Chapter 14

Cyclic Electron Flow in Cyanobacteria and Eukaryotic Algae

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In oxygenic photosynthesis light energy is largely captured in linear electron flow (LEF) between the photosystems and drives ATP formation *via* a thylakoid proton-driven ~~ATPase~~. In addition, for over 50 years there has been good evidence that an additional cyclic electron flow (CEF) around photosystem I (PSI) is harnessed to provide extra ATP in addition to that produced by LEF. The evidence comes from all oxygenic organisms, cyanobacteria, eukaryotic algae and embryophytic plants. However, the CEF mechanism has been difficult to investigate because of the cyclic nature of the EF and confusion with other pathways not using oxygen as a terminal electron acceptor, and the MAPS, flavodiiron and chlororespiration pathways to oxygen. This article discusses the current evidence for CEF in all oxygenic organisms and suggests future experiments by which the situation can be clarified.

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1. Introduction

The first description of photophosphorylation, *i.e*. light-energized ADP phosphorylation by isolated *Spinacia oleracea* thylakoids, was by Arnon *et al*. [1954], and "cyclic photophosphorylation" was coined by Arnon *et al*. [1958] for ATP production by thylakoids with no net synthesis of an oxidant and a reductant. Tagawa *et al*. [1963] demonstrated the occurrence of cyclic photophosphorylation by isolated thylakoids with ferredoxin as the only added redox co-factor; ferredoxin is now recognised as an *in vivo* co-factor in cyclic electron flow (CEF) coupled to H^+ flux into the thylakoid lumen and hence, *via* H^+ flux out of the lumen through the CF_OCF_1 ATP synthase, ADP phosphorylation [Peltier *et al.,* 2010; Labs *et al.,* 2016; Peltier *et al*., 2016; Yamori and Shikanai, 2016; Govindjee *et al.,* 2017].

Most of the subsequent mechanistic work on CEF has focused on a few species of flowering plants (*e.g*. *Arabidopsis thaliana*) and eukaryotic algae (*e.g*. *Chlamydomonas reinhardtii*) as well as some cyanobacteria [Peltier *et al.,* 2010, 2016; Yamori and Shikanai 2016]. Despite this, there are a number of areas of uncertainty, *e.g*. the contribution of CEF during steady state photosynthesis, the mechanism of proton gradient regulation-like photosynthetic phenotype 1 (PGRL1) proton gradient regulation 5 (PGR5) (see below), and the NDH pathway where it occurs as well as PGRL1-PGR5, the factors determining the relative importance of the two pathways. As well as considering these questions in the context of cyanobacteria and eukaryotic algae, this paper considers the algae in which CEF has been sought, the energy-requiring processes that CEF powers when linear electron flow (LEF) photosynthesis is blocked, and also the cases where other energy sources can substitute for CEF, or CEF is not involved. A further area that is considered is environmental influences on CEF by phenotypic acclimation and genetic adaptation.

2. *In Vivo* Evidence of Cyclic Photophosphorylation in Algae

2.1. *Overview*

The early and, more generally, non-molecular genetic work on cyclic electron flow and cyclic photophosphorylation in eukaryotic algae and cyanobacteria involved three main categories of *in vivo* studies. Most of the publications involve studies of ATP-requiring processes that were light stimulated and were characterized by one or more of (1) independence of the presence of CO_2 and O_2, (2) a higher ratio of the rate in radiation >700 nm to the rate in radiation <700 nm than is the case for photosynthesis and (3) less inhibition by DCMU than is the case for LEF

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photosynthesis [reviewed by Teichler-Zallen and Hoch, 1967; Simonis and Urbach, 1973; Raven 1976a, 1976b, 1984; Falkowski and Raven, 2007]. These studies involved a wide range of processes, and classes of algae (Table 1). A smaller number of papers deal with turnover of c-type cytochromes and, especially, P700 in radiation >700 nm and/or in a wide range of wavelengths in the presence of DCMU at a concentration that completely inhibits LEF photosynthesis (*e.g.* Biggins, 1973; Maxwell and Biggins, 1976; see Table 1). Three

Table 1. Non-molecular evidence of cyclic electron flow and associated phosphorylation in cyanobacteria and eukaryotic algae.

Division/Class	Electron flow	Photoacoustics	Photophosphorylation
Cyanobacteria	Teichler-Zahlen and Hoch [1967]; Berla *et al.* [2015]		Urbach and Simonis [1973]; Der-Vertanian *et al.* [1981]; Ogawa *et al.* [1985a, 1985b]
Rhodophyta: Bangiophyceae	Biggins [1973]; Maxwell and Biggins [1976]	Herbert *et al.* [1990]	Raven *et al.* [1990]
Rhodophyta: Floridiophyceae			
Chlorophyta: Prasinophyceae			
Chlorophyta: Chlorophyceae	Teichler-Zahlen and Hoch [1967]		Urbach and Simonis [1973]
Chlorophyta: Trebouxiophyceae		Cha and Mauzerall [1992]	Urbach and Simonis [1973]
Chlorophyta: Ulvophyceae		Herbert *et al.* [1990]	Urbach and Simonis [1973]
Strepotophyta: Charophyceae			Urbach and Simonis [1973]
Dinophyta			
Ochrophyta: Bacillariophyceae	Bailleul *et al.* [2015] Coldman *et al.* [2015]		
Ochrophyta: Eustigmatophyceae			
Ochrophyta: Phaeophyceae		Herbert *et al.* [1990] Fork *et al.* [1990]	
Cryptophyta			
Haptophyta	Zhang *et al.* [2014]		Paasche [1964]; Paasche [1966].

publications [Herbert *et al.,* 1990; Fork *et al.,* 1991; Cha and Mauzerall 1992] deal with photoacoustic estimates of energy storage under a range of wavelengths (see Table 1).

Considering *in vivo* ATP-requiring processes as an assay for cyclic photophosphorylation, the reviews cited above show that acetate and glucose uptake and metabolism, active (against an electrochemical potential gradient) ion transport, and diazotrophy in heterocystous cyanobacteria, photoreduction using H_2 in the absence of photosystem II (PSII), and inhibition of fermentation and respiration. Table 1 shows the diversity of cyanobacteria and algae in one or more of these processes. Raven [1976a, 1976b] showed that the capacity for cyclic photophosphorylation is similar to that of oxidative phosphorylation in the green algae examined.

However, none of the ATP-requiring processes examined in the charophycean (Characeae) *Chara corallina* (formerly *Chara australis*) used cyclic photophosphorylation as the ATP source [Smith and Raven, 1974]. In this alga light-stimulated ATP-requiring processes apparently involve LEF with oxygen as the terminal electron acceptor *via* the Mehler ascorbate peroxidase (MAP) or the flavodiiron reactions [Smith and Raven, 1974; Chaux *et al.,* 2017]. The processes examined include some that can be powered by cyclic photophosphorylation in another member of the Characeae, *Nitella translucens.* The reason for the inability (so far) to find processes energized by cyclic photophosphorylation in *Chara corallina* is not yet known. One major energy-requiring process in *Chara corallina* is the ATP-powered H^+ extrusion at the plasmalemma that is light-stimulated and inhibited by DCMU at the concentration that just inhibits LEF photosynthesis [Keifer and Spanswick 1978], and thence HCO_3^- use as part of the CO_2 concentrating mechanism [CCM] [Walker *et al.,* 1980]. In some other eukaryotic algae CCMs are powered by oxidative phosphorylation [*Nannochloropsis*: Huertas *et al.,* 2002] or pseudocyclic photophosphorylation, *i.e*. ADP phosphorylation coupled to H^+ gradients across the thylakoid lumen generated by LEF from H_2O to O_2 in the Mehler reaction involving both PSI and PSII [*e.g*. *Chlamydomonas*: Sultemeyer *et al.,* 1993]. In the cyanobacteria, *Anabaena variabilis* [Ogawa and Ogren, 1985] and *Anacystis nidulans* [Ogawa *et al.,* 1985a, 1985b] action spectra show a contribution of photosystem I (PSI) to the CCM, although interpretation is complicated by the involvement of energized, unidirectional, CO_2 to HCO_3^- conversion by the NDH-I_3 and NDH-I_4 as well as ATP-dependent active HCO_3^- influx in the CCM [Price, 2011].

Another major ATP-requiring process is N_2 fixation [Bottomley and Stewart, 1977]. Diazotrophy is restricted to archaea and bacteria, and cyanobacteria are the only N_2-fixing oxygenic organisms. The O_2 sensitivity of nitrogenase is accommodated in different cyanobacteria by limiting diazotrophy to the dark phase of the diel cycle, or having it occur in the light phase in colonial or multicellular

cyanobacteria in cells that have no, or very limited, oxygenic photosynthesis. This second possibility has been most investigated in the filamentous cyanobacteria that have diazotrophy confined to heterocysts, which are cells that lack PSII and autotrophic CO_2 assimilation and obtain organic carbon from the neighbouring symplasmically linked, vegetative cells [Bottomley and Stewart, 1977]. As would be expected, light-dependent N_2 fixation in heterocystous cyanobacteria is ATP-dependent [Bottomley and Stewart, 1977] and has the action spectrum of PSI with contributions from phycobilin light-harvesting complexes [Tyagi *et al.,* 1981; Staal *et al.,* 2003]. The symbiotic (with marine planktonic prymnesiophycean algae) diazotrophic unicellular cyanobacterium UCYN-A is effectively a heterocyst, lacking PSII and autotrophic CO_2 assimilation [Zehr *et al.,* 2008; Thompson *et al.,* 2012; Hagino *et al.,* 2013].

