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Published in:
New Phytologist

DOI:
[10.1111/nph.16541](https://doi.org/10.1111/nph.16541)

Publication date:
2020

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Raven, J. A. (2020). Chloride involvement in the synthesis, functioning and repair of the photosynthetic apparatus *in vivo*. *New Phytologist*, 227(2), 334-342. <https://doi.org/10.1111/nph.16541>

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Article type : Research Review

Research review

Chloride involvement in the synthesis, functioning and repair of the photosynthetic apparatus *in vivo*

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Received: 4 January 2020

Accepted: 5 March 2020

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/NPH.16541](https://doi.org/10.1111/NPH.16541)

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Abstract

Cl⁻ has long been known as a micronutrient for oxygenic photosynthesis resulting from its role as an essential cofactor for Photosystem II (PSII). Evidence on the *in vivo* Cl⁻ distribution in *Spinacia oleracea* leaves and chloroplasts show that sufficient Cl⁻ is present for the involvement in PSII function, as indicated by *in vitro* studies on, among other organisms, *S. oleracea* PSII. There is also sufficient Cl⁻ to function, with K⁺, of parsing the H⁺ electrochemical potential difference (proton motive force) across the illuminated thylakoid membrane into electrical potential difference and pH difference components. However, recent *in vitro* work on PSII from *S. oleracea* shows that oxygen evolving complex (OEC) synthesis, and re-synthesis after photodamage, requires significantly higher Cl⁻ concentrations than satisfy the function of assembled PSII O₂ evolution of the synthesised PSII with OEC. The low Cl⁻ affinity of OEC (re-)assembly could be a component of limiting the rate of OEC (re-) assembly.

Keywords

Chloride; oxygen evolving complex (OEC); photodamage repair; photosystem II; proton motive force

Introduction

The requirement for Cl⁻ as an essential element for oxygenic photosynthesis (Warburg 1948; Arnon and Whatley 1949; Gorham and Clendenning 1952; Bové et al. 1963; Homann 2002; Popelková and Yocum 2007; Raven 2017) before it was established as an essential micronutrient for oxygenic photosynthetic organisms to complete their life cycle (Broyer et al. 1954; Martin and Lavellay 1958; Raven 2017). The micronutrient role of Cl⁻ has focussed on an essential role in O₂ production by Photosystem II (PSII) (e.g. Popelková and Yocum 2007; Raven 2017), consistent with lower concentration of Cl⁻ (and also the other OEC elements, Ca and Mn) in the non-photosynthetic parasite *Cuscuta* than in the host *Pelargonium* (Förste et al. 2019). Although the occurrence, and mechanism, of inhibitory effects of high, salinizing, Cl⁻ concentration on glycophytic (crop) plants has received some attention, the main focus in salinity studies has been on the effects of Na⁺ (Raven 2017; Wege et al. 2017; Colmeno-Flores et al. 2019; Geilfus 2019). Recently there has been some emphasis on Cl⁻ at external concentrations between those satisfying the micronutrient requirement in PSII and those inhibiting the growth of glycophytes acting as a 'beneficial

nutrient' that can increase growth rate (Raven 2017; Wege et al. 2017; Colmeno-Flores et al. 2019; Geilfus 2019). The present paper analyses the quantitative role of Cl^- in O_2 production by PSII, as well as other photosynthetic roles such as synthesis and repair of the PSII complex, some of which require high intracellular Cl^- concentrations.

Cl requirement for assembly of PSII, including the oxygen evolving complex

Assembly of the PSII complex, including the oxygen evolving complex (OEC), is essential for the growth of oxygenic photolithotrophs, and for the replacement of photodamaged PSII (Vinyard et al. 2019; Weisz et al. 2019). Vinyard et al. (2019), in a paper focussing on the anomalous isotope effect with faster reaction with $^2\text{H}_2\text{O}$ rather than $^1\text{H}_2\text{O}$ as solvent, showed that the lag in the photoassembly of the Mn_4CaO_5 oxygen evolving complex (OEC: Vinyard and Brudvig 2017) of the PSII preparation lacking the luminal psbO subunit, universally present in oxygenic photosynthetic organisms (Koyama et al. 2008; Sovová and Ettrich 2007; Ifuku 2015; Pirella-Karlusich et al. 2015), was longer at $100 \text{ mol m}^{-3} \text{ Cl}^-$ than at $750 \text{ mol m}^{-3} \text{ Cl}^-$ when $^1\text{H}_2\text{O}$ was the solvent. Furthermore, the final yield (fraction of OECs assembled) was greater at the higher Cl^- concentration (Figure 4 of Vinyard et al. 2019). The half-saturation value for Cl^- for OEC assembly is about 120 mol m^{-3} when the solvent is $^1\text{H}_2\text{O}$; with $^2\text{H}_2\text{O}$ as the solvent, i.e. not relevant to *in vivo* conditions, the Cl^- affinity of OEC assembly, is higher than with $^1\text{H}_2\text{O}$ (Figure 4 of Vinyard et al. 2019). However, in the presence of the psbO subunit, assembly of the OEC in vitro with H_2O as solvent, a lower Cl^- concentration was needed for PSII-OEC assembly. Figure S5 of Winyard et al. (2019) shows that, after 900s of PSII OEC reassembly, the extent assayed as O_2 evolution was $41 \mu\text{mole mg}^{-1} \text{ chlorophyll h}^{-1}$ with $100 \text{ mole Cl}^- \text{ m}^{-3}$ and $29 \mu\text{mole mg}^{-1} \text{ chlorophyll h}^{-1}$ with $20 \text{ mole Cl}^- \text{ m}^{-3}$. After the shorter reassembly time of 300s, Figure S5 of Winyard et al. (2019) shows that the extent assayed as O_2 evolution was $20 \mu\text{mole mg}^{-1} \text{ chlorophyll h}^{-1}$ with $100 \text{ mole Cl}^- \text{ m}^{-3}$ and $9 \mu\text{mole mg}^{-1} \text{ chlorophyll h}^{-1}$ with $20 \text{ mole Cl}^- \text{ m}^{-3}$. The data for PSII preparations with the psbO subunit is much more likely to represent the *in vivo* Cl^- dependence of PSII-OEC assembly, with a half-saturation value for Cl^- of about 15 mole m^{-3} or about 40 mole m^{-3} for saturation (Table 1. As is discussed below, this is more than order of magnitude greater than the Cl^-

