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A new catalytic site in the cytochrome b_6 f complex of *Chlamydomonas reinhardtii*

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Introduction

The Q-cycle model for the operation of the cytochrome (cyt) b_6f 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 Fe_2S_2 center by cyt *f* (i.e. re-reduction of cyt *f*), 3) oxidation of PQH_2 to semiquinone by Fe_2S_2 center at the Q_o 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 ($t_{1/2}$ 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 ($t_{1/2}$ 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 Q_o is the reduction of the low potential (LP) chain (cyt *b*). This mechanism would require a conformational change at the Q_o site, induced by cyt *f* (photo)oxidation and without any role for cyt *f* reduction, to change the reductant's E_m at Q_o. 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 Fe_2S_2 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 b_6f complex and must propagate to (have limiting kinetic consequences in) the Q_o-inhibitor-sensitive P700 and PC reductions and to the steady-state electron transfer (Fernández-Velasco et al., 2001). ii) In PQH_2-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_2S_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_2S_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_2S_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_2S_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. Δ*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.

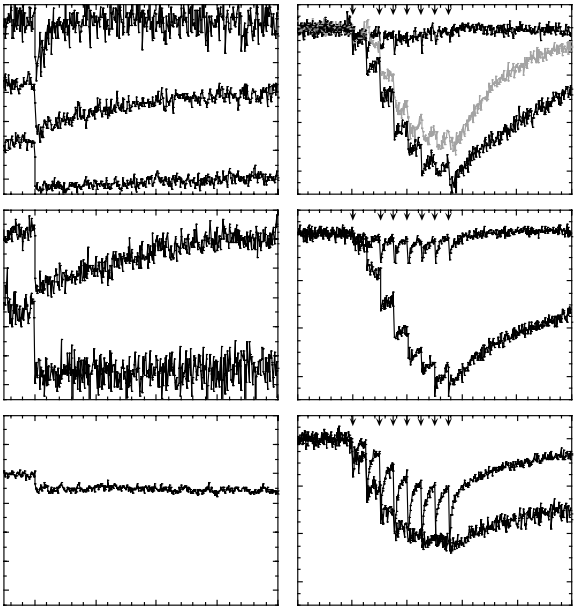

Fig. 1: Redox kinetics of cyt *f* (single 67%-P700-saturating flash) and P700 (train of 40% sat. flashes) with or without stigmatellin (conc. indicated). Intact cells of wt, P2V, and P2V/R156A, indicated; in anaerobiosis + FCCP. Cyt *f* (554-545 nm), RC= 200 μs. P700 (702-730 nm), RC= 10 μs. Arrows: flashes; dotted lines: time zero; dark time between repetitions: 30 s; downward deflections are oxidations.

To improve the time resolution of the kinetic coupling between the stigmatellin-sensitive electron donation of the cyt b_6f complex and the reduction of P700, two 92% P700-saturating flashes fired 250 μs 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 μs 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 μs) Fe_2S_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_6f complex and exclusively through cyt *f*, as in: $PQH_2 \rightarrow$Qo site$\rightarrow Fe_2S_2 \rightarrow$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_6f 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_6f 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 μM) 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 μM" in P2V/R156A vs. "22 μM" 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 ~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 (~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 μmol O_2 $mgChl^{-1}$ h^{-1} (errors $\leq$±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 ~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 μmol O_2 mg Chl^{-1} h^{-1}, respectively. Thus, mutations P2V and P2V/R156A have only moderate effects in the electron transfer from

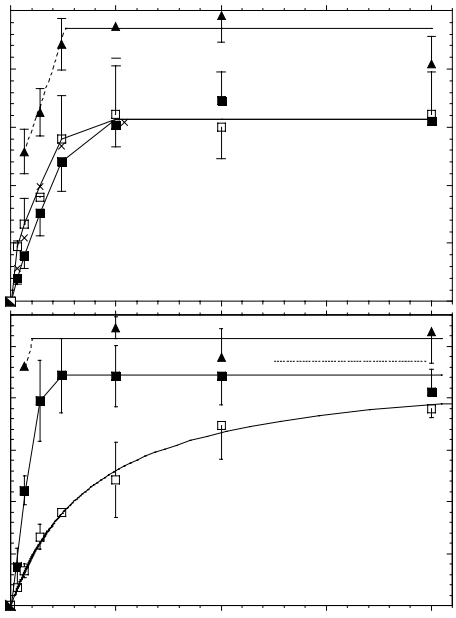

Fig. 2: The maximal amplitude of photooxidized cyt *f* and photoreduced cyt *b* in function of flash intensity for wt and P2V. The experiments are at Eh= 0±15mV and using permeabilized cells (30 μg Chl/mL). Default flash intensity is 100 %a.u. (a,b) Amplitudes of cyt *f* oxidation (554-545nm), in the presence of 10 μM HQNO and stigmatellin for wt, and 10 μM HQNO for P2V. (a) Maximal amplitude of cyt *f* oxidation with one flash (closed squares). (b) Maximal amplitude of cyt *f* oxidation observable within a train of 8 flashes fired at 60 ms apart (closed triangles). Curves (b) drop at <25 % a.u. because 8 flashes are not enough for full oxidation. (c) Maximal amplitude of cyt *b* reduction (564-575nm) with one flash, in the presence of 10 μM HQNO (open squares). With the same sample first is done (c) followed by (a,b). In the P2V panel, (c) was fitted with a hyperbolic function and the maximal amplitude of cyt *b* pho-toreduction (asymptote) is shown by (d). The wt panel also shows the calculated total cyt *b* that would be photoreduced (×) if both paths F and R would be operative (Jamshidi et al. 2001).

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_6f 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 *b6f* 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_6f complex that bypasses the cyt *f* heme. Therefore, we define a fourth catalytic site in the cyt b_6f 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 lumenal protruding domain bearing the Fe_2S_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_6f 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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