The "obvious" means of directly estimating CEF-related photophosphorylation and other forms of photophosphorylation, is to measure the net increase of ATP in a dark-light transition under appropriate conditions [Bottomley and Stewart, 1976, 1977]. However, the rapid turnover of ATP relative to the time needed to quench microalgae is a major problem; similar problems occur with the use of externally supplied $^{32}P\text{-}HPO_4^{2-}$ as a tracer [Raven, 1973].

Returning to the evidence of electron flow through P_{700}, cytochrome c_6 and cytochrome *f* in the presence of DCMU and/or under long wavelength irradiation mainly absorbed by PSI, an alternative explanation is net electron flow through PSI from organic carbon to an acceptor such as O_2. One indicator of this process is the Kok effect, *i.e.* a larger increment of net photosynthesis per unit increase in photon flux density at lower than at higher incident photon flux densities at less than half of the saturating photon flux density [Kok, 1949]. The Kok effect is widespread in cyanobacteria and eukaryotic algae [Healey and Myers, 1971; Peltier and Sarrey, 1988; Peltier *et al.,* 2010]. The Kok effect is a result of decreased respiration rather than an increase in gross photosynthesis [Hoch *et al.,* 1963; Bunt, 1965; Healey and Myers, 1971; Peltier and Sarrey, 1988]. Hoch *et al.* [1963] suggested that inhibition of (mitochondrial) respiration resulted from a higher free energy of hydrolysis of ATP generated by photophosphorylation than by oxidative phosphorylation, and oxidative phosphorylation close to equilibrium with the mitochondrial proton-motive force. However, the addition of an uncoupler (CCCP) at concentrations partially inhibiting photosynthetic and oxidative phosphorylation does not alter the Kok effect shows that the interaction is in terms of electrons from respiration being fed into PSI [Healey and Myers, 1971]. Subsequently, with the discovery of chlororespiration, this process rather than mitochondrial respiration is considered as the source of electrons [Peltier *et al.,* 2010]. O_2 as a sink for electrons from PSI could not explain the effect on net photosynthesis measured as O_2 production.

Interception by PSI of electrons from chlororespiration is, in the absence of additional evidence, a possible alternative to cyclic electron flow as an explanation of electron flow through PSI and the immediate electron donors to PSI [*e.g.* Huan *et al.,* 2014; Gao *et al.,* 2016]. However, the other lines of evidence, *i.e.* light dependent ATP-requiring processes and the absence of PSII and in the absence of electron acceptors, and photo-acoustic estimates of energy storage in the absence of PSII, do not suffer from the possibility of electron input to PSI from organic carbon.

A further interaction of cyclic photophosphorylation with dark energy metabolism is with glycolysis in the absence of O_2. Kandler and Haberer-Liesenkötter [1963] used *Chlorella*, Hirt *et al.* [1971] used *Scenedesmus* wild type and mutants 11 and 8 lacking, respectively, PSII and photophosphorylation, and Gfeller and Gibbs [1984], and Gfeller and Gibbs [1985] used *Chlamydomonas* and the inhibitors DCMU and FCCP. Light inhibits glycolytic fermentation by a process that is independent of PSII but is prevented by the protonophore uncoupler FCCP, showing that the inhibition involves cyclic photophosphorylation. This is analogous to the inhibition of fermentation by oxidative phosphorylation (Pasteur effect), but contrasts with the diversion of chlororespiratory electrons to PSI operating in a linear mode.

2.2. *Energetics of the assimilation of exogenous glucose and acetate powered by CEF and their mechanistic implications*

The relevant investigations here involved two genera of the Chlorophyta, *i.e. Chlorella pyrenoidosa* and *Chlorella vulgaris* (Trebouxiophyceae) and *Chlamydobotrys stellata* (Chlorophyceae), and two purple non-sulfur photosynthetic proteobacteria, *Rhododospirillum rubrum* and *Rhodopseudomonas capsulata.*

Tanner *et al.* [1965] measured the rate of glucose assimilation by *Chlorella pyrenoidosa* under aerobic conditions in the dark (energized by oxidative phosphorylation) and under N_2 in the light (energized by cyclic photophosphorylation). The dark-aerobic rate of glucose assimilation is 10–20% greater than the light-N_2 rate [Tanner *et al.,* 1965]. Tanner *et al.* [1968] measured the cost in absorbed photons of assimilation of glucose by *Chlorella vulgaris* under anaerobic conditions, and of photosynthetic $^{14}CO_2$ assimilation with illumination at 658 nm (absorbed by PSI and PSII) and 712 nm (almost all absorbed by PSI). Glucose uptake and assimilation requires 6.0 mol and 4.1 mol absorbed photons per mol glucose at 658 nm and 712 nm respectively; the corresponding values for photosynthesis are

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12.5 mol and 40 mol absorbed photons per mol carbon dioxide at 658 nm and 712 nm respectively [Tanner *et al.,* 1968]. Wiessner [1966a] examined the relative absorbed photon costs of anoxic glucose photoassimilation in *Chlorella pyrenoidosa*: the values are relative because, although the photon absorption was measured, the glucose assimilation was given as counts per minute of ^{14}C-labelled glucose rather than as mol glucose. The relative photon cost of glucose assimilation at wavelengths with minimal PSII absorption is 70% of that at wavelengths where both PSI and PSII absorb [Wiessner, 1966a]; these findings are in qualitative agreement with the findings of Tanner *et al.* [1968] on *Chlorella vulgaris.*

Assimilation of one mol glucose into the main products, oligo- and poly-saccharides, requires 2 mol ATP. Influx of glucose in *Chlorella vulgaris* is by 1 proton: 1 glucose symport [Komor and Tanner, 1974]. The proton electrochemical gradient across the plasmalemma is maintained by an energized, ATP-powered proton efflux with a mol proton: mol ATP ratio of 1 [Raven, 1984]. This adds 1 mol ATP per mol glucose to the cost of converting external glucose into oligo- and polysaccharide, making a total of 3 mol ATP per mol exogenous glucose assimilated; with 4.1 mol photon per mol glucose, each mol ATP corresponds to 1.37 mol absorbed photon. This can be accommodated by the following mechanism. One mol electron moved is moved from the oxidizing to the reducing end of PSI per mol photon absorbed by PSI. One mol electron moves from the reducing end of PSI through NDH1/2 and then PQ-cyt b_6/f (Figure 1A) and the oxidizing end of PSI with movement of 4 mol protons from stroma to thylakoid lumen [Raven *et al.,* 2014; Strange *et al.,* 2016]. Finally, 1 mol ADP phosphorylated per 4 mol protons moving from the thylakoid lumen to the stroma was measured [Peterson *et al.,* 2012] (Table 2). The outcome is 1 mol ATP synthesized per mol photons absorbed by PSI. This can readily accommodate the observed 1.37 mol photon absorbed per mol ATP. With the H^+:ATP of 14/3, or 4.67, predicted from structural biology 1 mol ATP from cyclic photophosphorylation requires 1.17 mol photons, again compatible with the observed 1.37 mol photon absorbed per mol ATP (Table 2).

However, NDH2 does not occur in the plastid genome of *Chlorella variabilis* [Cardol *et al.,* 2011]; this also seems to be the case for the other algae examined other than the Charophyceae and some members of the Prasinophyceae [Turmel *et al.,* 2009; Martín and Sabatier, 2010; Civán *et al.,* 2014]. The work of Tanner *et al.,* [1965] on *Chlorella pyrenoidosa* shows that CEF is inhibited by antimycin A, consistent with PGR5/PGRL1 (refer to Section 3.1. below) transferring electrons from ferredoxin to PQ [Labs *et al.,* 2016]. PGR5/PGRL1 occurs in *Chlorella variabilis* [Cardol *et al.,* 2011; Hertle *et al.,* 2012]. The PGR5/PGRL1 is not known to pump protons, and the proton:electron ratio of CEF lacking NDH2 or its H^+-pumping equivalent the mol photon per mol ATP is 2 [H^+:ATP of 4] or 2.33 (H^+:ATP of 4.67), neither of which is compatible with the *Chlorella* glucose

