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Review

The mechanism of cyclic electron flow

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ABSTRACT

Apart from the canonical light-driven linear electron flow (LEF) from water to CO_2, numerous regulatory and alternative electron transfer pathways exist in chloroplasts. One of them is the cyclic electron flow around Photosystem I (CEF), contributing to photoprotection of both Photosystem I and II (PSI, PSII) and supplying extra ATP to fix atmospheric carbon. Nonetheless, CEF remains an enigma in the field of functional photosynthesis as we lack understanding of its pathway. Here, we address the discrepancies between functional and genetic/biochemical data in the literature and formulate novel hypotheses about the pathway and regulation of CEF based on recent structural and kinetic information.

1. Linear and cyclic electron flow

Photosynthesis consists of a photoinduced linear electron flow (LEF), where electrons from water are transferred to $NADP^+$. The electron-coupled proton translocation generates a proton motive force across the thylakoid membrane that is used by the CF_1-F_0 ATP synthase to form ATP. Photoproduced ATP and NAD(P)H then allow CO_2 fixation by the Calvin-Benson-Bassham cycle. However, alternative electron transfer pathways have been documented in photosynthesis. Most notable among them are water-to-water cycles and cyclic electron flow (CEF) around Photosystem I (PSI). All these alternative pathways translocate extra protons into the lumen without producing reducing compounds, thus increasing the ATP/NAD(P)H ratio. The dynamic changes between the rates of LEF and alternative pathways are thus critical in conditions where i) NAD(P)H accumulates when the electron transfer chain is kinetically limited by the NAD(P)H oxidation pathways (carbon fixation and others), and ii) lumen acidification needs to be enhanced to protect the two photosystems *via* non-photochemical quenching (NPQ) and photosynthetic control at the level of cytochrome b_6f (cyt. b_6f).

Despite the well-recognized importance of CEF in the regulation of photosynthesis, information about its precise mechanism is still lacking. Not only is our understanding of the regulation of CEF and LEF pathways scarce, but even the cofactors involved in electron transfer from Ferredoxin (Fd) to plastoquinone (PQ) remain elusive. Here, we reassess current knowledge about CEF pathways, in particular from a kinetic and methodological standpoint in the light of our report that PGRL1 is not directly transferring electrons from Fd to PQ during CEF [1]. We further propose a mechanism that can explain how and why the rate of CEF is not tightly controlled by the redox state of the PQ pool.

2. Current view on cyclic electron flow

CEF was first identified by the Arnon group ([2] and references therein). These experiments pointed to the existence of cyclic photophosphorylation as a pathway of light-dependent ATP phosphorylation, where the net production or consumption of reducing power was null. Therefore, CEF was defined as a rerouting of reducing equivalents from the acceptor side of PSI back to its donor side. The implication of cyt b_6f in CEF, also proposed by Arnon and co-workers, was further confirmed by the sensitivity of CEF to Q_o site inhibitors [3]. Later attempts to identify specific CEF transporters - in particular through genetic approaches aimed at the isolation of mutants devoid of CEF - fell short but allowed to disclose molecular bases for CEF *regulation*. These studies led to propose two CEF routes, which are considered by most authors to be responsible for the reduction of PQ by PSI acceptors: the NDH-1/NDH-2-, and PGR5/PGRL1-dependent pathways (*e.g.* ref. [4]) (Fig. 1). For the first pathway, the components allowing the transfer of electrons from PSI acceptors to the PQ pool are homologous to the respiratory NAD(P)H:PQ oxidoreductases. In land plant chloroplasts, NDH-1 shares at least 11 subunits with the mitochondrial and bacterial NADH:UQ oxidoreductase (complex I) [5]. Chloroplast NDH-1 complex however lacks an