concentration required to saturate the Cl⁻ requirement for functioning of the PSII-OEC complex.

Cl⁻ requirement for function of the fully assembled PSII including OEC

Contrasting with the high Cl⁻ requirement for OEC assembly, the catalytic role of Cl⁻ in the fully assembled PSII-OEC is satisfied by much lower concentrations of Cl⁻ in glycophytic plants such as *S. oleracea*. Starting by considering the number of Cl⁻ ions bound to, and required for catalysis by, the assembled PSII-OEC complex, there have been a number of studies using a range of techniques, with the conclusion that there are 2 Cl⁻ binding sites per PSII-OEC complex (Lindberg and Andréasson 1996; Murray et al. 2008; Kawakami et al. 2009; Umena et al. 2011; Reiss et al. 2019). However, in the case of the cyanodiophycean red alga, the crystal structure only clearly revealed one Cl⁻ in the PSII-OEC (Ago et al. 2016). Lindberg and Andréasson (1996) found a half-saturation Cl⁻ concentration of 0.02 mol m⁻³ for the slow-exchanging (1 hour half-time) site, and 0.5 mol m⁻³ for the fast-exchanging (15 second half-time) binding site of *S. oleracea* PSII particles, when the Cl⁻ effects were assayed as O₂ production. Kühne et al. (1999) found a half saturation value of 0.47 mol m⁻³ Cl⁻ (their Table 1) for O₂ production in *S. oleracea* PSII particles. Table 2 of Popelkova et al. (2006) shows a Cl⁻ half saturation concentration of 0.41 mol m⁻³ for O₂ production, also for *S. oleracea* PSII particles. Accordingly, 2 mole Cl⁻ m⁻³ in solution would saturate O₂ production from assembled PSII-OEC (Table 1).

Contrasting with the data on glycophytes, higher Cl⁻ concentrations are needed to saturate O₂ production by halophytes such as the mangrove *Avicennia marina* (Critchley 1982; Critchley et al. 1982; Andersson et al. 1984) and the marine diatom *Cylindrotheca fusiformis* (Shieh et al. 2011). In the case of *A. marina*, the inside-out thylakoid vesicles had lost 16 kDa and 23 kDa peripheral (luminal) proteins, and addition of the 23 kDa protein from *S. oleracea* decreased the Cl⁻ requirement for O₂ production by *A. marina*, although not to as low as the requirement of *S. oleracea* (Critchley 1982; Andersson et al. 1984). No mention is made of the presence of the 30 kDa PsbO peripheral protein, found in all oxygenic photosynthetic organism (Ifuku 2015; Ifuku and Noguchi 2016; Ago et al. 2016). *Avicennia marina* has

glycophytic angiosperm ancestry, and the lower affinity of the assembled PSII-OEC for Cl^- may reflect relaxed selection for high affinity Cl^- affinity. The ancestral diatoms were almost certainly marine (Sims et al. 2006), and it would be interesting to determine the Cl^- affinity of PSII-OEC from the derived freshwater diatoms.

Relation of the Cl^- requirement for PSII-OEC (re-)assembly and function *in vitro* to the concentration and distribution of Cl^- in chloroplasts and cyanobacterial cell.

How do these Cl^- concentrations relate to possible *in vivo* Cl^- concentrations in the thylakoid lumen, the place where the photoassembly of the OEC accesses Cl^- ? For PSII-OEC (re-)assembly, it is assumed that Cl^- concentration is 40 mole m^{-3} , i.e. sufficient to saturate the Cl^- requirement (Figure S5 of Vinyard et al. 2015) Assuming that Cl^- is the only anion in the thylakoid lumen, and charge is balanced by univalent cations (including H^+), and the activity coefficients are 0.9 (Boyd and Gradmann 2002), $40 \text{ mole m}^{-3} \text{ cation}^+$ plus $40 \text{ mole m}^{-3} \text{ Cl}^-$ in the thylakoid lumen mean an osmolarity of 72 osmol m^{-3} . The default assumption is that all intracellular compartments are at osmotic equilibrium, although active water transport from the thylakoid lumen to the stroma, or cytosol of cyanobacteria, would allow a higher concentration of osmotically active solutes (including Cl^-) in the thylakoid lumen than in the rest of the cell of non-salinised glycophytes. Raven and Doblin (2014) discuss the possible mechanism, and energetics, of active water transport. Raven (2018) give 59 osmole m^{-3} as the lowest intracellular osmolarity of photosynthetic organisms; with Cl^- as the only anion in the thylakoid lumen, the lumen Cl^- would not saturate the Cl^- requirement for PSII-OEC (re-)assembly. As well as the involvement of active water transport mentioned above, Cl^- in the lumen could saturate the (re-)assembly process in the (perhaps) unlikely situation of divalent cations ($\text{Ca}^{2+} + \text{Mg}^{2+}$) as the only counter-ions for Cl^- , with lumen osmolarity of 60×0.9 or $54 \text{ osmole Cl}^- \text{ m}^{-3}$. This would saturate (re-)assembly of the PSII-OEC without the need for active water transport out of the thylakoid lumen. The dissolved Cl^- concentration of 2 mole m^{-3} required to saturate O_2 production is well within the range permitted by the minimal intracellular osmolarity.