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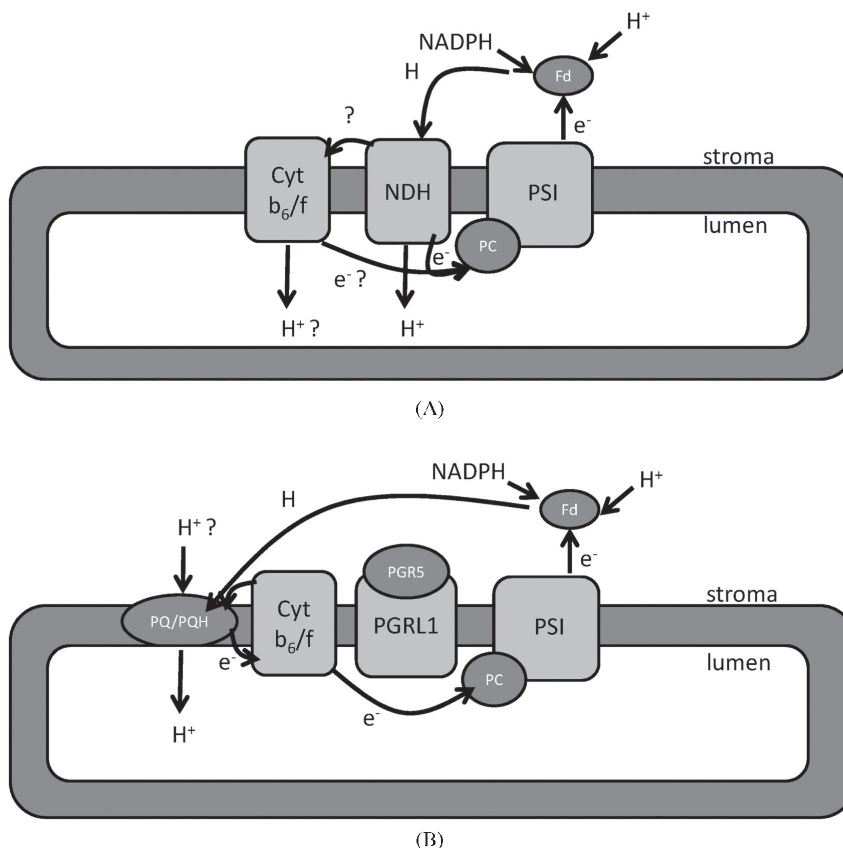

Figure 1. Diagrams of the proposed cyclic electron flow pathways in the thylakoid membranes of cyanobacteria and eukaryotic algae. (A) The NDH reductase pathway which is reduced by reduced ferredoxin, and which delivers protons to the thylakoid lumen. Note, if a Q cycle operates then extra protons are delivered to the thylakoid lumen. However, this would have to involve the operation of the cytochrome b_6/f complex. (B) The PGR5/PGRL-1 controlled pathway. Note that it is assumed that ferredoxin donates hydride to the plastoquinone pool, which then reduces the cytochrome b_6/f complex and delivers a proton to the thylakoid lumen. It is assumed that a Q cycle operates in which case 2 protons are delivered to the lumen for each hydride reducing PQ. In this formulation the PGR5/PGRL-1 complex is assumed to take on a sensing/controlling role.

assimilation value of 1.60 mol photon absorbed per mol ATP (4.67 mol H^+ per mol ATP in the CF_0CF_1 ATP synthase) or 1.37 mol photon absorbed per mol ATP (4 mol H^+ per mol ATP in the CF_0CF_1 ATP synthase) (Table 2).

If the mol proton:mol ATP ratio for the plasmalemma proton ATPase is 2, as is often thermodynamically possible [Raven, 1984], only 2.5 mol ATP per mol are required per mol exogenous glucose assimilated. With 4.1 mol absorbed photon per mol glucose, each mol ATP used in assimilation of exogenous glucose

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Table 2. Comparison of measured *in vivo* photon yield of ATP production from CEF with predictions from CEF mechanisms with NDH2 (eukaryotes) or with PGR5-PGRL1 (eukaryotes) and UQH as the first stable reduced product of photochemistry. See Section 2.2 of text for references.

Organism	*In vivo* PSI absorbed photons per ATP produced by CEF	PSI absorbed photons per ATP in CEF using NDH2	PSI absorbed photons per ATP in CEF using PGR5-PGRL1 (eukaryotes), UQH_2 first stable reduced product of photochemistry
Chlorella	1.37[1] [1.64][2]	1.0[4][1.17][3]	2.0[4][2.33][5]
Chlamydobotrys	16.7[1] [1.95][2]	1.0[4][1.17][3]	2.0[4][2.33][5]
Rhodospirillum Rubrum	4.4–5.13[3]	Not applicable	1.65[6]
Rhodopseudomonas ~~*Capsulate*~~	5.3–5.63[3]	Not applicable	1.65[6]

[1] H^+: ATP = 1 for plasmalemma H^+ ATPase
[2] H^+: ATP = 2 for plasmalemma H^+ ATPase
[3] No allowance for energized entry of acetate
[4] H^+: ATP = 4 for CF_0CF_1ATP synthase
[5] H^+: ATP = 4 67 for CF_0CF_1ATP synthase
[6] H^+: ATP = 3 33 4 for F_0F_1ATP synthase

References
Cardol *et al*. [2011]; Feniouk and Junge [2009]; Hertle *et al*. [2012]; Klamt *et al*. [2008]; Komor *et al*. [1968]; Labs *et al*. [2016]; Peterson *et al*. [2012]; Raven [1984]; Raven *et al*. [2014]; Soga *et al*. [2017]; Strange *et al*. [2016]; Tanner *et al*. [1965]; Tanner *et al*. [1968]; Wiessner [1966a]; Wiessner [1966b]; Wiessner and Gaffron [1964]; Yang *et al*. [2015].

corresponds to 1.91 mol photon absorbed per mol ATP (4.67 mol H^+ per mol ATP in the CF_0CF_1 ATP synthase) or 1.64 mol photon absorbed per mol ATP (4 mol H^+ per mol ATP in the CF_0CF_1 ATP synthase). These are still inconsistent with a non H^+-PGRL1-PGR5 catalysing all of the electron flow from ferredoxin to PQ, although the photon cost with the 4.67 mol H^+ per mol ATP in the CF_0CF_1 ATP synthase comes close (Table 2).

Wiessner [1965, 1966b] investigated the absorbed photon cost of the DCMU-insensitive aerobic acetate photoassimilation in *Chlamydobotrys*. With 723 nm and 716 nm incident irradiance the photon requirements are respectively 8.3 and 8.8. mol photon per mol acetate assimilated; at 450–680 nm incident irradiance the cost is 11.8–12.3 mol photon per mol acetate assimilated [Wiessner, 1965]. The ATP cost of acetate assimilation is 3.25 mol ATP per mol intracellular acetate, so the photon cost of the required ADP phosphorylation is 2.55–2.71 mol photon per mol ATP produced. This is consistent, assuming an H^+ per mol ATP ratio of 4 for the CF_oCF_1 ATP synthase, with the 2 mol absorbed photon per mol ATP if PGR5/PGRL1 catalyses all of the electron flow from ferredoxin to PQ, with no necessary

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involvement of NDH2. If the H^+/ATP ratio is 4.67 for the CF_oCF_1 ATP synthase, the 2.33 absorbed photons per mol ATP for the PGR5/PGRL1 is still compatible with data of Wiessner [1965, 1966b] (Table 2). However, Wiessner [1965, 1996b] did not include any energy costs associated with moving acetate across the plasmalemma. Yang *et al.,* [2015] suggest that acetate entry in chlamydomonad chlorophyceans is energized, adding another 0.5 or 1 mol ATP to the energy cost of assimilation of one mol of external acetate. This correction yields *in vivo* values of 1.95–2.34 mol photons per mol ATP for an H^+/ATP ratio of 4 for the CF_oCF_1 ATP synthase; the lower value is not, granted the variability in the data, significantly different from 2.0, so there is again no requirement for the involvement of NDH2 in CEF. For an H^+/ATP ratio of 4.67 for the CF_oCF_1 ATP synthase the lower of the *in vivo* values of 1.67–2.0 is more challenging for the absence of involvement of NDH2 in CEF (Table 2).

The situation for acetate assimilation in *Chlamydobotrys* seems more complicated than that for glucose assimilation in *Chlorella*; the rate of acetate photoassimilation in the absence of oxygen is only 24% of that with normal atmospheric oxygen, and the anoxic rate is restored to a rate approximating the rate with oxygen by the presence of carbon dioxide [Wiessner and Gaffron, 1964]. These findings are possibly related to redox balance in the glyoxylate cycle used in converting acetate into hexose [Wiessner and Gaffron, 1964].

Wiessner [1966b] also measured the absorbed photon cost of acetate photoassimilation in the purple photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata.* The bacteria use a PSII-like (but non-oxygenic) reaction centre to energize CEF involving a UQ cytochrome bc_1 proton pump but, mechanistically and energetically, no involvement of the NDH is found in these organisms [Nicholls and Ferguson, 2013]. The absorbed photon costs of acetate assimilation at wavelengths corresponding to the first excited state of the bacteriochlorophylls (795–893 nm) involved in photon harvesting and photochemistry in these organisms are as follows: for *Rhodospirillum rubrum* the values are 8.8–10.1 mol absorbed photons per mol acetate, and for *Rhodopseudomonas capsulata* they are 10.6–11.1 [Wiessner, 1966b]. In the purple bacteria assimilation of internal acetate requires 2 mol ATP per mol acetate [Wiessner, 1966b], so each mol ATP produced is associated with absorption of 4.4–5.6 mol photons (Table 2).