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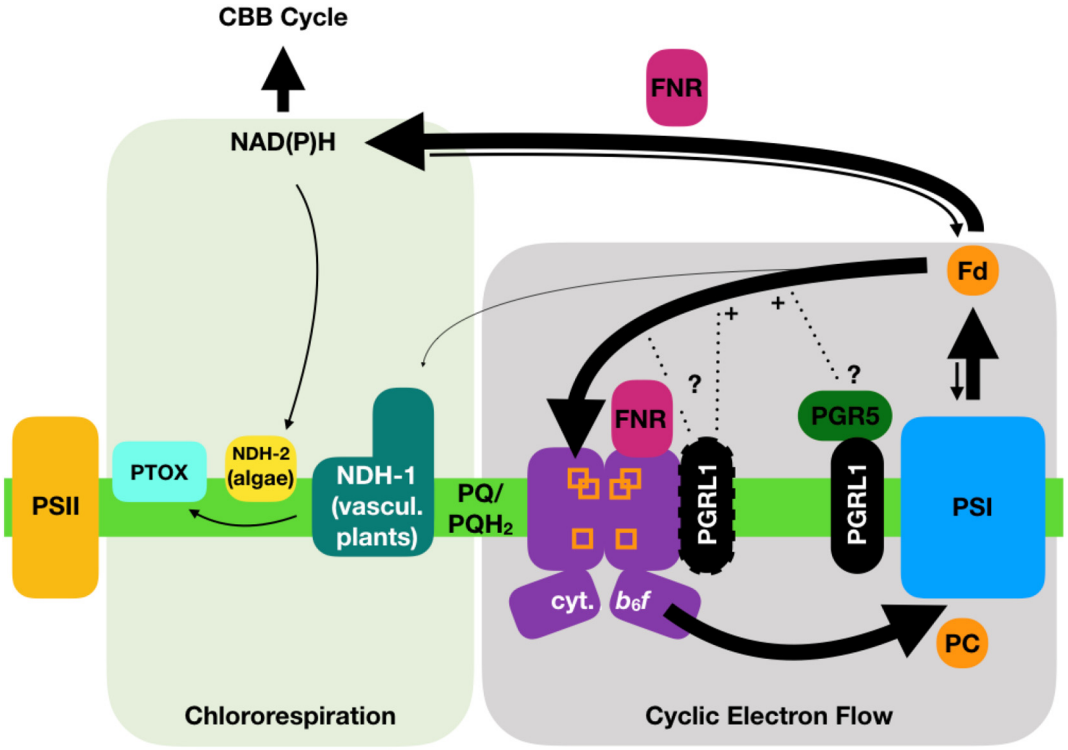

Fig. 1. A scheme of CEF and chlororespiration, and the molecular actors involved in these processes. PGRL1 (dashed) could almost stoichiometrically bind to cyt. b_6f to regulate CEF. Note that chlororespiration rates are so low that the only contribution of this process is observed in darkness [16].

NADH binding module and has been proposed to use Fd as a substrate [6]. It probably pumps additional protons per electron transferred [7], similarly to mitochondrial and bacterial complexes I. In some microalgae, NDH-2 is present instead of NDH-1. It resembles mitochondrial Ndi1 [8], and is a monotopic membrane protein [9] that inserts into the stromal leaflet of the thylakoid membrane, and therefore is unable to pump protons to the lumen upon oxidation of its substrate, NAD(P)H [10]. Yet, the enzymatic pathway allowing the electron transfer from PSI acceptors back to the electron transfer chain upstream cytochrome b_6f is still a matter of debate. Any legitimate candidate (enzyme or combination of enzymes) for the closing of the cycle must display a turnover rate in agreement with the highest rates of CEF measured *in vivo*, *i.e.* ~100 and ~60 electrons per second per PSI in plants [11,12] and in green algae (from the companion paper, [1]).

The role of NDH-1 in CEF is doubtful from many perspectives. It is highly sub-stoichiometric with regards to PSI (1:100 ratio [13]), therefore its rate would necessarily need to exceed $10^4\,e^-{\cdot}s^{-1}$ per complex to sustain transitory rates of CEF in plants in the order of a hundred of electrons per second per PSI [11,12], a value up to 4 orders of magnitude higher than experimentally measured *in vivo* for this enzyme [14]. Experimental data shows that the absence of NDH-1 does not alter the q_E (pH-dependent NPQ) – and therefore its proton-pumping contribution is negligible – in plants [15], consistent with its measured rate being in the order of only one tenth (0.1) $e^-{\cdot}s^{-1}{\cdot}PSI^{-1}$ *in vivo* [14]. Similarly, NDH-2 - the algal counterpart of NDH-1 – runs at 2.5 $e^-{\cdot}s^{-1}{\cdot}PSI^{-1}$ [7,16], a rate which is far too sluggish to sustain the rate of 60 $e^-{\cdot}s^{-1}{\cdot}PSI^{-1}$ for CEF that we report in the companion paper [1]. Finally, an NDH-2 mutant only showed limited changes in electron transfer with a slower PQ re-reduction in darkness [10] or in the light, exclusively when the competing PQ-reducing PSII activity was severely affected [17]. It is thus likely that the activity of NDH enzymes is limited to chlororespiration [7] (Fig. 1).