Turning to estimates of chloroplast Cl^- concentrations, many of the estimates of Cl^- requirements for PSII (re-)assembly (Vinyard et al., 2019) and function (Lindberg and Andréasson 1996; Murray et al. 2008; Kawakami et al. 2009; Umena et al. 2011; Reiss et al. 2019) were obtained for *S. oleracea*, a plant for which relevant data on chloroplast Cl^- concentration are available. This will now be discussed. Although *S. oleracea* is tolerant of moderate salinization, it grows well in non-saline media. Under non-salinised growth conditions the Cl^- concentration determined by anion chromatography in aqueously extracted chloroplasts is $1 \pm 1 \text{ mol m}^{-3}$ - $6 \pm 5 \text{ mol m}^{-3}$ (Schröppel-Meyer and Kaiser 1987; Schröppel-Meyer and Kaiser 1988; Speer and Kaiser 1991). The non-saline growth medium for *S. oleracea* had $50 \text{ mmol Cl}^- \text{ m}^{-3}$ (Johnson et al. 1957), i.e. in the micronutrient rather than the beneficial nutrient concentration range (Raven 2017). Earlier work found higher ($60 - 100 \text{ mol m}^{-3}$) Cl^- concentrations in aqueously isolated chloroplasts of *S. oleracea* (Demmig and Winter 1986; Kaiser et al. 1983; Robinson et al. 1983; Robinson and Downton 1984); these higher Cl^- concentrations was shown to be a result of interference of chloroplast components with amperometric titration assays of Cl^- (Schröppel-Meyer and Kaiser 1987). The methodology of aqueous chloroplast extraction produces chloroplasts with CO_2 - and light-saturated rates on a chlorophyll basis that were at least as high as those found for leaf slices of *S. oleracea* (Kaiser et al. 1981). Kaiser et al. (1981) also found a relatively low quantum requirement of 12 mol absorbed photons per mol O_2 produced by the isolated chloroplasts. Earlier work by Larkum (1968) used chloroplasts extracted by non-aqueous techniques from *Beta vulgaris*, from the same family (Amaranthaceae) as *S. oleracea*. Larkum (1968) found $130 \text{ Cl}^- \text{ mol m}^{-3}$ using amperometric titration assays of Cl^- , shown by Schröppel-Meyer and Kaiser (1987) to over-estimate the Cl^- concentration in aqueously extracted *S. oleracea* chloroplasts. Such over-estimation may also occur for the Cl^- concentration of non-aqueously isolated chloroplasts on the freshwater characean *Tolypella intricata* (Larkum 1968), and chloroplast-containing cytoplasm obtained by micro-dissection from freshwater giant cells of the characean *Nitella translucens* (MacRobbie 1964) and the chlorophycean *Hydrodictyon africanum* (Raven 1967).

Winter et al. (1994) found that thylakoids occupy 31.5% of the chloroplast volume of *S. oleracea*. Daum et al. (2010) determined the repeat distance of appressed thylakoid

membranes is 15.7 nm in *S. oleracea*, and cite earlier reports of values of 14.4-24.3 nm. With the Daum et al. (2010) value of 4.5 nm for lumen thickness, the lumen is 22% of the thylakoid volume, or 7% of the chloroplast volume. If all the chloroplast Cl^- concentration of 1 - 6 mol m^{-3} is in the thylakoid lumen, the concentration of Cl^- in the lumen is 14 - 86 mol m^{-3} (Table 1). Again in *S. oleracea*, Johnson et al. (2011) found that the lumen thickness of illuminated thylakoids is 17 nm, the thickness of appressed membranes in grana in the light is 12 nm, and the thickness of end membranes of grana, and presumably of stroma membranes, is 7 nm. Allowing for end membranes and stromal membrane, these thicknesses suggest that 58% of the thylakoid volume is thylakoid lumen, so 18% of the chloroplast volume is thylakoid lumen. If all of the chloroplast Cl^- is in the thylakoid lumen, the concentration of Cl^- in the thylakoid lumen is 5.5 - 33 mol m^{-3} (Table 1). The two values are about 35-215% (Daum et al. 2010) and 14-83% (Johnson et al. 2011) of the saturating Cl^- concentration for light-driven OEC assembly (Vinyard et al. 2019). While the upper limits on luminal Cl^- are saturating, the lower limit does not, especially for the value estimated from Johnson et al (2011). Furthermore, the assumption of all the chloroplast Cl^- occurs dissolved in the thylakoid lumen is inconsistent with the role of Cl^- in the variable parsing of the pmf across the thylakoid membrane into the pH difference and the electrical potential difference as conditions change (see below), and the Cl^- bound to the PSII-OEC, although this only amounts to 0.7 mole $\text{Cl}^- \text{m}^{-3}$ (see below).

