Plasticity in light reactions of photosynthesis for energy production and photoprotection

Jeffrey A. Cruz1, Thomas J. Avenson1, Atsuko Kanazawa1, Kenji Takizawa1, Gerald E. Edwards2 and David M. Kramer1,*

1 Institute of Biological Chemistry, Washington State University, Pullman, WA 99164, USA
2 School of Biological Sciences, Washington State University, Pullman, WA 99164, USA

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Abstract

Plant photosynthesis channels some of the most highly reactive intermediates in biology, in a way that captures a large fraction of their energy to power the plant. A viable photosynthetic apparatus must not only be efficient and robust machinery, but also well integrated into the plant’s biochemical and physiological networks. This requires flexibility in its responses to the dramatically changing environmental conditions and biochemical demands. First, the output of the energy-storing light reactions must match the demands of plant metabolism. Second, regulation of the antenna must be flexible to allow responses to diverse challenges that could result in excess light capture and subsequent photoinhibition. Evidence is presented for the interplay of two types of mechanistic flexibility, one that modulates the relative sensitivity of antenna down-regulation to electron flow, and the other, which primarily modulates the output ratio of ATP/NADPH, but also contributes to down-regulation.

Key words: ATP synthase, cyclic electron transfer, proton motive force, qE quenching, water–water cycle.

Photosynthesis and its down-regulation

Light is captured by a set of light-harvesting complexes (LHCs) that funnel light energy into photochemical reaction centres, photosystem (PS) I and PSII (Fig. 1) (see review by Ort and Yocum, 1996). Special subsets of chlorophyll molecules in these photosystems are excited by light energy, allowing electrons on them to be transferred through a series of redox carriers called the electron transfer chain (ETC), beginning from the oxygen evolving complex (OEC) of PSII (which oxidizes H2O and releases O2 and protons) (Diner and Babcock, 1996), through the plastoquinone (PQ) pool, the cytochrome (cyt) b6f complex (Sacksteder et al., 2000) and plastocyanin (PC), and finally through PSI (Malkin, 1996). Electrons from PSI are transferred to ferredoxin (Fd), which, in turn, reduces NADP+ to NADPH via ferredoxin:NADP+ oxidoreductase (FNR) (Knaff, 1996). This linear electron flux (LEF) to NADP+ is coupled to proton release at the OEC, and ‘shuttling’ of protons across the thylakoid membrane by the PQ pool and the Q-cycle at the cyt b6f complex, which establishes an electrochemical potential of protons, or proton motive force (pmf) that drives the synthesis of ATP by chemiosmotic coupling through the chloroplast ATP synthase (McCarty, 1996; Mitchell, 1966).

Following the absorption of photons by chlorophyll, the transfer of excitons to reaction centre chlorophyll and the initiation of electron transfer must be well regulated to prevent ‘over-excitation’ of the photosystems (i.e. more excitation than can be processed by the reaction centres), which favours the formation of highly reactive species and photoinhibition of the photosynthetic machinery (Anderson and Barber, 1996; Kramer and Crofts, 1996). In general, overexcitation of PSII is prevented largely by antenna down-regulation, which dissipates excess excitation energy

* To whom correspondence should be addressed. Fax: +1 509 335 7643. E-mail: dkrramer@wsu.edu

Abbreviations: CEF1, cyclic electron flow around PSI; cyt, cytochrome; ΔpH, transthylakoid pH gradient; Δψ, transthylakoid electric field; DIRK, Dark Interval Relaxation Kinetics; ECS, electrochromic shift; ECSinv, inverted ECS; ECSss, steady-state ECS; ECSf, full extent of the ECS decay; ETC, electron transfer chain; Fd, ferredoxin; FNR, ferredoxin:NADP+ oxidoreductase; gH+, proton conductivity; H+/e−, proton to electron stoichiometry; LEF, linear electron flux; LHCs, light-harvesting complexes; NPQ, non-photochemical exciton quenching; OEC, oxygen evolving complex; PC, plastocyanin; pmf, proton motive force; PQ, plastoquinone; PS, photosystem; qE, energy-dependent quenching; SOD, superoxide dismutase; τECS, ECS decay time; VDE, violaxanthin deepoxidase; WWC, water–water cycle.
as heat. This involves a series of processes, which are collectively termed non-photochemical exciton quenching (NPQ) and typically measured by the quenching of chlorophyll a fluorescence (reviewed by Maxwell and Johnson, 2000). Under most physiological conditions, the major form of NPQ is termed qE, for the ‘quenching’ of light energy in the antenna that is dependent on the ‘energization’ of the thylakoid membrane (reviewed by Horton et al., 1996; Müller et al., 2001; Owens, 1996; Yamamoto and Bassi, 1996). Activation of qE involves at least two processes (Fig. 1): (i) the conversion of the xanthophyll carotenoid violaxanthin to antheraxanthin and zeaxanthin, catalysed by violaxanthin de-epoxidase (VDE) (Eskling et al., 2001); and (ii) protonation of amino acid side-chains on an antenna-associated, chlorophyll binding protein, PsbS (Li et al., 2004). Both of these processes are activated by acidification of the lumen by the ΔpH component of pmf. In this analysis, the proton gradient is considered to equilibrate across the entire continuous lumenal space, i.e. it is not necessary to invoke proton domains to explain

