of the kinetic coupling between the stigmatellin-sensitive electron donation of the cyt $b_{6}f$ complex and the reduction of P700, two 92% P700-saturating flashes fired 250 $\mu$s apart were used (not shown). Following the second flash, a reduction of P700 (~50% of the total) that can be inhibited by stigmatellin is observed in all strains. This stigmatellin-sensitive phase has a $t_{1/2}$ = 700 $\mu$s in P2V, in the first phase of reduction in P2V/R156A, and also in the wt, being much faster than their corresponding cyt $f$ reductions, that in the case of wt is 3-4 fold faster. Therefore, a fast ($t_{1/2}$ 700 $\mu$s) Fe$_2$S$_2$ center-dependent but cyt $f$-kinetically-independent reaction can catalyze the reduction of P700 in all strains.

Thus, all the evidence from the pseudo-steady-states and reduction kinetics of P700 and cyt $f$ indicate that, in the mutants and also the wt, the kinetics of cyt $f$ reduction do not propagate to the reduction of P700. Similarly, in the mutants and also the wt, the kinetics of cyt $f$ reduction do not propagate to the stigmatellin-sensitive reduction of PC.

Systems with equivalent kinetics of cyt $f$ reduction do not have equivalent kinetics of P700 and PC reductions- If electron donation to PC and P700 is linked to PQH$_2$ oxidation only via the cyt $b_{6}f$ complex and exclusively through cyt $f$, as in: PQH$_2$$\rightarrow$Qo site$\rightarrow$Fe$_2$S$_2$$\rightarrow$cyt $f$$\rightarrow$PC$\rightarrow$P700, and because the PSII and PSI photochemistries and the PC shuttle activity are much faster than the cyt $b_{6}f$ complex reactions, then the (pseudo)steady state P700 and PC reduction kinetics should be some unique function of the electron flux through a rate limiting step in the cyt $b_{6}f$ complex (e.g. the cyt $f$ reduction in a cyt $f$-inhibited system). Thus, systems with comparable electron carrier stoichiometry and cyt $f$ reduction kinetics should also show other comparable P700 and PC reduction kinetics: the cyt $f$ reduction should determine the P700 and PC reductions. Violation of this prediction implies that the electron flow can bypass a rate limiting step at cyt $f$. We have mimicked in the wt the cyt $f$ reduction kinetics of P2V and P2V/R156A through the addition, in the wt, of subsaturating or saturating concentrations of stigmatellin, compare traces in Fig. 1. In spite of the equivalency in cyt $f$ reduction achieved in those ways, the kinetics of P700 reduction are very different: in P2V without inhibitors P700 is reduced as fast as in the wt (Fig. 1), whereas in wt with subsaturating stigmatellin (6 $\mu$M) P700 reduction shows a strong inhibition (Fig. 1). Therefore, the prediction of “uniqueness of kinetic dependence” is not confirmed. Mutant P2V/R156A enhances the contradiction (compare the P700 traces: “0 $\mu$M” in P2V/R156A vs. “22 $\mu$M” in...
wt or P2V). We conclude that the electron transfer activity through the cyt $b_6f$ complex HP chain (observable as the stigmatellin-sensitive reduction of P700) is not determined by the cyt $f$ reduction, see below. We extended this test to the reduction of PC and we concluded that the kinetics of stigmatellin-sensitive reduction of PC is also not determined by the cyt $f$ reduction.

The ratios of amount of cyt $b$ reduced per amount of cyt $f$ oxidized are not constant in function of flash intensity both in wt and mutant P2V. As the cyt $f$ reduction rates are independent of flash intensity it is expected that, after one flash, the ratios of amount of cyt $b$ reduced per amount of cyt $f$ oxidized in function of flash intensity should also be constant. The theory predicts a ratio $\sim 1:1$, independently of the flash intensity. The maximum amplitude of cyt $f$ oxidation with a train of 8 flashes (intensities $\geq 25\%$ a.u.) is the same in the wt and P2V (Fig. 2). The cyt $b$/cyt $f$ results however, are in contrast with the expectations of a constant ($\sim 1:1$) stoichiometry (Fig. 2). In the wt, at very low flash intensities (single flash), the amplitude of cyt $b$ photoreduction is clearly larger (2.5 fold) than the amplitude of cyt $f$ photooxidation. For P2V, at very low flash intensities the ratio is only 0.3, smoothly increasing with increasing flash intensity, and approaching 1 only at very high intensities. These results show for wt and P2V that the ratio of cyt $b$/cyt $f$ is highly sensitive to flash intensity and therefore, there is more than one light dependency in the operation of the redox reactions of the cyt $b_6f$ complex. The “cyt $f$-heme-dependent mechanism” hypothesis is contradicted and, instead, the hypothesis of a “cyt $f$-heme-independent mechanism” holds.