A possible role of Cl^- in limiting damage to the photosynthetic apparatus is suggested by the work of Zhang et al. (2014) who investigated the effect of 27 mole m^{-3} CaCl_2 (i.e. 54 mole m^{-3} Cl^-) on the decreased photosynthetic capacity resulting from low night temperatures in *Solanum lycopersicum* (as *Lycopersicum esculentum*). The investigation was designed, and interpreted, in terms of the effects of Ca^{2+} , consistent with the partial reversal of some effects by EGTA, but no other Ca^{2+} salts were tested, so a role for Cl^- has not been ruled out.

The data presented in the previous paragraph contributes to estimation of the Cl^- content in the thylakoids required to satisfy this structural and functional requirement for catalysis. With half saturation values of not more than 0.5 mol $\text{Cl}^- \text{m}^{-3}$ for the low-affinity Cl^- site, and 0.02 mol m^{-3} for the high-affinity site, 1.5 mol $\text{Cl}^- \text{m}^{-3}$ in the thylakoid lumen essentially

saturates the Cl^- for the high- and low-affinity sites. To compute the Cl^- bound to the high- and low-affinity sites in the thylakoid for comparison with free Cl^- in the lumen, we need to know the number of PSII sites in the thylakoid per unit lumen volume. We start with the 400 total chlorophyll a + b per total PSII units in the thylakoids (grana plus stroma) in *S. oleracea* (Malkin et al. 1981), and 500 nmol chlorophyll a+b per m^2 total grana plus stroma thylakoid per m^2 membrane area (Thomas et al. 1955) in *S. oleracea*. For other C_3 terrestrial plants there is a range of 670-1860 nmol chlorophylls a+b total grana plus stroma thylakoid area (Table 4.1 of Lawlor 1993). From the *S. oleracea* data there are 0.8 nmol PSII per m^2 total grana plus stroma thylakoid per m^2 membrane area, or, with 2 mole Cl^- per mole PSII-OEC as discussed above, 1.6 nmol PSII-bound Cl^- total grana plus stroma thylakoid per m^2 membrane area. From Johnson et al. (2011) used chloroplasts isolated in 450 mole m^{-3} and fixed in glutaraldehyde for transmission electron microscopy and found that the lumen thickness of illuminated *S. oleracea* thylakoids is 17 nm. This means that there is $8.5 \cdot 10^{-9} \text{ m}^3$ lumen volume per m^2 thylakoid area, hence $1.6 \cdot 10^{-9}$ mole bound Cl^- per $8.5 \cdot 10^{-9} \text{ m}^3$ lumen volume (close to the value cited by Cruz et al. 2001), or a concentration of $0.19 \text{ mole Cl}^- \text{ m}^{-3}$. Since $1.5 \text{ mole Cl}^- \text{ m}^{-3}$ in the thylakoid lumen essentially saturates the Cl^- requirement for the high-affinity and low-affinity Cl^- sites, the minimum total Cl^- concentration associated with the catalytic function of Cl^- in PSII of *S. oleracea* is $1.7 \text{ mole Cl}^- \text{ m}^{-3}$. From Daum et al. (2010), using chloroplasts isolated in 350 mole m^{-3} and cryofixed for transmission electron microscopy, the lumen thickness of illuminated *S. oleracea* thylakoids is 4.5 nm, so there is $2.25 \cdot 10^{-9} \text{ m}^3$ lumen volume per m^2 thylakoid area. This value corresponds to $1.6 \cdot 10^{-9}$ mole bound Cl^- per $2.25 \cdot 10^{-9} \text{ m}^3$ lumen volume, or a concentration of $0.71 \text{ mole Cl}^- \text{ m}^{-3}$. Since $1.5 \text{ mole Cl}^- \text{ m}^{-3}$ in the thylakoid lumen essentially saturates the Cl^- sites for PSII-OEC function, the minimum total Cl^- concentration associated with the catalytic function of Cl^- in PSII of *S. oleracea* is $2.2 \text{ mole Cl}^- \text{ m}^{-3}$ (Table 1).

A further estimate of the Cl^- availability to catalytic sites in PSII can be made for the basal cyanobacterium *Gloeobacter*. This organism lacks thylakoids and has all of its membrane-associated bioenergetics, i.e. H^+ -pumping photosynthetic and respiratory redox reactions and a H^+ -pumping rhodopsin, in the plasmalemma (Rexroth et al. 2011; Raven 2017). The OEC of *Gloeobacter* faces the periplasm and hence the growth medium, rather than the

derived state of facing the thylakoid lumen. Despite this, and the absence, or altered sequence, of some OEC-related proteins, and particularly sequence differences in the OEC-binding D1 protein, the *psbA* gene product (Cardona et al. 2015), what is known of the mechanism of O₂ production in *Gloeobacter violaceum* PCC 7421 shows it is closely similar to that of thylakoid-containing organisms (Koyama et al. 2008; Bernát et al. 2012; see also Sicora et al. 2008). The light- and inorganic C-saturated photosynthetic rate on a chlorophyll basis of *Gloeobacter violaceum* is just over half that of the thylakoid-containing *Synechocystis* sp, PCC 6803 (Koyama et al. 2008). *Gloeobacter* spp. naturally grow epilithically in damp non-saline subaerial habitats (Mareš et al. 2013; Saw 2013), and are generally cultured in the BG11 medium that contains 0.508 mole Cl⁻ m⁻³ (Rippka et al. 1979; Ritchie 1992). This Cl⁻ concentration is less than the calculated minimum dissolved Cl⁻ to saturate photosynthetic O₂ production in thylakoids of *S. oleracea*. Furthermore, cyanobacteria are gram-negative bacteria, and the periplasm of those gram-negative bacteria that have been examined bears negative charge on some macromolecules (Stock et al. 1977). The result is a negative Donnan potential of the periplasm relative to the medium (Stock et al. 1977), thus decreasing the Cl⁻ concentration in the periplasm relative to the medium.