Fig. 1. Primary routes of proton/electron flux and mechanisms of Type I and II flexibility. (A) Energy storage begins with the absorption of light energy (lightning bolts) by light-harvesting complexes (LHC) associated with photosystem (PS) II and I, respectively. Depicted is the linear electron flux (LEF, red arrows) of electrons derived from the oxidation of H₂O at the oxygen evolving complex (OEC) through PSII reducing sequentially plastoquinone (PQ) to a quinol (PQH₂). Bifurcated oxidation of PQH₂ occurs at the cytochrome b₆f complex (b₆f) where half of the electrons are linearly transferred to the NADP⁺/NADPH couple via plastocyanin (PC), PSI, ferredoxin (Fd), and ferredoxin-NADP⁺ oxidoreductase (FNR), and the other half of the electrons will return to the PQH₂ pool. Proton flux (blue arrows) originates from H₂O splitting at the OEC and the cyclic reduction and oxidation of PQ/PQH₂, establishing an electrochemical gradient of protons across the thylakoid membrane (pmf), comprised of pH (ΔpH) and electric field (Δψ) components. Total pmf drives ATP synthesis from ADP and Pₐ, as protons move down their electrochemical gradient through the CF₁-CFₒ ATP synthase. Energy dissipation by qE (purple arrow) is pH-dependent due to the pH-dependent activity of violaxanthin de-epoxidase (VDE), which sequentially reduces violaxanthin (V) to zeaxanthin (Z), and protonation of PsbS. Type II mechanisms (highlighted in red) involve variability in: (i) the response of the antenna to lumen pH, (ii) the conductive properties of the ATP synthase, and (iii) the relative partitioning of pmf into Δψ and ΔpH. Type I mechanisms (B) involve alternate routes of electron transfer at the reducing side of PSI, including the water–water cycle (WWC) and cyclic electron flow around PSI (CEF₁). The WWC uses the same electron transfer pathways as normal LEF except at the reducing side of PSI it reduces O₂ to O₂ which is subsequently detoxified to H₂O. As depicted, four carrier pathways have been proposed for the cycling of electrons from PSI back to the PQ pool (CEF₁): (1) a ferredoxin-PQ oxidoreductase (FQR), (2) a NADPH-PQ oxidoreductase (NDH), (3) oxidation of Fd by a FNR/b₆f super complex, and (4) oxidation of, for example, Fd by a newly discovered haem associated with the stromal side of the b₆f complex.
these data. Thus, pmf not only drives the synthesis of ATP, but is also a key signal for feedback regulation of the light reactions.

The need for modulation of down-regulatory sensitivity (qE-modulation)

$q_E$ sensitivity is defined as the responsiveness of $q_E$ to LEF, because both parameters are readily and frequently measured using chlorophyll fluorescence measurements. Under most conditions, NPQ may be substituted for $q_E$, since $q_E$ makes up a significant fraction of NPQ. If the light reactions behaved in a static fashion, $q_E$ sensitivity would be constant, i.e., $q_E$ would be a continuous function of LEF. However, such rigidity in down-regulation of the photosynthetic apparatus would leave it prone to catastrophic failure (Asada, 1996; Heber and Walker, 1992; Kanazawa and Kramer, 2002). For example, if photosynthesis became limited by the lack of PSI electron acceptors, as might be expected under conditions of metabolic stress, LEF and its proton pumping will be attenuated. A static model would predict a decrease in $q_E$, precisely under the conditions where photoprotection is needed most to prevent the build-up of reduced intermediates, which could lead to ‘acceptor side’ photoinhibition (Anderson et al., 1997). Clearly, a more flexible model must be invoked to account for the response of antenna regulation to the fluctuating physiological status of the plant (Horton, 1989; Horton et al., 1999).

Indeed, such flexibility has been demonstrated in C₃ plants (Avenson et al., 2004; Kanazawa et al., 2001; Kanazawa and Kramer, 2002). Rather than a continuous relationship, as the static model would predict, a series of distinct curves was observed, with $q_E$ becoming increasingly more sensitive to LEF as $[CO_2]$ was lowered (Kanazawa and Kramer, 2002). Physiologically, this is desirable because the availability of PSI electron acceptors, and thus overall LEF, is expected to decrease with decreasing $CO_2$ to maintain reasonable levels of photoprotection, $q_E$ should become more sensitive to LEF.