The other most studied putative CEF route is described as "PGR5/PGRL1-dependent". The two molecular actors of this pathway were discovered in mutagenized plants exhibiting distinctly low steady-state q_E, and thus ΔpH across the thylakoid membrane, leading to their name (proton gradient regulation 5 and pgr5-like 1, respectively) [18,19]. However, despite a plethora of proof of their involvement in the regulation of CEF [4,18,20–24], there is still no clear evidence of their *direct* role in the transfer of electrons from the PSI acceptors to the PQ pool. On the contrary, several observations make this very unlikely. PGR5 is a small, stroma-soluble protein, binding no cofactors [25], and it is found tethered to the thylakoids by PGRL1. The maximal rate of CEF does not change in the absence of the former at the beginning of the induction of photosynthesis, showing that while PGR5 is involved in fine-tuning of CEF, its presence is not required for a fully efficient Fd:PQ electron transfer [20]. PGRL1 has been proposed to fulfil the role of the elusive FQR [26], directly reducing quinones, and could almost stoichiometrically bind to the fraction of cyt. b_6f found in the stroma lamellae [27]. However, the FQR role is difficult to reconcile with its sub-stoichiometric ratio relative to PSI, with about 0.3 proteins per PSI, or 0.15 PSI^{-1} if it indeed dimerises *in vivo* [26]. Crucially, we show in the accompanying article [1] that a *Chlamydomonas pgrl1* mutant exhibits no differences in the maximal transitory rate of CEF, which would be expected if PGRL1 was a *bona fide* CEF Fd:PQ oxidoreductase. Instead, we observed that the PSI acceptor side was altered in its contribution to charge recombination within PSI and to the rerouting of reductants toward CEF, both of which being lower than in the WT. These traits rather argue for an indirect role of PGRL1 in CEF, similarly to that of PGR5 [28], by regulating either the fate of PSI acceptors or the redox state of the stroma. Such a role would go in line with the observations that PGRL1 is required for recruitment of PGR5 into PGRL1/PGR5 complex [28].

3. Cyt. b_6f is a well-known plastoquinone reductase

The lack of evidence for sustained CEF through the two pathways above strongly suggests another pathway for rerouting reductants produced by PSI to its donor side: namely a direct PQ reduction by cyt. b_6f (Figs. 1, 2A). Originally proposed by Mitchell and reiterated