However, it must be borne in mind that there are significant differences in the sequence of the D1 protein (*psbA* gene product) of PSII that binds the OEC between the basal cyanobacteria and other oxygenic photosynthetic organisms. For a freshwater cyanobacterium with thylakoids, *Synechococcus* PCC 7942, growing in BG11, there is a intracellular Cl⁻ concentration of 12.2 ± 0.85 mole m⁻³ in the light and 1.27 ± 0.11 mole m⁻³ in the dark (Ritchie 1992). Intracellular Cl⁻ was determined using ³⁶Cl⁻ (Ritchie 1992), so the measurement was not subject to the problems with coulometric measurements. Partitioning of Cl⁻ between cytosol and thylakoids depends on (a) Cl⁻ channel(s) or Cl⁻ transporter(s) in the thylakoid membrane; such methods of Cl⁻ movement are incompletely characterised in cyanobacteria (Carraretto et al. 2016).).

Other photosynthetic roles of Cl⁻

Other roles of thylakoid Cl^- are as a cofactor of PSII-associated carbonic anhydrase(s), in the allocation of the proton motive force (H^+ electrochemical potential difference) across the thylakoid membrane into electrical potential and pH difference. More widely, Cl^- may be involved in HCO_3^- influx in photosynthesis in some algae.

For the PSII-associated carbonic anhydrase(s) (Ignatova et al. 2019a,b), Cl^- -depleted thylakoids and PSII particles of *Zea mays* have their carbonic anhydrase activity increased by the addition of Cl^- up to 30 mole m^{-3} , with inhibition at higher concentrations (Stemler 1986; Lu and Stemler 2007). Lu and Stemler (2007) suggest that carbonic anhydrase activity is associated with the oxygen evolving complex 33 (OEC33 = psbO) protein, although this was not confirmed by Ignatova et al. (2019a,b) for C_3 flowering plants, including *S. oleracea*.

Another known role of thylakoid Cl^- is in parsing the proton motive force across the thylakoid membrane into an inside-positive electrical potential difference and inside-low pH difference (van Kooten et al. 1986; Cruz et al. 2001; Tang and Wei 2001; Herdean et al. 2016a,b; Davis et al. 2017; Spetea et al. 2017; Szabò and Spetea 2017). Light-driven pumping of H^+ into the thylakoid lumen immediately results in an electrical potential difference across the membrane. This outcome is a function of the relative values of the thylakoid membrane capacitance and the pH buffer capacity of the thylakoid lumen on a thylakoid area basis (Cruz et al. 2001). For (swollen) thylakoids of *S. oleracea* and *Lactuca sativa* at pH 8.1, the capacitance is 9.3 mF m^{-2} (Arnold et al. 1985), i.e. $9.3 \cdot 10^{-9} \text{ mole charge m}^{-2} \text{ V}^{-1}$; in the case of H^+ this is $9.3 \cdot 10^{-9} \text{ mole H}^+ \text{ m}^{-2} \text{ V}^{-1}$. For the thylakoid lumen buffer capacity of $30 \text{ mole H}^+ \text{ m}^{-3}$ lumen volume pH^{-1} used by Cruz et al. (2001), and the thylakoid lumen volume per thylakoid membrane area of $8.5 \cdot 10^{-9} \text{ m}^3 \text{ m}^{-2}$ calculated above which is close to the value of $8 \cdot 10^{-9} \text{ m}^3 \text{ m}^{-2}$ used by Cruz et al. (2001), the pH buffer capacity of the thylakoid lumen on a thylakoid membrane area basis is $30 \text{ mole H}^+ \text{ m}^{-3} \text{ pH}^{-1} \times 8.5 \cdot 10^{-9} \text{ m}^3 \text{ m}^{-2} = 2.55 \cdot 10^{-7} \text{ mole H}^+ \text{ m}^{-2} \text{ pH}^{-1}$. A potential difference across the membrane of 0.3 V results in electrical breakdown of lipid bilayer membranes (Benz et al. 1979); the plasmalemma of protoplasts from guard cells of the stomata of *Vicia faba* has an electrical breakdown voltage of 1 V (Zimmermann et al. 1980). There are no estimates of electrical breakdown voltage for thylakoids, and a value of 1 V is assumed. For light-driven H^+ transport into the thylakoid lumen, with no other ion

fluxes across the thylakoid membrane, the electrical breakdown voltage of 1V is achieved when $9.3 \cdot 10^{-9}$ mole H^+ m^{-2} has entered the thylakoid lumen, granted the capacitance of $9.3 \cdot 10^{-9}$ mole H^+ $m^{-2} V^{-1}$. With the area-based pH buffering $2.55 \cdot 10^{-7}$ mole H^+ $m^{-2} pH^{-1}$ the pH decrease in the thylakoid lumen at the electrical breakdown voltage is only 0.036 pH units. By analogy with mitochondria, H^+ recirculation through the CF_0CF_1 ATP synthase could operate with a membrane potential as the dominant H^+ driving force generated by light-drive H^+ entry, and for H^+ flux through the ATP synthase, powering the phosphorylation of ADP. Granted the measured H^+ :ATP of 4 of the CF_0CF_1 ATP synthase rather than the prediction of 4.67 from structural biology and an in vivo free energy of ATP hydrolysis of 55 kJ mol^{-1} (Raven et al. 2014), a minimum electrical potential difference across the thylakoid membrane of 140 mV is required. In the absence of ion fluxes other than active H^+ influx and H^+ efflux through the ATP synthase, 140 mV electrical potential difference corresponds to a pH decrease in the thylakoid lumen of only 0.005 pH units.