The need to balance ATP/NADPH ratios

With LEF to NADP⁺, ATP synthesis and NADPH production are coupled, and within a static model the output ratio of ATP to NADPH would be fixed. However, this would work only in a system where consumption of ATP and NADPH occurs at the same fixed ratio; that is, their relative consumption by chloroplast metabolism (including fixation of carbon, nitrogen, phosphorus, and sulphur) and other plastid maintenance processes continuously matches output by energized thylakoids. Yet each individual process imposes a different demand for ATP/NADPH. Again, this leaves a static model susceptible to failure in cases where differential flux is required to respond to the changing demands on the chloroplast. If shortage of a single metabolite decreases relative metabolic flux through the pathway that fixes it, then the demand for ATP versus NADPH may change. Also, the resulting mismatch between production and consumption ratios would create ‘back pressure’ on the light reactions from excess product (ATP or NADPH) or lack of substrate (ADP+Pᵢ, NADP⁺), sensitizing the photosynthetic apparatus to photoinhibition. Therefore, contrary to a static model, a certain measure of flexibility in the LEF output ratio is expected in order to compensate for changes in demand.

The need for balancing mechanisms is further exemplified by potential mismatch between the LEF-dependent output and the demand of CO₂ fixation. If one considers only LEF, the ATP/NADPH ratio is defined by the proton coupling stoichiometries for the ETC (H⁺/e⁻) and that for ATP synthesis (H⁺/ATP, termed $n$) (Allen, 2002; Kramer et al., 2003). There is strong evidence that $H^+/e^-$ for LEF remains at 3 under physiological conditions (Sacksteder et al., 2000). New information about the structure and mechanism of the ATP synthase implies that $n$ is likely to be 4.67 (reviewed by Allen, 2002; Kramer et al., 2003). With these stoichiometries, ATP/NADPH should be 1.3, which as discussed later, would provide insufficient ATP to support $CO_2$ fixation in C₃ plants. Without flexible responses, even larger supply–demand mismatch would occur in species using modified CO₂ fixation strategies, for example, in plants with some types of C₄ photosynthesis.

As discussed in (Kanazawa and Kramer, 2002) and extended here, there are several models that could, together or separately, account for $q_E$ modulation (Fig. 1), some of which will also affect the output ratio of ATP/NADPH, and these were termed Type I flexibility mechanisms. Other mechanisms will have no effect on the ATP/NADPH output ratio, and these were termed Type II flexibility mechanisms. This distinction is critical for understanding the relative roles of these processes.

Type I: Flexibility mechanisms affecting the ATP/NADPH ratio

In accordance with the general model for electron and proton transfer, any process increasing the rate of proton translocation into the lumen will tend to activate $q_E$ by increasing pmf. If such processes supplement proton flux supplied by LEF, they will increase $q_E$ sensitivity as it has been defined here. They will also tend to increase the ATP/NADPH ratio, because the resulting increase in proton flux will drive more ATP synthesis (Fig. 2), without a net increase in the reduction of NADP⁺. There have been several proposals for this type of mechanism.

Changes in the $H^+/e^-$ ratio for LEF

The Q-cycle is a catalytic mechanism which couples electron transfer through the cyt $b_6/f$ complex to the
translocation of protons from the stroma to the lumen (reviewed by Kurisu et al., 2003; Sacksteder et al., 2000). For each electron transferred through LEF, one proton is released into the lumen from water oxidation, and one proton is taken up during PQ reduction at the QB site of PSII and released when PQH2 is oxidized at the Qo site of the cyt b\textsubscript{6}f complex. An additional proton is translocated by the Q-cycle, making the overall H+/e\textsuperscript{+}/C\textsubscript{255} stoichiometry for LEF 3.

Although several authors have proposed that the Q-cycle is facultative (reviewed by Berry and Rumberg, 1999; Cornic et al., 2000; Ivanov, 1993; Kramer and Crofts, 1993; Sacksteder et al., 2000), disengaging it (see review by Sacksteder et al., 2000) would lower the H+/e\textsuperscript{+} ratio to 2, thereby lowering the pmf generated by LEF and, consequently, the ATP/NADPH output ratio and q\textsubscript{E} sensitivity.

On the other hand, in vitro mechanistic studies of the cyt b\textsubscript{6}f complex indicated that the Q-cycle was very likely obligatory (Kramer and Crofts, 1993; Rich, 1988). Furthermore, comparisons of estimated fluxes of protons with LEF and with electron flux through the cyt b\textsubscript{6}f complex in vivo suggested a constant H+/e\textsuperscript{+} ratio from low to saturating light intensities (Sacksteder et al., 2000). It was concluded that the Q-cycle is probably continuously engaged under normal, non-stressed photosynthetic conditions.