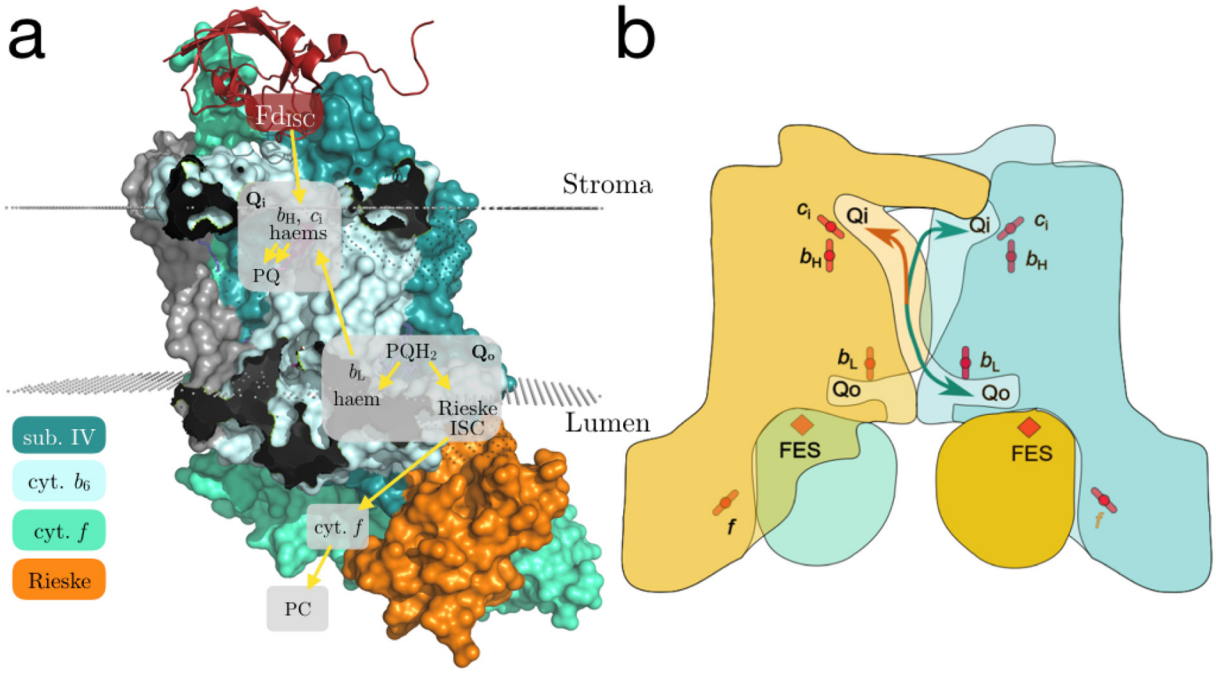

Fig. 2. Electron and plastoquinone transfer within cyt. b_6f. (a), A scheme of possible cyclic electron transfer within cyt. b_6f. A side view of the cyt. b_6f monomer from the dimerization surface perspective. The stromal and luminal extremities of the thylakoid membrane are depicted in grey (OMP). Separate subunits of the monomer are coloured individually as indicated in the figure. The electron transfer pathways are shown as yellow arrows. Ferredoxin in its putative binding mode is shown in a red cartoon form. (b) PQ/PQH2 transfer within cyt. b_6f dimer. Side view of the complex. One cyt. b_6f monomer is in orange, while the other one is blue. Possible route for the quinone between the Q_o and Q_i side are represented in green for transfer in the same monomer, which implies crossing the narrow portion of the crevice, or in green/orange for transfer in the opposite monomer, which uses a broader and shorter path. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

afterwards [11,29], the classical Q-cycle involves a double reduction of PQ at the Q_i site. Initially, such a mechanism included one electron provided by the b_H haem (following a PQH_2 oxidation at the Q_o site) and the second electron provided by a stromal donor, such as Fd, possibly through the later discovered redox-active c_i haem which is absent in mitochondrial and bacterial cyt. bc_1 [11,30–32]. This early mechanism was mostly abandoned at the expense of the modified Q-cycle, in which the electrons used for PQ reduction at the Q_i site are stored on the two *b* haems [33–35]. Nonetheless, for the purpose of rapid CEF (see above), it is possible to envisage the abovementioned scenario of Fd^--[stromal haems] electron transfer similar to the classical Q-cycle. This is because once Fd is placed in a putative binding site on the stromal side of cyt. b_6f (Fig. 2A), its iron-sulfur cluster has an edge-to-edge distance to the PQ or the haems in the Q_i site that is short enough to allow efficient electron transfer between them, as defined by the Moser-Dutton ruler [36]. Accordingly, Fd:NAD(P)H oxidoreductase (FNR) was shown to bind to the b_6f and at least to regulate CEF [12,37,38]. It could therefore act as a tether for Fd in the vicinity of stromal haems of the b_6f [38]. One could further argue that FNR is involved directly in CEF as a cofactor and mediates the electron transfer between Fd and stromal haems: indeed Fd in an Fd:FNR complex has a redox potential of −400 to −500 mV, when FNR alone stands at −350 mV [39], and the b_H and c_i haems are at −30 mV and +100 mV, respectively (note that in dark-adapted algae the redox difference between the latter two is much smaller with the c_i haem being more reduced than the b_H haem; see ref. [40] for a discussion). Functional measurements of CEF *in vivo* are in agreement with a strong involvement of FNR in the process [12,41].

If indeed the cyt. b_6f is the FQR involved in CEF, there might not be a single cofactor specific to CEF - the c_i and b_H haems seem to form an ensemble in any type of Q-cycle. Crucially, this is in line with (i) the absence of mutants fully devoid of CEF but not of LEF; (ii) site-directed mutagenesis in the vicinity of the ci haem heavily affecting overall cyt. b_6f function (personal communication, C. de Vitry, F.-A. Wollman); and (iii) specific binding of CO to the c_i haem strongly impeding LEF [42].