Purple photosynthetic bacteria have the equivalent of NDH2 that is involved in oxidative phosphorylation under dark aerobic growth conditions, and the reduction of NAD^+ by UQH_2 driven by the proton gradient generated by CEF during autotrophic CO_2 assimilation [Klamt *et al.,* 2008; Nicholls and Ferguson, 2013]. However, the NDH2 equivalent is not involved in CEF in purple photosynthetic bacteria [Klamt *et al.,* 2008; Nicholls and Ferguson, 2013]. With a CEF photon:electron ratio of 1, a proton:electron ratio of the purple bacterial UQ cytochrome bc_1 proton pump of 2 [Klamt *et al.,* 2008; Nicholls and Ferguson,

2013], and a proton:ATP ratio of 3.3 for the bacterial ATP synthase [Klamt *et al.,* 2008; Feniouk and Junge, 2009; Nicholls and Ferguson, 2013; Soga *et al.,* 2017] the predicted photon cost of ATP synthesis is 1.65 mol absorbed photon per mol ATP. This predicted photon cost is much less than the observed *in vivo* 4.4–5.6 mol absorbed photons per mol ATP for assimilation of intracellular acetate, so any energy cost of acetate flux into the cells could be very readily accommodated (Table 2). This contrasts with the much closer similarity of predicted and measured values in *Chlamydobotrys.*

3. Detailed Mechanism of Cyclic Electron Transport and ATP Formation in Oxygenic Photosynthetic Organisms

3.1. *Overview*

Having established the strong historical evidence for cyclic photophosphorylation and cyclic electron transport around PSI in cyanobacteria and eukaryotic algae, proposals for the detailed mechanism are now discussed.

Two well-established mechanisms for CEF exist across all the oxygenic photosynthetic organisms, both of which probably involve the cytochrome b_6/f complex and plastoquinone, but the exact mechanism is not well-established (see Figure 1):

(A) An NDH dehydrogenase (NADH/NADPH dehydrogenase) system, driven by electrons from reduced ferredoxin, which ferries electrons and protons inwards to the inner compartment of the thylakoid, the lumen (Figure 1A). Most commonly this is an NDH-1 system but in eukaryotic algae apparently there is an NDH-2 system. The NDH-1/2 system is the dominant one in cyanobacteria, is found in some green eukaryotic algae, especially streptophytes (see Section 4.3) and is a minor protein component in most embryophytic plants but lacking in several clades [Ruhlman *et al.,* 2015].

(B) An electron/proton transport system involving at least two protein complexes with many subcomplexes (Figure 1B). The two complexes are Proton Gradient Regulation 5 (PGR5) and Proton Gradient Regulation-like Photosynthetic phenotype 1 (PGRL1) complexes (see definitions on the first page of the Introduction). Reduction of PGR5 on the stroma/plasma side of the thylakoid membrane is by ferredoxin. There is some evidence for these complexes in cyanobacteria, but there they play a minor role [Allahverdiyeva *et al.,* 2013]. They are found in some eukaryotic algae particularly in the green algal line e.g. *Chlamydomonas reinhardtii* and are more abundant than in cyanobacteria. In land plants the NDH system apparently plays only a minor role and the PGR5/PGRL1 system predominates. Thus there seems to be an evolutionary drift from the NDH dehydrogenase system (utilizing Fd[H]) to the PGR5/PGRL1 system as one goes from cyanobacteria to land plants but the reason(s) for this are at present not clear.

3.2. *Non-cyclic (= linear) electron flow, cyclic electron flow, the Q cycle and ATP generation*

In non-cyclic flow or LEF, electrons from the oxidation of water are passed from the reaction centre of PSII to Q_A/Q_B in a semiquinone/quinone reaction. The electrons are then passed on to plastoquinone, which is reduced to plastoquinol by a plastoquinone reductase on the stroma side of the thylakoid membrane or the cytoplasmic side of the cyanobacterial plasma membrane. This is then coupled to electron donation to the Q_1 site of the cytochrome b_6/f complex and simultaneous deposition of a H^+ in the lumen of the thylakoids (of algal plastids and many cyanobacteria) or the periplasmic space of the cyanobacterium *Gloeobacter*. From there the cytochrome b_6/f complex takes part in a modified (activated) Q cycle [Mulkidjanian, 2010] whereby an electron is returned to the outer side of the membrane picks up a proton and hydrogenates a second molecule of plastoquinone. The second cycle moves another H^+ from the cytoplasm/stroma to the lumen for each e^-, so that a total of 2 H^+ are moved per e^- participating in the cycle or 8 H^+ per 4 e^-. To summarize, for each pair of water molecules split in the oxygen evolving complex 4 e^- are moved by PSII and 4 H^+ are deposited in the lumen, which with the operation of the Q cycle deposits 12 protons in the thylakoid lumen per 4 e^-. The number of ATP that are generated depends on the number of H^+/ATP involved in the CF_oCF_1 ATP synthase in the thylakoid/cyanobacteria plasma membrane; this ratio is usually taken to be ~4, although the structural biology prediction is 14/3 or 4.67 [Steigmiller *et al.*, 2008].

In cyclic electron flow much less is clear. We know that there are membrane dehydrogenases. Some of these are clearly allied to Type 1 dehydrogenases and drive 3 – 4 protons across the active membrane per 2e^-. These almost certainly interact with the cytochrome b_6/f complex but it is not clear whether a Q cycle is involved [Joliot and Johnson, 2011; Johnson *et al.*, 2014], and if it is whether it is an activated Q cycle [Mulkidjanian, 2000]. The PGR system of green algae and embryophyte plants, seems more reliably to interact with the cytochrome b_6/f complex (Figure 1B) and there is better evidence that a Q cycle is involved there (and e^- flow is generally but not always inhibited by antimycin A (see below)).

Recently, the role of PGR5/PGRl-1 in controlling CEF has been questioned. As the name "proton gradient regulator" implies these protein complexes were discovered, first in *Arabidopsis thaliana* and then in *Chlamydomonas reinhardtii*, (see Section 4), as essential for CEF to occur. However, doubts have been raised as to whether they actually control or modify the ΔpH gradient. Or whether they play a much more subtle role in influencing the thylakoid ATPase and in modulating the tie-up between ΔpH and NPQ in photoprotection [Armbruster *et al.*, 2017; Kanazawa *et al.*, 2017]. As a result of these new ideas it is also necessary to place on the table the question of the exact role of the NDH proteins in CEF.

In summary, CEF can generate ATP. How much ATP is generated from CEF has not been fully established and would depend on which dehydrogenase system is used, the interconnections of the membrane complexes and the ATP synthase involved (and this is considered in Section 2.2). To further complicate the situation, reductive processes close to PSI are also the result of a balance of metabolic processes and if CEF is deleted by genetic manipulation mitochondrial respiration, the MAP pathway, chlororespiration and other processes can potentially come into play [Dang *et al*., 2014; Johnson *et al*., 2014; Chaux *et al.,* 2017].

3.3. *Early evolution of NAD dehydrogenases — Bacteria, cyanobacteria, plastids and mitochondria*

The complex protein assemblage, which constitutes the NDH complex of cyanobacteria and plastids clearly shares an evolutionary history with complex 1 of bacteria and mitochondria. Much more is known of the structure and functioning of complex 1 than of the NDH complex, although the detailed structure of the NDH complex, which has been elucidated, links it intimately with complex 1. It is known that complex 1 has a two-part structure: (1) a peripheral hydrophilic arm abutting the aqueous phase outside the membrane, which mediates electron transfer through an FMN molecule and 10 Fe/S clusters; (2) a hydrophobic membrane arm that mediates proton translocation across the membrane by an unknown mechanism [Friedrich, 2014]. In complex 1, the binding site for the NADH is a ubiquinone, which lies at the junction of the hydrophilic and hydrophobic parts. It was previously thought that one NADH oxidation couples the transfer of two electrons to the translocation of $4H^+$; however, Wikstrom and Hummer [2012] argued cogently for a coupling of $2e^-$ per $3H^+$. In any case the respiratory complex 1 is an energy converting NADH-ubiquinone oxidoreductase.

Phylogenetic trees based on the NuoH polypepetides [*e.g*. Moparthi and Hagerhall, 2011] indicate that complex 1 has a long history in ancient cells back at least to LUCA (the last common universal ancestor), in which the complex 1 genes share affinities with archaea and bacteria and where the genes of anoxygenic photosynthetic bacteria are well separated from cyanobacteria.

3.4. *Mechanisms for CEF in cyanobacteria*

As cyanobacteria are the oldest known oxygenic photosynthesizers, it is logical to assume that the major dehydrogenase, NDH dehydrogenase complex (now utilizing Fd[H]), is the major mechanism for cyclic electron transport, and came by lateral gene transfer from similar dehydrogenases in other non-photosynthetic

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organisms [Vermaas *et al*., 2010; Shih *et al*., 2016; Soo *et al.,* 2017]. Whether or not NDH was the earliest CEF system to evolve is not known, but certainly it is the most abundant system and occurs in all extant cyanobacteria that have been assayed [Peltier *et al*., 2016]. However it should be remembered that the thylakoid membrane of cyanobacteria is, evolutionarily, an invaginated extension of the plasma membrane (and indeed cyanobacteria in the genus *Gloeobacter* lack thylakoids altogether): and respiratory enzymes, for example, are present in both membranes [Mullineaux, 2014] and were all probably evolved by lateral gene transfer borrowing (see Vermaas *et al.,* 2010).