These arguments are bolstered by recent structural studies of the mitochondrial cyt bc\textsubscript{1} complexes (Zhang et al., 1998) (which are homologous to the chloroplast cyt b\textsubscript{6}f complex) and cyt b\textsubscript{6}f complexes (Kurisu et al., 2003; Stroebel et al., 2003) which have led to proposals that the ‘Rieske’ iron-sulphur protein ‘gates’ electron transfer by undergoing large-scale conformational changes during catalysis, essentially forcing the complex to shuttle protons via the Q-cycle (reviewed by Roberts et al., 2001; Zhang et al., 1998). It was concluded that there is a strong

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**Fig. 2.** Relationships between energy-transduction and q\textsubscript{E} sensitivity. As determined by its sensitive components, PsbS and VDE, q\textsubscript{E} (and thus NPQ) will be a function of luminal pH. As pH drops from \(-6.5\) to \(-5.8\), q\textsubscript{E} will continuously increase to saturation. If the steady-state pH of the stroma is constant, then q\textsubscript{E} will be a function of ΔpH. Therefore, factors affecting the extent to which ΔpH forms will influence q\textsubscript{E} induction. Depicted are simplified schematics of chloroplastic energy transduction with proton and electron fluxes indicated in blue and red, respectively. The table indicates relative changes in ATP output, NADPH output, pmf, and ΔpH (NC indicates no change). The pmf (and by extension ΔpH) will depend, in part, on the steady-state rate of proton accumulation. Supplementing the rate of proton accumulation through CEF1 (A) or WWC (B) will increase pmf, the rate of proton efflux and, consequentially, the rate of ATP synthesis. However, since electrons on the reducing side of PSI return to the PQ/PQH\textsubscript{2} pool via CEF1 or to water via WWC, NADPH output does not change. Since at steady-state, the rate of efflux will equal the rate of accumulation, pmf will also depend on how conductive the membranes are to proton flux. Thus, decreasing conductivity (C) will require an increase in pmf to balance proton accumulation with efflux. Since the steady-state rate of proton flux does not change in proportion to electron flux to NADPH, the relative outputs of ATP and NADPH remain constant. Finally, if, under most conditions the ΔpH partition is approximately 50% of pmf, collapsing the electric field component through counterion movements (D) would require an increase in ΔpH to sustain steady-state proton flux. In all cases, the sensitivity of q\textsubscript{E} to LEF (q\textsubscript{E}/LEF) increases.

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<td>(C) ↓ gH\textsuperscript{+}</td>
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mechanistic basis for a constant $H^+/e^-$ ratio at the cyt $b_{6f}$ complex and that differential engagement of the Q-cycle probably does not account for ATP/NADPH balancing or for variable sensitivities of down-regulatory processes.

Alternate electron transfer cycles
Various light-driven cyclic electron transfer pathways have been proposed to translocate protons across the thylakoid and thus drive ATP production or initiate $q_E$ in the absence of NADP$^+$ reduction. Two of the pathways, cyclic electron flow around PSI (CEF1) and the water-water cycle (WWC), have gained support in recent years and are discussed here.

Cyclic electron flux around PSI
CEF1 bypasses the photosynthetic Z-scheme by involving only one of the two photosystems, PSI. Light excites PSI, resulting in reduction of its FeS centres and oxidation of its primary chlorophyll donor, $P_{700}$. Just as in LEF, the oxidized $P_{700^{+}}$ is reduced by electrons from the PQ pool, via the cyt $b_{6f}$ complex and PC (Bendall and Manasse, 1995). Electrons on the reducing side of PSI eventually reduce PQ, completing the cycle. There is no net reduction of Fd or NADP$^+$ but flux of electrons through the cycle will translocate protons to the lumen, resulting in pmf, which can drive ATP synthesis and activate $q_E$.

At least four pathways have been proposed to link the reducing side of PSI with the PQ pool (Fig. 1B, paths 1–4). First, a linkage may occur via a ferredoxin-PQ oxidoreductase (FQR) (path 1), a pathway that has been shown to be sensitive to antimycin A (Bendall and Manasse, 1995). Recently, Shikanai and coworkers (Munekage et al., 2002) identified an Arabidopsis mutant, pgr5, lacking antimycin A-sensitive Fd reduction of the PQ pool, preliminary evidence that the PGR5 gene product may be involved in FQR-mediated CEF1. Second, an enzyme homologous to complex I of mitochondria and bacteria (Edwards and FQR-mediated CEF1. Second, an enzyme homologous to evidence that the PGR5 gene product may be involved in A-sensitive Fd reduction of the PQ pool, preliminary

In vivo estimates of CEF1 rates
Care must be taken before accepting in vitro rates as reflecting those that can occur in vivo, especially since CEF1 is known to be well-regulated and a measurable change in its relative rate may appear only under special conditions. There is strong evidence for participation of CEF1 in ATP synthesis in green algae (e.g. Chlamydomonas) and cyanobacteria (Depege et al., 2003; Finazzi et al., 2002), as well as in C4 plant bundle sheath chloroplasts (Kubicki et al., 1996). However, the situation in C3 vascular plants is clearly unresolved, with the bulk of the evidence pointing to only minor contributions of CEF1 under steady-state conditions.

Several groups have estimated CEF1 rates in C3 vascular plants under steady-state conditions. These measurements are difficult because the electrons flow in a cycle, and no readily measurable, stable products are formed. One approach to indicate the activation of CEF1 is to estimate steady-state transthylakoid $\Delta pH$ using pH-indicator dyes, or the onset of $q_E$ with LEF. The argument is that at a given LEF, CEF1 will increase pmf, thereby decreasing lumen pH, and thus increasing $q_E$ (Cornic and Briantais, 1991; Heber, 2002). However, it is argued below that such effects can equally result from the engagement of Type II mechanisms, which have been shown to alter the relationship between LEF and steady-state pmf, as well as between pmf and $q_E$.