In vascular plants the maximal rate of CEF is comparable with that of LEF, making it difficult to determine which exact step of those two interdependent pathways is limiting *in vivo* [12]. It remains a possibility that the PQH_2 oxidation in the Q_o site constitutes the slowest step of the electron transfer in both CEF and LEF. On the other hand, we have shown that in *Chlamydomonas* the maximal CEF rate is lower, around 60 instead of $\sim 100\, e^- \cdot s^{-1} \cdot PSI^{-1}$ [1]. We speculate that in this case, the Fd^--[stromal haems] transfer could be limiting for the overall rate. Given the differences in architecture of the cyt. b_6f in *Chlamydomonas* due to the presence of regulatory/associated proteins [43–45] and the borderline distance for electron tunnelling between Fd and the stromal haems, some minor changes of the stromal side of the cytochrome could affect the rate of the Fd^--[stromal haems] electron transfer.

4. Dimeric structure of cyt. b_6f complex and its functional implications

Most functional studies are satisfactorily interpreted in the framework of a monomeric complex, but tridimensional structures showed that the two cyt. b_6f monomers are strongly intertwined in the dimer [31,32,46] (Fig. 2B). On one hand, the soluble domain of the Rieske protein of one monomer interacts with the other one. On the other hand, the interface between monomers spans the transmembrane space and forms a V-shaped cavity filed with lipids. The tip of this V-shaped cavity is on the lumenal side, next to the entrance of the Q_o site of one monomer whereas the Q_i site of the other monomer is positioned toward the stroma where the crevice becomes wider.

Crucially, these structural features may deeply impact cyt. b_6f activity. The PQ/PQH_2 bound to either site could easily slide between the Q_i and the Q_o sites of opposite monomers. The helix D of cyt. b_6 also forms the narrowest part of the crevice next to the two-fold symmetry

axis and creates a potential hindrance to the transfer of quinones between the Q_o and Q_i sites of the same monomer. Interestingly, the two b_L haems have an edge-to-edge distance of 14–15 Å, sufficiently close to allow interdimer electron transfer [47,48]. All these features are shared not only by all cyt. b_6f complexes, but also by all cyt. bc_1 complexes and are probably a general feature of most other Rieske/cytochrome *b* complexes. However, some features, potentially critical for the CEF function discussed here, differ between the cyt. b_6f and proteobacterial-related cyt. bc_1 complexes. The Q_o site entrance in the cyt. b_6f complex harbors the phytyl chain of a chlorophyll molecule that modifies the accessibility of the active site [49]. Most importantly, the Q_i site of the cyt. b_6f complex has the additional haem c_i that pushes the Q_i pocket toward the stroma by about 6 Å, the position of haem b_H staying identical. The amphipathic helices of cyt. b_6, delimiting the cavity from the stromal side, and most likely the membrane boundaries, are shifted by an equivalent distance. Despite these changes, the hydrophobic residues of the amphipathic helix that faces the crevice are well conserved, underlying the critical importance of this inter-monomer space.

5. Regulation of CEF/LEF partitioning – involvement of the dimeric cyt. b_6f

The model for CEF, proposed by Mitchell and others [11,29] and further discussed here in the light of structural information, has strong implications regarding the competition between LEF and CEF. Due to its transmembrane interfacial cavity, the cytochrome b_6f complex offers a niche for quinones, which should be in slow equilibrium with the rest of the PQ pool. Indeed, it has been shown earlier by Joliot and Joliot [50] that, in reducing conditions, a quinone readily produced (oxidized) at the Q_o site has an absolute priority to shuttle to the Q_i pocket of the very same protein complex rather than to escape and diffuse to other Q_i sites in its vicinity. In anoxic *Chlorella* cells, subsaturating laser flashes lead to the oxidation of a small proportion of cyt. *f* and to the rapid generation of an electric field caused by a quinone reduction by the low potential chain [50]. This indicates that the quinone generated at the Q_o site successfully shuttles to the Q_i site *of the same* cyt. b_6f complex to act as an electron acceptor from the reduced b_H and b_L haems. However, transfer of the nascent quinone between the Q_o and Q_i sites from a same monomer may be challenging due to the obstacle formed by D helices of cyt. b_6 (see above and in Fig. 2). We thus further propose that a direct electron transfer between the b_L haems, experimentally detected in cyt. bc_1 complexes [47], may alleviate this difficulty by “coupling” the two monomers within the dimeric complex. The nascent quinone at Q_o, which has reduced the b_L haem in monomer A (“b_L(A)”) could be next transferred to the Q_i site of monomer B, Q_i(B), which is adjacent to the Q_o site (A), through the internal cavity of the dimer (see Fig. 2b). Electron tunnelling between haems b_L(A) and b_L(B) could then occur: its rate, according to the Moser-Dutton ruler [36,51] is within the measured rates of CEF in reducing [22] and oxidizing conditions [1] in the order of $60\ e^- \cdot s^{-1} \cdot PSI^{-1}$, especially because of the lack of an electron acceptor in the Q_i(A) pocket. Would this happen, the two haems of the low potential chain of the B monomer would be reduced, thus allowing a reduction of the Q_i(B)-bound quinone and the observation of the electrogenic $b_L(B) \rightarrow b_H(B)$ electron transfer despite an initial oxidation of the high potential chain in the A monomer. Such intermonomer electron transfer would not necessarily need to continuously compete with the more efficient monomeric turnover [52] if one considers it a mean to ‘prime’ the system during a transition from reducing to oxidizing conditions. Unfortunately, a molecular dynamics study, which could reveal preferential intracomplex quinone shuttling, similar to diffusional exploration of quinone channels in PSII supercomplex [53], has not been conducted yet.