The current nomenclature for the chloroplast NDH-dehydrogenase-like complex (utilizing Fd[H]) was first given by Ifuku *et al.,* [2011] and is centred on the complex in eukaryotic organisms, but this nomenclature can be used for cyanobacteria with just a few exceptions.

The development of proteomic procedures and single particle electron microscopy led to the first indications of the molecular structures of complexes involved in CEF. NDH complexes are present in a wide variety of photosynthetic organisms from cyanobacteria to embryophytic plants [Peltier *et al.,* 2016]. However, in eukaryotic algae and in embryophytic plants the role and abundance of NDH complexes is currently obscure. NDH complexes seem to have evolved to regulate and control the electron transport reactions of photosynthetic organisms [Howitt *et al*., 1999; Howitt *et al*., 2001; Peltier *et al*., 2016; Strand *et al*., 2016] and it seems that these reactions drew on reactions, which had been established in bacteria for respiratory purpose (see previous section).

In cyanobacteria the NDH-1 complex would appear to be the major protein complex involved in CEF [Arteni *et al*., 2006; Battchikova *et al*., 2011a; Deák *et al*., 2014: Ohkawa *et al*., 2001: Xu *et al*., 2008; Zhang *et al*., 2004, 2005]; a protein complex that even at the most basic level (NAD-1M) is made up of 14 subunits. For the physiological function, which may be control of electron flow or redox regulation [Peltier *et al*., 2016] it is necessary to have several other subunits, which form a hydrophilic domain and several subunits, which form a hydrophobic domain. One NDH subunit of the hydrophilic domain, Ndhs, is responsible for interacting with ferredoxin, which has recently been shown to be responsible for reducing the active complex and initiating CEF [Battchikova *et al*., 2011b; He *et al*., 2015], rather than NADH or NADPH.

In cyanobacteria there are two forms of NDH-1: (1) NDH-1$_3$ contains the so-called NdhD3 and NdhF3, with, in addition, two additional subunits, which together form a region in the distal arm of the complex. (2) NDH-1$_{4,}$ has the same NDH-1m component but, however, incorporates different subunits in the distal arm of the complete complex.

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Cyanobacteria also harbor another set of protein complexes (PGR5/PGRL1 or homologues), which are discussed below [Allahverdiyeva *et al.,* 2013]. There seems to be no evolutionary relationship between these two sets of proteins [Peltier *et al*., 2010]. Both NDH and PGR proteins seem to have found their evolutionary way through the green algae, which colonized the land, to embryophytes [Hori *et al*., 2014].

3.5. *Detailed mechanism, and inhibitors, of the NDH system*

The detailed mechanism of CEF in cyanobacteria involves an NAD complex with a hydrophilic arm incorporating several FeS ligands interacting through a quinone (ubiquinone) with NADH. This interaction results in a concomitant translocation of the two protons for every electron across the membrane [Peltier *et al.,* 2016], implying that the NDH-1 complex has a function in electron flow as well as in regulation and control.

There is a lack of good inhibitors for NAD-1 and NAD-2. Based on the similarity of complex 1 of other bacteria, and mitochondria, with those complexes of cyanobacteria, then piericidin A, an inhibitor of quinone binding sites, should be appropriate. However, there is no report of an effect of this inhibitor, which interacts at the site of coenzyme Q reduction, *i.e.* the quinone-binding sites in mitochondria (along with amytal and rotenone); in chloroplasts, piericidin interacts with the reduction of Q_B in PSII [Ikegawa *et al*., 2002] and this may have deterred any attempts to look for effects on CEF; rotenone does inhibit the cyanobacterial complex 1 [Berger *et al*., 1999], which should encourage the use of rotenone and piericidin in studies of cyanobacterial CEF.

The lack of functional inhibitors for NDH-1 or NDH-2 has limited research into the detailed mechanism of CEF in cyanobacteria, in comparison with the strong inhibitory action of antimycin A on CEF in green algae and higher plants. A single point mutation in PGR5 in *Chlamydomonas* can confer resistance to this inhibitor [Sugimoto *et al.,* 2013]; and there is evidence that a similar base shift in *Synechocystis* sp. PCC6808 (ssr2016) confers resistance/sensitivity to antimycin A [Yeremenko *et al*., 2005].

An alternative line of investigation is to use strains in which these complexes have been impaired by the use of deletion mutants; this was first achieved in the M55 strain of *Synechocystis* 36803 [Ogawa, 1991] and since then in a range of other mutants [Gao *et al*., 2016a, 2016b]. Use of these mutants has shown that deletion of the linker protein CpcG2 is strongly inhibitory of CEF under strong light, indicating that the NDH-1L-CpcG2- PSI supercomplex facilitates PSI-CEF

via NDH-1L. So far, however, these mutants have been little used to probe the mechanism of CEF. Initial results [Fitzpatrick, 2016] agree with previous inferences [Howitt *et al*., 2001; Battchikova *et al*., 2011a; Jia *et al*., 2015; Chen *et al*., 2016] that CEF is used in Cyanobacteria to generate extra ATP through a proton pumping activity across the thylakoid membrane. One caveat here is that the supercomplex may also be involved in other reductive processes, when the electron transport [ET] from PSII is inhibited, *e.g.* by high temperatures [Fitzpatrick, 2016].

Another line of investigation has been to delete PSII and respiratory oxidases in a cyanobacterium. This was done by Howitt *et al*. [2001] where respiratory and PSII mutants of *Synechocystis* sp. PCC6803, grown on glucose showed growth proportional to photosynthetically active radiation up to 25 μmol m^{-2} s^{-1} and which suggested that CEF around PSI was the major provider of energy under these circumstances. Unfortunately, there has been little follow up of these interesting experiments.

To summarize, in cyanobacteria there is a major NDH-1 complex which is strongly implicated in CEF. There is also an NDH-2 complex for which there is much less evidence of its function. In addition there seem to be PGR (Proton Gradient Regulation complexes; discussed below) but again the evidence for their general presence and functional significance is generally lacking; and there is even evidence that inhibition by antimycin A, a major feature of PGR complexes in land plants, is only prevented by a single point mutation. In eukaryotic algae PGR5/PGRL1 proteins are widespread [Peltier *et al.,* 2010; Thamatrakoln *et al.,* 2013]. NDH-1 and NDH-2 are restricted to many of the charophycean members of the Streptophyta, and some basal Chlorophyta [Peltier *et al.,* 2010; DePriest *et al.,* 2013; Leliaert *et al.,* 2016].

3.6. *Towards a better understanding of cyanobacterial CEF*

In spite of decades of research, accurate measurements to determine rates of CEF under steady state, physiologically relevant conditions, in cyanobacteria and eukaryotic algae, have proven elusive [Fan *et al.,* 2016].

Work on CEF in cyanobacteria has been hindered by the lack of a specific CEF inhibitor and in eukaryotic algae, apart from *Chlamydomonas reinhardtii*, where a specific inhibitor is also lacking, work has been very sparse. Recently, Fitzpatrick [2016] and Fitzpatrick *et al*. (in preparation) have worked to integrate membrane inlet mass spectrometry (MIMS) [Beckmann *et al.,* 2009] with a P700-based method to estimate CEF, published by Kou *et al*. [2013], and also described in Chapter 12 of this book, to measure CEF in cyanobacteria. The CEF estimate, termed ΔFlux, is based on the difference in rates of electrons that pass through P680, the reaction center chlorophylls of PSII (determined using rates of gross O_2

evolution and requiring the addition of the stable $^{18}O_2$ isotope to discriminate O_2 consumption from O_2 evolving reactions) and P700, the reaction center chlorophylls of PSI. The integrated system was used to investigate the response of *Synechocystis* PCC6803 cells to acute high temperature stress. Although the dataset is preliminary, it has been included as an example of a cyanobacterial response to high temperature stress. Presented in Figure 2, clear differences in trends were observed when comparing the WT cells, with those of the M55 NDH-1 mutant [Ogawa, 1991], which lacks the predominant cyanobacterial CEF pathway. This mutant was used to determine *f*1, the fraction of absorbed light partitioned to PSI (required for calculating electron transport through PSI, ETR1), in the absence of a specific CEF inhibitor in cyanobacteria, as *f*1 must be determined under conditions where CEF is absent.