Most commonly, the relative fluxes of electrons through different parts of the electron transfer chain are compared to estimate the relative engagements of LEF and CEF1. In steady-state LEF, the rates of electron transfer through PSII should equal that through PSI (Genty et al., 1990; Klughammer and Schreiber, 1994; Kramer and Crofts, 1996; Ort and Baker, 2002) or the cyt $b_{6f}$ complex (Klughammer and Schreiber, 1994; Sacksteder and Kramer, 2000). The engagement of CEF1 should increase electron flux through PSI over that through PSII. Likewise, the ratio of proton translocation to LEF should increase with the engagement of CEF1 (Sacksteder et al., 2000). The fraction of overall photosynthetic energy storage attributable to PSI will change with the engagement of CEF1 (Herbert et al., 1990). Unfortunately, each of these techniques measures CEF1 only as a fraction of LEF and is only sensitive to changes in the ratio of CEF1:LEF (Bendall and Manasse, 1995; Kramer and Crofts, 1996), and low rates are not readily detected. A number of studies using such assays have found little evidence for changes in the fractional turnover of
CEF1 in steady states as conditions were altered, and thus the general consensus appears to be that, in C₃ vascular plants, CEF1 is either negligible or a fairly constant fraction of steady-state LEF (Genty et al., 1990; Herbert et al., 1990; Klughammer and Schreiber, 1994; Kramer and Crofts, 1996; Ort and Baker, 2002; Sacksteder and Kramer, 2000). On the other hand, in more recent papers other groups have reported substantial rates of CEF1 (15–100% of LEF) during photosynthetic induction (Joliot and Joliot, 2002) or anaerobiosis (Joet et al., 2001) or under high light, low temperature conditions (Clarke and Johnson, 2001) or drought stress (Golding and Johnson, 2003).

The water-water cycle (WWC)

In the WWC, electrons extracted from H₂O by PSII are transferred through the ETC to PSI, where O₂ acts as the terminal acceptor forming superoxide (O₂⁻, Fig. 1B, WWC). O₂⁻ is dismutated to hydrogen peroxide and dioxygen, a reaction that is catalysed by superoxide dismutase (SOD), and the hydrogen peroxide is reduced to H₂O by ascorbate peroxidase, thereby completing the cycle. Since O₂ is reduced more slowly by Fd than FNR, the concentrations of NADP⁺ are low. Although the WWC suggests that, at most, WWC operates at 10% of LEF of C₃ plants (Backhausen et al., 2000; Heber, 2002) and thus it is not surprising that issues concerning the relative contribution of the WWC to overall electron transfer have not yet been resolved. Much of the literature (Foyer and Noctor, 2000; Heber, 2002) suggests that, in vivo, the WWC is a relatively minor contributor to LEF. An estimate based on a survey of more recent work (Badger et al., 2000) suggests that, at most, WWC operates at 10% of LEF of C₃ photosynthesis, even under conditions of extreme stress. Moreover, other have observed little to no WWC under conditions that should favour NADPH accumulation, such as lowered RUBISCO levels (Ruuska et al., 2000) or low temperatures (Clarke and Johnson, 2001).

By contrast, higher flux capacities for WWC have been observed in isolated chloroplasts of C₃ plants (Backhausen et al., 2000; Badger et al., 2000), suggesting that conditions which favour WWC may not be simple to produce in vivo. However, there is evidence for the active engagement of the WWC in conjunction with CEF1 in rice leaves, during photosynthetic induction (Makino et al., 2002). It was suggested that the supplemental proton flux was required to generate additional ATP for the initiation of the Calvin–Benson cycle from a dark-adapted state. Furthermore, suppressed expression of thylakoid-associated Cu/Zn-SOD in Arabidopsis suppressed photosynthetic activity and growth, which is consistent with the need for detoxification of O₂ generated by photosynthesis (Rizhsky et al., 2003).

While this observation supports the presence of the WWC in vivo, it does not necessarily support a role for the WWC supplementing pmf during steady-state photosynthesis.

Type II: Flexibility without altering ATP/NADPH output ratio

While Type I mechanisms could be modulators of qₑ, effective engagement would require them to comprise a large fraction of total electron flux, leading to mismatch in supply and demand for ATP and NADPH. By contrast, Type II mechanisms, as depicted in Fig. 1, allow the regulation of qₑ sensitivity without perturbing the ATP/NADPH ratio.