Consequently, we further propose that *in vivo*, nascent PQH_2, reduced at the Q_i site, have a high probability to be reoxidized at the adjacent Q_o site of the same cyt. b_6f dimer, *i.e.* without entering the “PQ pool”. Such a mechanism provides heterogeneity in the pool of PQ/PQH_2, and uncouples LEF and CEF at this step. Indeed, the LEF and CEF routes to the Q_o site involve different pools of PQ/PQH_2: the LEF route from PSII involves the pool of PQ/PQH_2 outside of the cytochrome b_6f, whereas the CEF route would only require the plastoquinol in the cavity of the cyt. b_6f complex. The reduction of a quinone at the Q_i site would be *independent* of the overall redox state of the PQ pool and thus would not compete for PQ reduction by PSII. In such a CEF model, previous proposals [54,55] that the highest efficiency of CEF is achieved when 50% of the PQ pool is oxidized, and 50% in reduced state would not be required. It would allow CEF to be efficient also under high light, when the PQ/PQH_2 pool is strongly reduced, as supported by experimental data [56].

Therefore, we consider that a control of the routing of electrons in the stroma is sufficient for a modification of CEF/LEF partitioning, without a need for considering the redox state of the plastoquinone pool. Competition between CEF and LEF for reduced Fd is, in our view, the hub where those two pathways require some regulatory processes. As outlined above, Fd^- is readily oxidized by the CBB cycle and also by Flv proteins in microalgae and cyanobacteria [57]. We showed that such an oxidation is decreased at low oxygen concentrations when CEF increases. Furthermore an increase in the *duration* of CEF also was observed in an Flv mutant of moss [58], yet it was interpreted as a compensatory increase in CEF rather than a decrease in electron leak.

The regulation between CEF and LEF could be achieved by increasing the probability that Fd stays in proximity of the stromal side of the cyt. b_6f, through its anchoring by the cyt. b_6f-bound FNR, as had been proposed earlier [12]. Recently, FNR was shown to be a target of the redox-sensitive STN kinase, a transmembrane enzyme which interacts with cyt. b_6f [59,60]. STN being activated upon reduction of the PQ pool, one can imagine a tentative model for an FNR phosphorylation-dependent CEF regulation - but there is a need to produce functional and biochemical data regarding the regulation of Fd-FNR-b_6f binding before such a model can be critically assessed. Fd is at the very crossroads of photosynthetic electron transfer, donating electrons not only to CEF and LEF, but also to other metabolic pathways such as nitrite and sulphide reduction [61]. Furthermore, there are multiple Fd isoforms, with varying redox potentials and concentrations, which interact with multiple isoforms of FNR, some of which are soluble and some membrane-bound [61,62]. It is obvious that a strict regulation of this hub is necessary both for photosynthesis and for poising the redox state of the entire stroma, making it easy to imagine that CEF is also governed at this level.

6. Supercomplexes: can they really *drive* CEF?

A dynamic heterogeneity among electron transfer complexes that would favour CEF also has been sought based on the formation of PSI-cyt. b_6f supercomplexes [63]. Originally proposed to account for the elevated rates of CEF observed in anoxic conditions in *Chlamydomonas*, it is poorly consistent with our recent study where we compared maximal rates of CEF in conditions that would favour - or not - such supercomplex formation [1]: we found no evidence for changes in CEF maximal rates although we did observe that a lower oxygen availability increased the duration of CEF due to a slowdown of the electron leakage from the electron transfer chain.