In Figure 2 the rates of ETR2(O_2) between the WT and the mutant exhibited very similar trends in response to higher temperature incubations: dropping at 50°C and showing almost complete inhibition of ETR2(O_2) at 55°C. However, a large difference could be observed between the two samples in terms of the calculated rates of ΔFlux. Whereas the M55 samples showed no ΔFlux, the WT samples exhibited increasing rates of ΔFlux with increasing temperature, with a marked

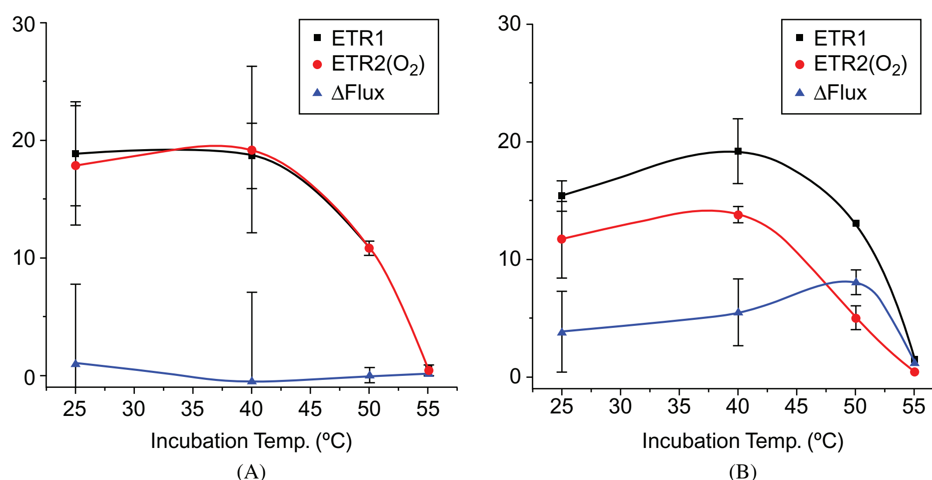

Figure 2. ΔFlux, an estimated rate of cyclic electron transport around PSI in samples of (A) the NDH-1 inhibited mutant of *Synechocystis* PCC6803, [M55], and (B) wild type samples of the same species, after samples were incubated for 10 minutes in the dark at each of the specified temperatures. DFlux was determined as the difference between two simultaneously collected values: ETR2[O_2] based on gross O_2 evolution rates measured with MIMS utilizing $^{18}O_2$ and ETR1, based on the Y[I] values from $P700^+$ measurements. Values were collected during steady state photosynthesis at 100 μmol photons.$m^{-2}.s^{-1}$ with cyanobacterial cells immobilized on glass fiber discs with saturating total carbon. Cells were cultivated at 30°C in BG-11 medium pH 8.0 in 2% CO_2 enriched air Error bars = standard deviation of three repeats.

increase as ETR2(O_2) became inhibited after exposure to elevated temperature (50°C). Although some aspects of the method are still being optimized, the data suggest that this integrated approach holds some promise for estimating rates of CEF under steady state conditions in samples where it is possible to estimate the partitioning of absorbed energy between the two photosystems (*i.e.* using inhibitors or a mutant to inhibit CEF without impacting upon linear electron transfer between PSII and PSI).

Significantly, the use of MIMS is an improvement on original published methods, especially for experiments with cyanobacteria where rates of gross O_2 evolution can be underestimated by as much as 50% due to the activity of flavodiiron 1,3 proteins [Allahverdiyeva *et al.,* 2013]. The power of MIMS to utilize stable isotopes to measure true gross gas fluxes may also be useful to deconvolute potential alternative donors to the thylakoid membrane, which could complicate the interpretation of $P700^+$ reoxidation kinetics, a commonly used estimate of CEF rates.

It is to be hoped that use of the MIMS technique combined with P700 and other measurements will allow precise quantification of CEF activity in cyanobacteria and eukaryotic algae in the future; and furthermore allow for the establishment of the interaction of this pathway with other pathways, such as LEF, respiration, glycolytic reactions, reactions to oxygen such as the MAP and flavodiiron pathways and chlororespiration.

4. Cyclic Electron Transport Complexes of Chlamydomonas and Other Green Algae

4.1. *Introduction*

While it is clear that NDH complexes do occur throughout the eukaryotic algae and in higher plants (in some eukaryotic algae it has been argued that NDH-2 replaces NDH-1 [Peltier and Cournac, 2002]) and were obviously inherited through the endosymbiosis of a cyanobacterial line that led to the evolution of plastids [Larkum *et al*., 2006], another set of membrane protein complexes shares a major role in the plastids of many eukaryotic algae and higher plants. These are the PGR5 and PGRL1 complexes [Yamori and Shikanai, 2016].

As mentioned in the early sections of this review, CEF was discovered in higher plant thylakoids in a study of ferredoxin-dependent ATP synthesis that could be inhibited by antimycin A [Tagawa *et al*., 1963]. Antimycin A, the key to this early work on embryophyte chloroplasts, was discovered as an inhibitor of mitochondrial respiration, specifically electron transport and proton transport, powering oxidative phosphorylation. In eubacteria and mitochondria, antimycin A binds to the Q_i site of the cytochrome bc_1 cytochrome *c* reductase; this inhibits the

oxidation of ubiquinone at the Q_i site of ubiquinol and prevents turnover of the Q cycle [Labs *et al*., 2016]. In photosynthetic LEF a similar cytochrome oxido-reductase operates between the two photosystems and in embryophytes antimycin A plays a similar role inhibiting this cytochrome b_6/f complex, which also takes part in a Q cycle [Cramer *et al*., 2011]. The cytochrome b_6/f complex is also the probable route for the PGR dependent CEF (see below and Figure 1). Thus, antimycin A is generally a potent inhibitor of CEF in those organisms that have a strong component of PGR-dependent CEF and, where it is active, can be used to measure the involvement of CEF in photosynthetic reactions. However, a single point mutation in cyanobacteria and *Chlamydomonas* confers resistance to antimycin A [Yermenko *et al*., 2001; Sugimoto *et al.,* 2013]; and in other eukaryotic algae the inhibition of AA is sporadic.

4.2. *PGR5/PGRL1 complexes in thylakoids*

As mentioned above, the naming of these complexes relates to their role in sensing and/or controling the proton gradient (ΔpH) set up by the reduction of the plastoquinone pool and electron flow through the cytochrome b_6/f complex: hence PGR across the thylakoid membrane of chloroplasts (and which in turn drives the formation of ATP) [Fisher and Kramer, 2011]. Their relationship to NDH-generated proton gradients still needs to be determined.

The machinery of the PGR5/PGRL1 complexes has been elucidated using molecular genetics, mainly in *Arabidopsis* and *Chlamydomonas reinhardtii* [Yamori and Shikanai, 2016]. PGR5 was first identified from a series of *Arabidopsis* mutants found to suffer a major disruption in their ability in generating ΔpH across the thylakoid membrane. Later PGR5 was identified in *Chlamydomonas* and other algae and also in (where it is present in the form of a homologue, Ssl0352) [Yeremenko *et al*., 2005]. Another functionally related complex PGRL1 was also been identified first in *Arabidopsis* [Dal Corso *et al*., 2008], and then in *Chlamydomonas* [Merchant *et al.,* 2007; Johnson *et al*., 2014]; but PGRL1 is only found in eukaryotes and is absent from cyanobacteria [Dal Corso *et al*., 2008; Peltier *et al*., 2010].

The structure of the PGR5 and PGRL1 complexes is still not fully resolved. It is clear that PGR5 is a small protein component on the outer (stromal) side of the thylakoid membrane, abutting ferredoxin and the calcium sensing (CAS) phosphoprotein. The major transmembrane component is made up of the PGRL1 protein, for which there are usually two gene copies. The PGR5/PGRL1 complex forms a supercomplex with PSI and the cytochrome b_6/f complex in *Chlamydomonas*. This differentiates it from *Arabidopsis* where the cytochrome b_6/f complex is separate. Nevertheless proton pumping occurs in both organisms through the

cytochrome b_6/f complex. The exact mechanism of AA inhibition in PGR5/PGRL1 is still a matter of speculation [Leister and Shikanai, 2013; Labs *et al.*, 2016]: in *Arabidopsis* evidence suggests that AA acts in conjunction with PGRL1 in preventing plastoquinone from interacting with the cytochrome b_6/f complex and inhibiting CEF (see below). In *Chlamydomonas* the situation with a very large supercomplex is less clear, although AA is not inhibitory of CEF here. However, this can be due to a single point mutation and does not have wide evolutionary significance [Sugimoto *et al.*, 2013].

The evolutionary phylogeny of PGR genes is unclear. They occur in green algae and higher plants [Peltier *et al.*, 2010], and homologues occur across the algal divisions and even in cyanobacteria: PGR5 genes or homologues (Ssl0352) occur in cyanobacteria. So it appears that the two systems, NDH and PGR, both evolved at the earliest stages of oxygenic photosynthesis and have been inherited through the algal and embryophyte systems. Even the action of antimycin A can be traced back to cyanobacteria [Yeremenko *et al.*, 2005]. PGR5 is the smaller of these complexes and there are no clear indications of how this protein evolved. It has some short sequence homology with PGRL1, the larger protein [Larkum, unpublished], which may indicate an evolutionarily shared origin, but the broader origins and even the complete function of these two protein complexes are frustratingly unresolved.

4.3. *Reductases of thylakoid membranes and their role in CEF*

It was earlier assumed that NADPH was the coenzyme of choice for reductase reactions of cyanobacteria and plastids, especially in interactions with the reducing side of PSI. In terms of CEF it was always assumed that an NADPH dehydrogenase would interact with a thylakoid membrane component to donate electrons through a membrane ET carrier towards the intrathylakoid space (or the plasma space of some cyanobacteria). This enzyme has recently been named NADH dehydrogenase-like complex because it has been discovered that it accepts electrons from ferredoxin [Yamamoto and Shikanai, 2013].