Physiological evidence- If cyt $f$ is the only electron donor to PC, mutants with strongly decreased cyt $f$ reduction rates should show correspondingly inhibited photosynthetic electron transfer steady-state rates. The total (respiration-corrected) light-saturated photosynthesis in intact cells of wt, P2V, and P2V/R156A is 158, 90, and 92 $\mu$mol O$_2$ mg Chl$^{-1}$ h$^{-1}$ (errors $\leq \pm 13\%$), respectively. In all cases the steady-states are stable for $>25$ min. Thus, in spite of the 5-fold lower rate of cyt $f$ reduction and a factor of 2 lower cyt $b_6f$ complex/P700 stoichiometry in P2V/R156A respect of P2V, their rates of light-saturated O$_2$ evolution are identical and only $\sim 45\%$ smaller than in the wt. Most remarkably, the inhibited rates in the mutants are much faster (13 to 90-fold) than the calculated electron fluxes through the cyt $b_6f$ complex if the cyt $f$ reduction is considered the rate limiting step. For wt, P2V, and P2V/R156A these values are 166, 6.9, and 1.0 $\mu$mol O$_2$ mg Chl$^{-1}$ h$^{-1}$, respectively. Thus, mutations P2V and P2V/R156A have only moderate effects in the electron transfer from

![Fig. 2](image-url)
PQH$_2$ to PSI, adding further proof that electrons can be transferred from PSII to PSI in a cyt f-independent way, see Fernández-Velasco et al. (2001).

New catalytic site in the cyt b$_6$f complex- All our results falsify any hypothesis explaining the kinetic uncoupling between the cyt f and cyt b reductions involving a “cyt f-dependent mechanism” type of model: a strongly inhibited cyt f re-reduction in the mutants does not become the rate limiting step in the electron transfer catalyzed by the cyt b$_6$f complex. Instead, all the results are consistent with the hypothesis involving a “cyt f-independent mechanism”. This points to a PQH$_2$:PC redox activity of the cyt b$_6$f complex that bypasses the cyt f heme. Therefore, we define a fourth catalytic site in the cyt b$_6$f complex: a cyt-f-heme-independent PC reductase. We name this new site and path as “site R” and “path R”, respectively, in order to identify it as “only-(R)ieske-center dependent”. We distinguish it from “site F” and “path F”, that are the cyt f:PC oxidoreductase site and route involving the cyt (f) heme. The upper limit for the t$_{1/2}$ of “site R” is 700 µs. The Rieske protein, with a luminal protruding domain bearing the Fe$_2$S$_2$ center (Carrell et al., 1997), could be the extra docking site for PC and the alternative electron transfer point. In P2V and P2V/R156A, most of the electron transfer activity would take place through path R and cyt f would be reduced slowly, either directly by the Rieske center, or, indirectly, via PC previously reduced by path R. The affinity of PC for site R would be 2-3 fold weaker than the same for site F, as indicated by the higher K$_m$ for light of curve (c) for P2V in Fig. 2. Electron transfer through path R is also possible in the wt, as evidenced by the stigmatellin-sensitive P700 and PC reductions, which are 3-4 fold faster than the corresponding cyt f reduction. The functionality of site R in the wt can also be evidenced through the cyt b/cyt f ratio at very low flash intensities; indeed the cyt b/cyt f ratio of 2.5 necessarily means that, in those conditions, there are cyt b$_6$f complexes where cyt f is not oxidized but cyt b is reduced anyhow (Jamshidi et al., 2001). The functional meaning of the coexistence of paths F and R and their steady-state operating ratios in the wt remain to be defined. Thus, although cyt f is redox-active, the precise role of this conserved protein needs clarification. Cyt f could be part of the proton path connecting Q$_o$ with the thylakoid lumen (Martinez et al., 1996).

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References