Nevertheless, the biochemical identification of supercomplexes associating cyt. b_6f, PSI and a number of CEF-related proteins has stimulated much speculations as to the way CEF may function [43,45,63–66]. The hypothesis of a supercomplex-borne CEF process raises several mechanistic and stoichiometric issues. Cyt. b_6f being a dimer, a cyt. b_6f dimer-PSI supercomplex uses twice as much cyt. b_6f as PSI. The overall cyt. b_6f:PSI stoichiometry is about 0.7 [67], with less than half of cyt. b_6f present in the appressed regions of the membrane [68]. In consequence, less than 20% of PSI are available for supercomplex formation.

From a structural standpoint, PSI from Chlamydomonas placed in

state II in anoxia, where supercomplex formation would be favoured, binds multiple PSII antenna on the side where its own peripheral antenna, LHCa, are not attached [69]. This leaves a place for binding of a cyt. b_6f dimer only on the edges of the PSI complex. Such putative supercomplex between the b_6f and PSI was present in less than 1% of PSI particles as assessed with single-particle cryo-EM after thylakoid solubilisation ([70], Supporting information). Furthermore, in cyt. b_6f-PSI supercomplex particles isolated from land plants, it was found that a majority of the complexes bound a cyt. b_6f in a monomeric form [64]. Recent biochemical data finally calls into question the significance of green bands of high molecular weight in sucrose density gradient preparations from *Chlamydomonas* thylakoids. These should not be mistaken for cyt. b_6f-PSI supercomplex formation, since various PSI complexes can migrate in these sucrose density regions. [44].

As for the kinetic rationale for supercomplex formation, one can take the well-documented supercomplex in mitochondria, the NADH dehydrogenase-cyt. bc_1, as a case study. Through flux control analysis, this complex was suggested to provide a kinetic advantage for NADH oxidation due to a purported trapping of the lipid-soluble electron carrier, ubiquinone (UQ), allowing its oxidation before it diffuses away within the mitochondrial membrane [71]. However, recent structural data argues against the UQ trapping in a mitochondrial respirasome and provides an "open" structure, at least for the UQ reduction site in the NADH dehydrogenase (see [72] and the discussion within). Moreover, the distance between the cyt. *c* reduction site on cyt. bc_1, and its oxidation site on cyt. *c* oxidase (COX), is too large to propose an electron transfer without a release of cyt. *c* from the respirasome. Finally, the lack of kinetic advantage from complex formation between bc_1 and COX has been experimentally demonstrated in yeast [73]. The conclusions from respiratory system indicate that a presence of supercomplex does not infer a kinetic advantage for electron transfer.

We suggest that a similar situation does hold for CEF supercomplexes between PSI and cyt. b_6f [63,64]. A trapping of water-soluble CEF carriers, Fd and PC would be required to prevent an electron leak to LEF. Such a trapping is unlikely because the distances between cyt. *f* and the PC binding site in PSI, and between F_A/F_B iron-sulfur clusters and the hypothetical Fd-binding site close to the Q_i site are too large to allow intracomplex electron transfer without a release and diffusion of these soluble carriers (at least 100 Å, see [64,74]). Moreover, the kinetic limitation in both cases (presence or absence of PSI-b_6f complex) still would be set by the rate of PQH_2 oxidation within the membrane, at the Q_o site of the cyt. b_6f. Such a system could at best contribute to the regulation of the CEF/LEF ratio, but it would not control the maximal rate of CEF. Finally, the complexes between PSI and NDH are even more unlikely to have functional relevance for CEF not only due to the extremely low rates of NDH, as discussed above, but critically due to the absence of the PQ:PC oxidoreductase within such complex [64,75,76].

To conclude, the mechanism and actors of cyclic electron flow around Photosystem I remain far less well established than the photosynthetic community generally assumes. It is crucial to better consider the kinetics of this process in order to propose appropriate assumptions as to its mechanism and regulation. While the contribution of auxiliary CEF proteins such as PGR5 and PGRL1 is now firmly grounded, the actual mechanism of CEF is probably very different from what is presently discussed in the literature. It is of note that the functional insights provided by the 3D structure of cyt. b_6f have been overlooked up to now. The quinone cavity inside the cyt. b_6f dimer stands like a treasure trunk that has not yet revealed its CEF secrets. This is a thrilling time for the bioenergetics of photosynthesis.

Transparency document

The Transparency document associated with this article can be found, in online version.

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