There has been much research effort involved in identifying just what enzyme system is involved. Two hypotheses emerged over the years. On the one hand there is a proposal for a plastoquinone reductase (PQR), as proposed by Bendall and coworkers [Moss and Bendall, 1984; Cleland and Bendall, 1992; Bendall and Manasse, 1995]. According to their hypotheses antimycin A did not inhibit a cytochrome, *i.e.* cytochrome b_6, as in mitochondria, and therefore they suggested that AA inhibited the PQR. Unfortunately, testing of this hypothesis has been difficult, especially the route for reducing equivalents across the thylakoid membrane

towards the intrathylakoid space [Labs *et al*., 2016]. Alternatively, Hertle *et al*. [2013] suggested that the route is through PGRL1 and that this is the elusive PQR. However, this proposal has also not been universally accepted to date. In *Chlamydomonas*, there is a supercomplex made up of PGRL1/PGR5, cytochrome b_6/*f*, plastoquinone, PSI and Lhca5 and 6 reduced by ferredoxin [Peltier *et al*., 2016]; and somewhere in there the reducing equivalents cross the membrane to the inside. However, the complexity of this supercomplex makes the establishment of the exact route problematical. It has even been suggested that the PGRL1/PGR5 proteins act as regulatory agents rather than as oxidoreductase components [Peltier *et al*., 2016].

The action of NADPH-like dehydrogenase, which in fact has been shown to utilize Fd(H), as the primary reductant has found much greater support; however, even here much more evidence is needed. And it is well-established that this route even when it occurs accounts for only a small fraction of the CEF activity [Yamori and Shikanai, 2016].

Both hypotheses might be accommodated based on the proposal from studies on tobacco and *Arabidopsi*s that there are two CEF pathways. One involving the NAD-1 complex and the other involving an antimycin A sensitive Fd-PQR [Joet *et al*., 2001; Hashimoto *et al*., 2003]. It has been proposed that the PGRL1 acts as the antimycin A sensitive pathway; and the discovery that NDH-1 complex receives electrons from Fd suggests that it is the antimycin-insensitive Fd-PQR pathway [Peng *et al*., 2011; Yamamoto *et al*., 2011]. Recently studies of knockout-mutants have concluded that lack of NDH-1 significantly lowers the generation of ΔpH in *Arabidopsis* at low light [Yamori at al., 2011], while the PGR5/PGRL1 pathway operates at high light [Wang *et al*., 2015]. Two CEF pathways also seem to operate in *Chlamydomonas* [Ravenal *et al*., 1994]. Since the mutant *Chlamydomonas* in these studies uses NADH, it is speculated that a transhydrogenase, catalyzing NADH/NADPH interconversion and driven by ΔpH, exists in the thylakoid membrane.

Cyanobacteria, lack the PGR5/PGRL1 complex, although, as mentioned already a homologous protein to PGR5 but not to PGRL1, exists in some cyanobacteria [Allahverdiyeva *et al.,* 2013]. Here CEF seems to rely on the NDH-1 complexes: NDH-1_1 and NDH-1_2, whilst NDH-1_4 complex seems to be involved in a CO_2 concentrating mechanism [Peltier *et al*., 2016].

The situation is complicated further in cyanobacteria because the plasma and thylakoid membranes are coextensive. However, it seems that while some proteins may be initiated on the plasma membrane, such as respiratory proteins, and can migrate to the thylakoid membrane, the reverse is not true and chlorophyll proteins for example are restricted to the thylakoid membrane, except in *Gloeobacter* which has no thylakoid membranes. Nevertheless this means that thylakoid

membranes of cyanobacteria can simultaneously be carrying: (i) respiratory proteins, (ii) photosynthetic proteins and (iii) chlororespiratory proteins of the PTOX-dependent system as well as other important enzymes [Mullineaux, 2014]. The situation is somewhat simpler in plastids but in many algal divisions there is still much "cross talk" between cytosol, mitochondria and plastid and there is a need for further investigation and clarification. The presence of mitochondria, which can generate reduced compounds such a succinate, malate (and other acids of the tricarboxylic acid cycle), NADH, NADPH, especially in the dark under aerobic conditions, and which can be imported into plastids, needs special consideration. These issues are discussed in the following sections.

5. Evidence for Cyclic Electron Flow in Eukaryotic Algae

5.1. *Pyrrophyta, Dinophyta (Dinoflagellata)*

5.1.1. *Symbiodinium sp., the coral endosymbiont algae*

The first observations indicated that *Symbiodinium* exhibits remarkable light-dependent O_2 uptake activity [Jones *et al*., 1998; Leggat *et al*., 1999] which has been assigned later on as a Mehler reaction as the most important alternative electron transport and photoprotective process in several *Symbiodinium* clades [Tchernov *et al*., 2004; Suggett *et al*., 2008; Roberty *et al*., 2014] and recently it was found that light-dependent O_2 uptake along with other photophysiological parameters reflects the acclimatory capacity to vertical light gradients in corals [Einbinder *et al*., 2016]. However, other studies indicate that the MAP pathway is not the only significant alternative electron transfer pathway in *Symbiodinium*, as chlororespiration [Reynolds *et al*., 2008], and CEF around PSI [Aihara *et al*., 2016] likely play photoprotective roles, *e.g.* under heat stress, recently reviewed by Warner and Suggett [2016]. Measured on intact corals, it was found that PSI is quite robust under various stress conditions as compared to PSII, and the elevated PSI activity under these conditions may play a role in CEF [Hoogenboom *et al*., 2012]. The existence of strong chlororespiratory activity in corals [Jones and Hoegh-Guldberg, 2001; Hill and Ralph, 2008] and the inhibition of Calvin–Benson cycle [Hill *et al*., 2014] results in reduction of the PQ pool, which might also be the initiator of CEF, although direct evidence for CEF has not been shown in these studies.

Although several hypotheses have been suggested on the role of CEF as a stress avoidance mechanism in *Symbiodinium*, currently there is no evidence about the existence and operation of the "typical" CEF components (such as PTOX, PGR5 and NDH) in *Symbiodinium*. [Aihara *et al*., 2016] reported that amino acid sequence homologues of PGR5/PGRL1, PTOX and NDH2 in *C. reinhardtii* exist

in the genome of *Symbiodinium*. However, to date there is no physiological confirmation which of these components/pathways may be operational in *Symbiodinium*. Moreover, considering the high diversity of the *Symbiodinium* genetic types, it is likely that the significance and components of alternative electron transfer pathways and other photophysiological processes vary in different *Symbiodinium* types [Suggett *et al*., 2015; Aihara *et al*., 2016]. Therefore the clarification of the role and function of CEF warrants further proteomics, molecular biology and bioinformatics investigations, possibly in various genetic types. A promising new direction is functional genomics, which might facilitate our knowledge on several physiological/metabolic functions in dinoflagellates [Murray *et al*., 2016]. Creating knock-out mutants of specific CEF components would facilitate our understanding of the role of alternative electron flow in *Symbiodinium*, as a response to environmental stress.

5.2. *Haptophyta*

5.2.1. *Prymnesiophyceae*

Zhang *et al.* [2014] found differences in the CEF:(CEF + LEF) ratio at high light between two closely related strains of *Isochrysis galbana.* Paasche [1964, 1966] showed that light-dependent production of the $CaCO_3$-containing coccoliths in the coccolithophorid *Coccolithus* (now *Emiliana*) *huxleyi* was much less inhibited by DCMU than was photosynthesis, consistent with cyclic photophosphorylation involvement in transport of Ca^{2+} and HCO_3^- as substrates for calcification in an intracellular compartment. Interpretation is complicated by the involvement of polysaccharides, whose production needs DCMU-inhibited LEF, in the structure of coccoliths [Lavoie *et al.,* 2016; Monteiro *et al.,* 2016].

5.3. *Ochrophyta*

5.3.1. *Bacillariophyceae*

Bailleul *et al.* [2015] and Goldman *et al*. [2015] showed that CEF (+DCMU) was about 5% of CEF + LEF (–DCMU) in the non-psychrophilic marine *Fragilaria pinnata*, *Phaeodactylum tricornutum, Thalassiosira pseudonana* and *Thassiosira weissflogii* at light saturation, but up to 30% at low light. Goldman *et al*. [2015] showed that CEF (+DCMU) was about 35% of CEF + LEF (–DCMU) in the psychrophilic marine *Fragilariopsis pinnata*. Thamatrakoln *et al.* [2013] report predicted genes with sequence similarity to PGR5 in the genomes of *Fragilariopsis cylindrus*, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, but also have "death-specific proteins" (DSPs) that may also act as proton gradient

regulators in the "red" lineage of chloroplasts. Thamatrakoln *et al.* [2013] show that over-expression of the *Thalassiosira pseudonana* DSP (*trDSP1*) has no effect of CEF in Fe-replete cells, but increases CEF by 61% when cells are deficient in Fe.