Alteration of qₑ response to lumen pH

One way to alter qₑ sensitivity would be to change the response capacity of qₑ to lumen pH. Over developmental time-scales, the differential accumulation of antenna and xanthophyll components has been shown to alter qₑ sensitivity (Demmig-Adams and Adams III, 1996). Hypothetically, more dynamic changes in qₑ sensitivity could occur though alterations in the pH response of the molecular components of qₑ. For example, covalent modification of VDE or PsbS could shift either pH dependence of VDE or pKₐ of protonatable groups on PsbS, respectively. Alternatively, components in the membrane could be modified, affecting the propensity of LHCs to aggregate or associate with the xanthophyll components, processes which have been linked to exciton dissipation by qₑ (reviewed by Horton et al., 1996). The predicted outcome, in all cases, would be a range of sensitivities of qₑ to ΔpH. However, in tobacco, a constant relationship was observed between qₑ and estimates of light-driven pmf changes, over conditions where qₑ sensitivity was substantially altered by changing CO₂ levels (Kanazawa and Kramer, 2002), while under extreme acceptor limiting conditions qₑ was a continuous function of ΔpH (Avenson et al., 2004). These observations suggest that a constant relationship exists between lumen pH and qₑ and that modifications in antenna response do not account for short-term changes in qₑ sensitivity, under these conditions.

The importance of pmf composition for modulating qₑ response

Since qₑ is triggered by the ΔpH, but not the Δψ (electric field) component of thylakoid pmf, one way to change qₑ sensitivity would be to alter the manner in which pmf is stored. The chemiosmotic mechanism, first described by Peter Mitchell, states that pmf is thermodynamically composed of the sum of the ΔpH and Δψ potentials (Mitchell, 1966). Many of the earlier characterizations of pmf were performed by monitoring ATP synthesis in intact thylakoids.
as a function of ΔpH produced by pH jump and/or by measuring ΔpH-dependent uptake of radiolabelled or fluorescent amines (Davenport and McCarty, 1986; Junesch and Grüber, 1985; Schuldiner et al., 1972). While useful for defining the thresholds of activation and other energetic parameters, these studies ignored and actively suppressed the Δψ component of pmf through the use of uncouplers and/or relatively high concentrations of counterions. Direct measurements of Δψ, made using salt-filled microelectrodes (Vredenberg and Tonk, 1975), helped to popularize the notion that it contributed little or negligibly to steady-state pmf, despite changes observed in vivo in the electrochromic shift (ECS) (Finazzi and Rappaport, 1998; Joliot and Joliot, 1989; Sacksteder et al., 2000) or measurement of Δψ-dependent ATP synthesis (Hangarter and Good, 1982; Junesch and Grüber, 1991), which suggested the contrary. Lately, it has been argued that under permissive conditions, it is unlikely that ΔpH solely comprises pmf (reviewed in Cruz et al., 2001; Kramer et al., 1999). In essence, a ΔpH requirement of 2–3 to activate ATP synthesis (Kramer and Crofts, 1989) yields a lumen pH that is inconsistent with the pH sensitivities of PSII and PC and with the pH-dependent rates of VDE and cyt b6f, observed in vivo.

In much of the authors’ recent work, the ECS has been exploited as an endogenous probe for changes in thylakoid Δψ during light-to-dark transitions (Avenson et al., 2004; Cruz et al., 2001; Kanazawa and Kramer, 2002; Sacksteder et al., 2000). The relevance of the ECS to pmf was first reported by Junge and Witt (1968). ECS refers to a Δψ-induced ‘shift’ in the absorption spectrum of pigments (i.e. chlorophyll and carotenoids) embedded in the thylakoid membrane. The peak of the difference spectrum occurs at 515–520 nm and has been shown to be a linear indicator of the strength of the thylakoid Δψ (Witt and Zickler, 1973). One particular advantage of using the ECS is that it is non-invasive, allowing in vivo measurements on intact leaves. Generally, two techniques were employed when using ECS to probe pmf, both of which are variations of Dark Interval Relaxation Kinetic (DIRK) analysis (Sacksteder and Kramer, 2000). The DIRK technique uses brief (<500 ms) dark intervals to create reproducible perturbations in steady-state electron and proton fluxes. The initial rate of the ECS decay has been attributed to proton flux through the ATP synthase (Kramer and Crofts, 1989), and initial rates have been argued to reflect steady-state LEF or proton flux linearly (Sacksteder et al., 2000). From steady-state conditions, these rapid ECS decay kinetic traces are fit to mono-exponential decays (Fig. 3A), giving decay times (τECS) inversely proportional to proton conductivity (gH+) across the membrane (i.e. predominantly through the ATP synthase). The full extent of the decay (ECS) should be proportional to the light-induced, steady-state pmf (Avenson et al., 2004; Kanazawa and Kramer, 2002; Sacksteder and Kramer, 2000).
With longer dark intervals (i.e. minutes), following the rapid, initial decay the ECS relaxes to a dark level (Fig. 3B). Determination of the dark baseline allows tentative separation of Δψ- and ΔpH-driven decays of pmf (Cruz et al., 2001). Since the ECS is a linear indicator of Δψ, the extent of its decay from steady-state to baseline (ECS_{ss}) should be proportional to the light-induced Δψ. However, the ECS continues to decay below baseline, indicating ‘inversion’ of the electric field with respect to steady-state levels. This effect arises from the continued efflux of protons from the lumen, driven by ΔpH (and beyond that driven by Δψ alone). Since the ECS decay should continue until ΔpH essentially reaches equilibrium with the inverted Δψ, the extent to which ECS drops below baseline (ECS_{inv}) should be proportional to the light-induced ΔpH, at least under appropriate conditions (Cruz et al., 2001). The sum of the amplitudes for ECS_{ss} and ECS_{inv} (i.e. ECS_{t}) should be proportional to the light-induced pmf. Using this ‘partition analysis’, the fraction of pmf stored as Δψ (or ΔpH) may be expressed as the ECS_{ss} (or ECS_{inv}) divided by ECS_{t}.