However, the available evidence shows that the H^+ electrochemical potential difference across the illuminated thylakoid membrane has significant contributions from both an electrical potential difference and a pH difference component (van Kooten et al. 1986; Cruz et al. 2001; Herdean et al. 2016a,b; Davis et al. 2017; Spetea et al. 2017; Szabò and Spetea 2017; Dukic et al. 2019). The conversion of the electrical potential difference to a pH difference involves either K^+ efflux from the thylakoid lumen via a Mg^{2+} channel, K^+ channel or a K^+ : H^+ antiporter, and/or Cl^- influx through (a) Cl^- channel(s) (Hind et al. 1974; Krause 1977; Ben-Hayyim 1988; van Kooten et al. 1986; Pottosin and Schönknecht (1988); Schönknecht et al. (1995); Cruz et al. 2001; Tang and Wei 2001; Kirchhoff et al. 2011; Bernát et al. 2012; Duan et al. 2016; Herdean et al. 2016a,b; Kaňa and Govindjee 2016; Davis et al. 2017; Lyu and Lazár 2017a,b; Spetea et al. 2017; Szabò and Spetea 2017; Dukic et al. 2019; Galvis et al. 2020; see also Demmig and Gimmler 1983).

The Cl^- channel option would increase the thylakoid lumen Cl^- concentration relative to that in the stroma, qualitatively consistent with catalytic roles of Cl^- in the lumen. For the lower measured concentration of Cl^- in the chloroplast of *S. oleracea*, 1 mole m^{-3} , a thylakoid lumen concentration of 2 mole m^{-3} (sufficient to saturate the Cl^- requirement for PSII operation)

leaves a Cl^- concentration of 0.66 mol m^{-3} in the stroma if the lumen is 20% of the chloroplast volume, and 0.86 mol m^{-3} in the stroma if the lumen is 7% of the chloroplast volume. These lumen: stroma Cl^- ratios of 3.0 and 2.3 correspond, respectively, to Cl^- passive equilibrium with inside-positive electrical potential differences of 28 mV and 21 mV. Even for this very low whole-chloroplast Cl^- concentration (as little as 1 mol m^{-3}), catalytic functions of Cl^- in the thylakoid can be satisfied with not more than 20% of the H^+ electrochemical potential across the thylakoid membrane occurring as an electrical potential difference. The involvement of downhill Cl^- influx as part of mechanism converting the part of the PMF generated as an electrical potential by light-energized H^+ movement into the thylakoid lumen is consistent with the observed Cl^- flux into isolated thylakoids of *S. oleracea*, as well as the swelling as (buffered) H^+ and Cl^- are accumulated, when illumination begins (Hind et al. 1974). It is also consistent with the in vivo comparison cryo-transmission electron microscopic sections prepared from 'dark-adapted' and from illuminated leaf sections of *Arabidopsis thaliana* showing an increase of thylakoid lumen width from 4.7 nm in the dark to 9.2 nm in the light (Kirchhoff et al. 2011). However, in *Pisum sativum* chloroplasts in vivo the lumen volume is decreased by 22% in the light relative to the dark (Miller and Nobel 1972), i.e. not consistent with HCl accumulation in the lumen in the light. This is also the cases for the bangiophycean marine red alga *Porphyra* (from the Pacific, for which the newer generic name is *Pyropia*) and the ulvophycean marine green alga *Ulva* there is also in vivo evidence of decreased thylakoid lumen volume in the light (Murakami and Packer 1970). For the flowering plants there are also K^+ channels in the thylakoid membrane (see above), and cyanobacteria the available evidence only shows a role for K^+ channels in regulating the contribution of electrical potential difference and pH difference to the proton motive force across the thylakoid membrane (Checchetto et al. 2012).

For the Cl^- option, there is the possibility that the anion channels are able to allow passage of singly charged anions other than Cl^- that occur in the chloroplast stroma. Duan et al. (2016) showed that a bestrophin-like protein is involved in modulating the transthylakoid membrane proton motive force in *Arabidopsis thaliana*, While bestrophin channels in Metazoa are typically considered to be Cl^- channels, they are also highly permeable to HCO_3^- (Qu and Hartzell 2008), and a bestrophin-like protein is the HCO_3^- channel in the thylakoid

membrane of *Chlamydomonas reinhardtii* that is involved in the CO₂ concentrating mechanism (CCM) (Mukherjee et al. 2019). The bestrophin-like channels in *C. reinhardtii* are localised in the thylakoid tubules in the pyrenoid, colocalised with the luminal carbonic anhydrase Cah3, generating CO₂ which diffuses through the thylakoid membrane to Rubisco (ribulose biphosphate carboxylase-oxygenase) localized in the pyrenoid (Mukherjee et al. 2019). For C₃ terrestrial flooring plants such as *A. thaliana*, HCO₃⁻ influx to the thylakoid lumen would, in the presence of soluble luminal carbonic anhydrase (as in *A. thaliana*: Ignatova et al. 2019b) act as an uncoupler by reacting with HCO₃⁻ with H⁺ to produce membrane-permeable CO₂ and H₂O). Returning to potential competition between HCO₃⁻ and Cl⁻ in transport through 'Cl⁻ channels' in the thylakoid membrane, while we do not know the selectivity of the channels for HCO₃⁻ relative to Cl⁻, the concentration of HCO₃⁻ (Werdan and Heldt 1972) and Cl⁻ (Werdan and Heldt 1972; Raven and Glidewell 1981; Schröppel-Meyer and Kaiser 1987; Schröppel-Meyer and Kaiser 1988; Speer and Kaiser 1991) in the stroma in saturating light can each be as low as 1 mol m⁻³.