5.3.2. *Eustigmatophyceae*

Simionato *et al.* [2013] used P700 turnover measurements to show that control (N-replete) *Nannochloropsis gaditana* CEF [+DCMU] was 0.04 of CEF plus LEF (–DCMU). Also using P700 turnover in the presence and absence of DCMU, CEF in *Nannochloropsis gaditana* occurs at up to 0.08 of the rate of CEF plus LEF [Meneghesso *et al.,* 2016]. The function of CEF in *Nannochloropsis* is not known; HCO_3^- entry in the CO_2 concentrating mechanism is energized by mitochondrial respiration rather than ATP produced by CEF [Huertas *et al.,* 2002].

5.3.3. *Phaeophyceae*

There is evidence of the occurrence of a CEF in brown algae photoacoustic measurements on *Macrocystis pyrifera* [Herbert *et al.,* 1990; Fork *et al.,* 1991], and from measurements of PSI electron transport rates in the presence and absence of two DCMU concentrations indicating that CEF is 10% or less than CEF + LEF in *Sargassum fusiforme* [Gao *et al.,* 2016]. Coughlan [1977] found that 10 mmol m^{-3} DCMU inhibited light-stimulated active influx of SO_4^{2-} in *Fucus serratus* to almost the dark rate, though the absence of other concentrations of DCMU and of comparisons with DCMU inhibition of photosynthesis means that the absence energization of SO_4^{2-} by CEF needs further testing.

5.4. *Rhodophyta*

5.4.1. *Bangiophyceae*

The available data concern estimates of electron transport through PSI in the absence of PSII, *i.e*. CEF, and of CEF + LEF, so that CEF:[CEF + LEF] can be computed for growth under conditions yielding high growth rates [Biggins, 1973; Maxwell and Biggins, 1976; Gao and Wang, 2012], and when growth is limited by desiccation [Gao and Wang 2012]. The Bangiophyceae, and the Cyanidiophyceae, lack chloroplast NDH [references in DePriest *et al.,* 2013].

5.4.2. *Floridiophyceae*

The Floridiophyceae (references in DePriest *et al.* [2013]) lack chloroplast NDH.

6. The Role of CEF in Eukaryotic Algae in a Range of Habitats

6.1. *Desiccation*

Gao *et al.* [2011] investigated the effect of water loss from the thallus of the ulvophycean marine green alga *Ulva* on the electron transport rate through PSII and PSI. The PSI electron transport rate in the presence and absence of 10 mmol DCMU m^{-3} shows that the cyclic electron flow through PSI as a fraction of total electron flow is 0.08 in the fully hydrated controls (100% hydration), 0.21 at 65% hydration, and 0.93 at 22% hydration. Gao and Wang [2012] found similar outcomes in the bangiophycean red alga *Porphyra* [now *Pyropia*] *yezoensis,* with PSI as a fraction of total electron flow of 0.09 in the fully hydrated controls [100% hydration], 0.15 at 69% hydration, and 0.98 at 2% hydration.

6.2. *Variations in salinity*

Gao *et al.* [2016] found a higher PSI activity in the presence of DCMU to block PSII under higher and lower salinity than in controls of the low intertidal brown alga *Sargassum fusiforme.* The measured PSI turnover cannot necessarily be related to CEF in view of the possibility of net electron flow through PSI when DCMU blocks PSII, with electrons supplied by chrysolaminarin breakdown *via* NADPH [Gao *et al.,* 2016]. Huan *et al.* [2014] examined the effect of increased salinity in the intertidal ulvophycean green alga *Ulva prolifera* on PSI turnover in the presence of DCMU. The increased PSI turnover at high salinity seems to involve net electron flow rather than CEF in view of the decreased starch and sugar content of the thalli and the activity of the oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase.

6.3. *Low temperature*

The low temperature-adapted *Chlamydomonas raudensis* UWO241 from Lake Bonney in Taylor Valle, Antarctica is a psychrophile that cannot grow above 16°C, and is halotolerant [Dolhi *et al.,* 2013]. *Chlamydomonas raudensis* SAG49.72 isolated from a pond in the Czech Republic has an identical ITS sequence UWO241 but is a mesophile with an optimal growth temperature of 29°C [Dolhi *et al.,* 2013]. This phylogenetic analysis supercedes the taxonomy used in earlier work on the CEF [*e.g.* Morgan-Kiss *et al.,* 2002]. The psychrophilic *Chlamydomonas raudensis* UWO241 has CEF rate three times that of the mesophilic *Chlamydomonas raudensis* SAG49.72 [Morgan-Kiss *et al.,* 2002; Dolhi *et al.,* 2013; Szyska-Mroz

et al., 2015]. A similar difference is found in marine diatoms; the psychrophilic *Fragilariopsis cylindrus* has a CEF:(CEF + LEF) ratio more than four times that in the mesophilic *Thalassiosira weissflogii* [Goldman *et al.,* 2015]. Borla *et al.* [2015] show that CEF is 7% and 12% of CEF + LEF in wild-type cells of *Synechocystis* PCC6803 at 30°C and 20°C respectively; for an alkane-producing strain the values are, respectively, 10% and 21%.

6.4. *High temperature*

Aihara *et al.* [2016] showed that temperature above the normal tolerance range induced what is probably antimycin A-insensitive PSI CEF in the symbiotic dinoflagellate *Symbiodinium.* This apparent increase in CEF seemed to be an inevitable consequence of the higher temperatures rather than a protective or repair response. The main alternative photosynthetic electron transport pathway in *Symbiodinium* is the PSI-dependent MAP pathway [Roberty *et al.,* 2014].

6.5. *High light*

Niu *et al.* [2016] showed the role of CEF in decreasing non-photochemical quenching during high light treatments of the bangiophycean red alga *Pyropia* (formerly *Porphyra*) *yezoensis.*

6.6. *Nitrogen deficiency*

N-deficient *Nannochloropsis gaditana* had CEF [+DCMU] that was 0.25 of CEF + LEF (–DCMU) as compared to 0.08 in N-replete cells [Simionato *et al.,* 2013].

6.7. *Iron deficiency*

PSI has a higher Fe content than other thylakoid complexes and the PSI content is usually decreased relative to the other Fe complexes under Fe deficiency [Raven *et al.,* 1999; Ivanov *et al.,* 2000, 2004; Marchionetti and Maldonado, 2016; Raven and Beardall, 2017] and cells adapted genetically to low Fe habitats also have a lower PSI content [Strzepek and Harrison, 2004]. Ivanov *et al.* [2000] showed that the PSII:PSI ratio increased from 0.53 to 0.9 under Fe deficiency and that CEF increased relative to $NADP^+$ reduction in *Synecchococcus* sp. PCC 7942. The decreased electron flux to $NADP^+$ under Fe deficiency is (partly at least) offset by electron flow from PSII to O_2 *via* PTOX rather than the Mehler reaction involving

PSI [Bailey *et al.,* 2008]. Thamatrakoln *et al.* [2013] found that CEF per PSI reaction centre was unchanged by Fe limitation in *Thalassiosira pseudonana.* A further CEF-related aspect of the response to Fe deficiency is that PGRL1, involved in one mechanism of CEF, is also related to remodeling the photosynthetic apparatus in *Chlamydomonas reinhardtii* [Petroutsos *et al.,* 2009].

7. Summary and Conclusions

This article outlines the strong evidence, in cyanobacteria and eukaryotic algae for CEF in the generation of extra proton motive force, and therefore extra ATP from PSI activity, *i.e.* an increase in the amount of ATP produced per e^- transported from PSII to PSI. The early *in vitro* evidence for this was established over 50 years ago, but despite strong evidence *in vivo* early on this has remained inferential. The use of mutants over recent years in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* has strengthened the evidence for CEF, but because of overlapping pathways of electron transport close to PSI the exact proof has remained frustratingly elusive. The situation is not helped by the fact that there are at least two dehydrogenase pathways involved (NDH and PGR) in all oxygenic PS organisms (cyanobacteria, eukaryotic algae and embryophytes). Also there is no good inhibitor for the NDH pathway, and the only known inhibitor for the PGR pathway antimycin A is patchy in its effectiveness (active in PS bacteria, inactive in cyanobacteria, inactive in many algae including *Chlamydomonas,* and active in embryophytes).

Cyanobacteria have a strong NDH-linked CEF which clearly contributes significantly to ATP production for the Calvin–Benson Cycle and other energy dependent processes such a nitrogen fixation, nitrogen metabolism and chlorophyll synthesis. The eukaryotic algae have a great variety of structural and metabolic variation; and there is evidence for CEF in all of the algal divisions where it has been put to the test.

The case for CEF is strongest where stress effects lower the input of energy from LEF: in such situations as temperature stress, salt stress, and dehydration stress (such as in intertidal algae), *etc*. A few examples of such stresses are given but these examples will undoubtedly grow in the coming years.

Finally, we need better techniques to elucidate exactly what mechanisms contribute to CEF and what other electron transport pathways, such as the MAP pathway, oxidative respiration, glycolysis, and chlororespiration, intersect with CEF and how these interactions are controlled. Use of the MIMS and stable isotopes of oxygen and carbon is a promising means of improving precise knowledge of the CEF pathway.

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