**Modulation of pmf by proton conductivity**

Above the threshold of activation for ATP synthesis, a linear relationship exists between pmf and proton efflux until the ATP synthase pool reaches the maximum rate of turnover (Hangarter and Good, 1982; Junesch and Gräber, 1985; Kramer and Crofts, 1989). The slope of this relationship, g_{H+}, is a measure of the response of transthylakoid efflux to changes in driving force (Fig. 3C). It is important to note that changing g_{H+} will not change the steady-state rate of H+ efflux at a given LEF, but it will change the pmf required to sustain this rate (Fig. 2). Since qE should be a continuous function of pmf, provided that ΔpH is a constant fraction of pmf, qE sensitivity could be modulated by changes in g_{H+}. Indeed, correlative changes in qE sensitivity and g_{H+} have been observed in tobacco. Through DIRK analysis of the ECS, large decreases in g_{H+} were observed as CO2 levels were lowered from 2000 to nearly 0 ppm with coincident increases in qE sensitivity (Kanazawa et al., 2001; Kanazawa and Kramer, 2002). Further analysis suggested that essentially all changes in qE sensitivity could be explained by modulation of g_{H+} alone.

**Variable partitioning of pmf**

Variable pmf partitioning allows a more flexible relationship to exist between steady-state ATP synthesis and qE. For example, increasing the fraction ΔpH at a given pmf will increase the qE response without affecting the ATP synthetic rate (Fig. 2). Such effects would be important in cases where the physiological status of the leaf requires qE to be large, but LEF is too low to support a large enough pmf. Variable pmf partitioning will lead to discontinuities in relationships between pmf and qE as well as LEF and qE, and recently, this effect was observed in tobacco leaves under low O2 and low CO2 (Avenson et al., 2004). Partition analysis of the ECS kinetics indicated a relative increase in the fraction of pmf stored as ΔpH, suggesting that variable partitioning of pmf was responsible for enhancing the sensitivity of qE. Furthermore, decreases in g_{H+} were observed as well, suggesting that variable pmf partitioning and g_{H+} modulation may act in concert to increase qE sensitivity.

Previous work (Cruz et al., 2001) suggested that, if the thylakoid membrane contains only passive channels to allow counterions to dissipate Δψ, the extent to which pmf will be stored as Δψ and ΔpH will depend largely on the proton buffering capacity of the lumen and the concentration of counterions. The collective work of several groups suggest that buffering capacity will depend on the concentration of fixed buffering groups (Ewy and Dilley, 2000; Junge et al., 1979; van Kooten et al., 1986) and that in vivo this is unlikely to change to a large extent (Junge et al., 1979; van Kooten et al., 1986). Thus, postulating the existence of active ion transport mechanisms in the thylakoid (e.g. coporters, antiporters, ion-transporting pumps), the most likely mechanism to alter pmf partitioning is the concentration of counterions, and it is not difficult to imagine that these could be regulated in some way in vivo to adjust qE sensitivity.

**An integrated model for photosynthetic flexibility**

As discussed earlier, without Type I flexibility, LEF would yield a ratio of 1.3 ATP/NADPH (Allen, 2002). As estimated from the combined energy requirements for CO2 fixation under photorespiring conditions and nitrate assimilation to glutamate, a ratio of ~1.43 ATP/NADPH (Edwards and Walker, 1983) would be needed through photochemistry in the chloroplast, yielding a deficit of about 0.13 ATP per NADPH. The net contributions of Type I mechanisms to increasing relative ATP output will depend on their H+/e- coupling stoichiometries. The WWC, which uses essentially the same reactions as LEF, probably produces an H+/e- of 3. In the case of CEF1, the H+/e- will be partly determined by the pathway for PQ reduction, which is not well understood, especially in C3 vascular plants. Conservative estimates, based on various models, place the lower-upper bounds for H+/e- between 2 and 4. Using these values, the WWC would need to run at a rate of about 12%, or CEF1 between 18% and 9%, that of LEF to fill the ATP deficit between the light reactions and downstream metabolism. While current estimates of WWC and CEF1 capacity are close to and in some cases exceed this requirement, as noted by Makino et al. (2002), to balance output it is probable that they run in concert, possibly with other electron sinks such as the malate valve (Fridlyand et al., 1998) or chlororespiration (Cardol et al., 2003) or with mitochondrial respiration (Noctor and Foyer, 1998). Although this contribution may seem small, the impact of Type
I mechanisms on C₃ photosynthesis might be quite significant. Indeed, *Arabidopsis* double mutants for PGR5 and NDH show substantial decreases in capacity for NPQ, probably due to the observed decreases in LEF (and subsequent pmf formation), created by an uncorrected imbalance in the supply and demand ratios for ATP and NADPH, leading to metabolic congestion (Munekage *et al.*, 2004). Thus, these data are consistent with the view presented here, in that the relatively small contributions indirectly affect proton translocation.