Another anion that has been examined for movement through thylakoid channels termed 'Cl⁻ channels' is NO₃⁻. Schönknecht et al. (1998) and Pottosin and Schönknecht (1995) measured Cl⁻ conductance in thylakoids of *Peperonia metallica* and *Nitellopsis obtusa* respectively. Duan et al. (2016) and Herdean et al. (2016a,b) examined genetically identified two bestrophin-like proteins in the thylakoid membrane, one of which was much more highly expressed than the other and which were functionally redundant as anion channels; the data are discussed by Szabò and Spetea (2017). While Schönknecht et al. (1998) found that the channel they examined was 25% more permeable to NO₃⁻ than to Cl⁻, Herdean et al. (2016a) showed that the bestrophin-like channel was more than three times as permeable to Cl⁻ than NO₃⁻. As well as the intrinsic permeability of the channels, the ion fluxes are determined by the anion concentrations in the chloroplast stroma; relevant measurements were made by Wellburn (1985) and Schröppel-Meyer and Kaiser (1988). Wellburn (1985) examined non-aqueously extracted chloroplasts of *Hordeum vulgare*: from the ion chromatography trace the Cl⁻ concentration is much greater than that of NO₃⁻, but no calibration is provided. Schröppel-Meyer and Kaiser (1988) examined aqueously extracted intact and photosynthetically competent chloroplasts of *S. oleracea* growing with 50 mmole Cl⁻ m⁻³.

and 6 mole $\text{NO}_3^- \text{ m}^{-3}$ in the growth medium (Johnson et al. 1957) , and found concentrations of 1 – 6 mole $\text{Cl}^- \text{ m}^{-3}$ and concentrations of 3 – 8 mol $\text{NO}_3^- \text{ m}^{-3}$ in the chloroplasts.

Considering all these data, it would seem that NO_3^- cannot be ruled out as a contributor to anion equilibration across the thylakoid membrane, except in the globally significant situations of oxygenic photolithotrophs are growing without NO_3^- , i.e. with NH_4^+ or organic N was the supplied N source, or symbiotically with diazotrophic bacteria (Raven et al. 1992a,b).

Returning to the larger Cl^- requirements for OEC assembly with a saturation value of about 40 mole m^{-3} (Figure 5C of Vinyard et al., 2019), this is about 20 times the minimum free Cl^- concentration in the lumen needed for saturation of the function of assembled PSII, and 10 times the concentration needed for poising the electrical potential difference and pH difference contributions to the pmf, in *S. oleracea*. While higher concentrations of Cl^- in the thylakoids of non-salinised *S. oleracea* are possible, it is also possible that Cl^- limitation of the assembly of the OEC contributes to slowing the rate recovery from photodamage to PSII (Miyata et al. 2015). A further constraint on the Cl^- availability for OEC assembly is the low intracellular osmolarity (as low as 62 osmole m^{-3}) of some freshwater algae (Raven 2018; see also Tepper et al. 2013), meaning (with an osmotic coefficient of 0.9), an upper limit of 34 mole m^{-3} Cl^- in the thylakoid lumen. While the occurrence of OEC assembly in isolated *S. oleracea* chloroplasts has not been demonstrated, OEC assembly must occur during growth of the freshwater algal cells with very low intracellular osmolarity. A relevant aspect of OEC re-assembly is the role of decreased lumen pH in increasing non-photochemical quenching at high irradiances , decreasing the potential for photodamage to the PSII-OEC and hence the need for thylakoid Cl^- for OEC reassembly, although the luminal Cl^- increases if charge balance is maintained by Cl^- entry from the stroma.

For *Gloeobacter* the relevant Cl^- concentration is that in the growth medium, i.e. 0.508 mole m^{-3} in BG11 (Ritchie 1992), would give very low rates of OEC assembly if the Cl^- dependence is the same as in *S. oleracea* in the presence, as is the case in vivo, of the PsbO subunit (Figure S5 of Vinyard et al. 2019), i.e. 1.3% of the rate with the saturating concentration of 40 mole m^{-3} . Even lower rates would occur if the fixed negative charges on the gran-negative

peptidoglycan that yield an inside-negative electrical potential across the outer membrane (Cayley et al. 2000), yielding lower Cl^- concentrations in the periplasmic space than in the growth medium. Such low rates are compatible with, or perhaps in part a cause of, the low specific growth rate of *Gloeobacter* relative to freshwater cyanobacteria with thylakoids. *Gloeobacter violaceus* PCC 7421 grown on BG11 at 26°C has a specific growth rate of 0.276 d^{-1} at the highest irradiance tested 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (Nguyen et al. 2012), while light-saturated specific growth rate of eight species of freshwater cyanobacteria (with thylakoids) at 25°C range from 0.58 – 0.93 d^{-1} (Lürling et al. 2012). While the comparison of *Gloeobacter* with the other cyanobacteria requires further work (e.g. allowing for cell size), the conclusion that *Gloeobacter* grows less rapidly than freshwater cyanobacteria with thylakoids seems robust.

Overall, the data discussed above are consistent with a role for Cl^- undersaturation in limiting the *in vivo* rate of PSII and OEC synthesis and resynthesis (Raven 2011).

A further chloroplast enzyme in Chlorophyta and Streptophyta is alpha-amylase; while some alpha-amylases are Cl^- dependent, those from the chlorophyte and charophyte algae, and embryophytes, are Cl^- -independent (D'Amico et al. 2000). Coleman (1990) does not list alpha-amylase in a list of Cl^- -binding proteins other than PSII-OEC; the proteins listed are not relevant to photosynthesis. Asparagine synthetase is a plant enzyme that is activated by Cl^- (Rognes 1980) has not been shown to occur in chloroplasts (Gaufichon et al. 2016).

A final potential photosynthetic role of Cl^- is in antiport entry of HCO_3^- at the plasmalemma in the freshwater cyanobacteria *Microcystis aeruginosa* and *Synechocystis* PCC6803 (Chen et al. 2009) and the marine eustigmatophycean microalga *Nannochloropsis oculata* (Merrett et al. 1996), based on dependence of HCO_3^- on external Cl^- at 'beneficial' nutrient concentration. On molecular genetic evidence, $\text{Cl}^-:\text{HCO}_3^-$ antiport could occur in some other marine algae (Van de Waal et al. 2013; Taylor et al. 2017). However, in the freshwater cyanobacterium *Synechococcus* R-2 PCC7942 the active (probably 2 Cl^- per ATP) Cl^- influx in the light is 4 $\text{nmole m}^{-2} \text{s}^{-1}$, while the HCO_3^- influx is 57 $\text{nmole m}^{-2} \text{s}^{-1}$ (Ritchie 1998), so the energetics and magnitude of the Cl^- influx are clearly inadequate to energize an inorganic carbon

concentrating mechanism (CCM) by $\text{Cl}^-:\text{HCO}_3^-$ antiport except with the implausible $\text{Cl}^-:\text{HCO}_3^-$ ratio of 80. For the marine algae the cytosol Cl^- concentration is lower than in seawater, so a 1:1 antiport $\text{Cl}^-:\text{HCO}_3^-$ antiport would export Cl^- from the cytosol. Enlisting the inside-negative electrical potential difference to drive Cl^- efflux and concentrate HCO_3^- in the cytosol would require a $\text{Cl}^-:\text{HCO}_3^-$ of 2 or more.

Conclusions

The status of Cl^- as a micronutrient for glycophytes, primarily through its role at low concentration as a cofactor of the PSII-OEC in photosynthesis, has recently been supplemented by the status of Cl^- at higher external concentrations as a beneficial nutrient. The higher concentration of Cl^- recently shown to be needed for the maximum rate of assembly of PSII OEC in the ontogeny of thylakoids, and the repair of photodamaged thylakoids, could be regarded as a beneficial role of Cl^- , speeding up the rate of OEC assembly relative to the rate achieved with micronutrient concentrations of Cl^- . Further investigation is needed of these two roles of Cl^- , as well as the other functions such as regulating the allocation the transthylakoid PDF into electrical potential difference and pH difference components, and a possible role in HCO_3^- entry in marine photosynthetic organisms.

Acknowledgements

The comments and suggestions of G M Geilfus and two other, anonymous, reviewers have been very helpful in improving the manuscript. Discussions with Jan Anderson, Jim Barber, Mary Beilby, Mary Bisson, Jack Dainty, Geoff Findlay, Alex Hope, Tony Larkum, Enid MacRobbie, Hugh Saddler, F Andrew Smith and N Alan Walker have been very useful. The University of Dundee is a registered Scottish charity, No 015096.

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Table 1

Estimates for glycophytes of the Cl^- concentration needed to saturate function, and assembly/re-assembly, of PSII-OEC, and the Cl^- concentration found around PSII-OEC, in *Spinacia oleracea* and *Gloeobacter violacea*.

Organism	<i>Spinacia oleracea</i>	<i>Gloeobacter violacea</i>
Cl^- concentration needed to saturate Cl^- requirement for PSII-OEC function	2 mole $\text{Cl}^- \text{ m}^{-3}$ (2.2mole Cl^- , converting Cl^- bound to the PSII-OEC to equivalent in solution using PSII-OEC per unit lumen volume.	?
Cl^- concentration needed to saturate Cl^- requirement for PSII-OEC assembly/reassembly	40 mole $\text{Cl}^- \text{ m}^{-3}$	?
Cl^- concentration surrounding OEC	$\leq 5.5 - \leq 86$ mole $\text{Cl}^- \text{ m}^{-3}$ (thylakoid lumen of plants grown in medium with 0.55 mole $\text{Cl}^- \text{ m}^{-3}$; upper limit to allow for Cl^- in stroma as reservoir for parsing pmf across thylakoid membrane into $\Delta\Psi$ and ΔpH)	≤ 0.508 mole $\text{Cl}^- \text{ m}^{-3}$ (growth medium concentration of 0.598 mole Cl^- is higher than in the periplasm between inner and outer cell membranes with fixed negative charge, hence a periplasm-negative Donnan potential partially excluding Cl^-)

For references and details see 'Cl⁻ Requirement for assembly of PSII including the oxygen evolving complex', 'Cl⁻ requirement for function of fully assembled PSII including OEC', and 'Relation of the Cl⁻ requirement for PSII-OEC (re-(assembly and function *in vitro* and to the concentration and distribution of Cl⁻ in chloroplasts and a cyanobacterium.'

Abbreviations: OEC = Oxygen Evolving Complex; ΔpH = pH difference across the thylakoid membrane; pmf = Proton Motive Force across the thylakoid membrane; $\Delta\Psi$ = electrical potential difference across the thylakoid membrane.