Assignment of this specific role to Type I mechanisms is supported by the observed evolutionary adaptations of the photosynthetic apparatus. For example, if carbon fixation is looked at specifically, there is considerable evidence that steady-state CEF1 is small in C₃ vascular plants, where the expected output balance of LEF is close to, but does not precisely match, biochemical demand. The exception is induction of photosynthesis when priming of the carbon cycle might also require additional ATP (Poolman *et al.*, 2003), as inferred from high CEF1 rates reported by Joliot and Joliot (2002) and Cardol *et al.* (Cardol *et al.*, 2003). However, some C₄ plants require a ratio of ATP/NADPH of 5:2. In species like maize and sorghum, mesophyll chloroplasts generate most, if not all, of the reductive power while bundle sheath chloroplasts function to produce ATP, likely via a CEF1 pathway (Edwards and Walker, 1983; Ivanov *et al.*, 2001). Similarly, cyanobacteria and green algae, for which robust CEF1 is well-documented, need a higher PSI/PSII ratio to fix CO₂ than do C₃ vascular plants, in part because they possess ATP-driven CO₂ concentrating mechanisms (Ogawa and Kaplan, 2003; Turpin and Bruce, 1990).

However, extremes in acceptor limitation (namely O₂ and CO₂) can induce rather dramatic (up to ~6-fold) changes in the sensitivity of qₑ to LEF (Avenson *et al.*, 2004; Kanazawa and Kramer, 2002). To account for such a robust response by themselves, CEF1 or WWC would have to occur at rates about 5-fold larger than that of LEF. Even the largest estimates of CEF1 and WWC capacity fall far short of this. Thus, it is argued that CEF1 cannot by itself account for the observed large changes in qₑ sensitivity to LEF, and that contributions from Type II mechanisms are therefore necessary.

It is important to keep in mind that all protons translocated into the lumen, either via LEF or a cyclic process, pass back across the thylakoid membrane, mainly through the ATP synthase. This implies that any increase in pmf generation by CEF1 or WWC (Type I mechanisms) will result in additional ATP synthesis, as long as uncoupling or ‘slip’ in the ATP synthase reaction is negligible, as shown by Junge and coworkers (Groth and Junge, 1993). Moreover, the augmented pmf should also increase regulatory sensitivity via qₑ, as long as static pmf partitioning etc., accompany changes in Type I mechanisms. However, as discussed previously, large increases in Type I flux could result in an excessive increase in the supply ratio of ATP/NADPH. While, in principle, the problem could be solved by dissipating ATP non-productively in a futile cycle, no such futile ATPase activity has yet been identified. By contrast, Type II mechanisms increase regulatory sensitivity without altering ATP/NADPH output ratios, and rather strong evidence has been presented that when an increase in qₑ sensitivity is *all* that is needed, for example, under LEF-limited conditions, Type II mechanisms are activated.

These observations form the basis of an integrated model, where Type I mechanisms (CEF1 and WWC) provide plasticity at the level of ATP/NADPH, no doubt also impacting qₑ sensitivity, while Type II mechanisms play a more significant role in adjusting qₑ sensitivity without altering the ATP/NADPH output ratio. Furthermore, these mechanisms are not mutually exclusive and may act separately or in parallel to modulate qₑ sensitivity.

One implication of the integrated model is that, for both types of mechanisms, regulation will be mediated through metabolic pools of the reactants or products of the light reactions. For example, for Type I mechanisms to operate effectively, the levels of their induction would need to be dictated by fluctuations in ATP consumption relative to NADPH. One possible model is that induction will be sensitive to the redox poise of the NADP⁺/NADPH couple or the intermediate carriers of the ETC. Indeed, there is strong evidence that CEF1 must be properly redox poised to operate in vascular plants (Bendall and Manasse, 1995; Joet *et al.*, 2001; Joliot and Joliot, 2002). Moreover, in *Chlamydomonas*, state transitions, which have a large effect on excitation energy distribution between PSI and PSII (Delosme *et al.*, 1996), appear to trigger CEF1 (Depege *et al.*, 2003; Finazzi *et al.*, 2002), and this process is regulated by the phosphorylation of antenna complexes and is initiated by changes in the redox state of the PQ pool (reviewed in Allen and Forsberg, 2001; Haldrup *et al.*, 2001).

Similarly, engagement of Type II mechanisms would be expected to be linked to proportionate changes in overall flux of NADPH and ATP. In fact, these mechanisms appear to be induced under conditions where LEF and ATP synthesis are limited by the availability of electron acceptors and Pᵢ acceptors (e.g. low CO₂). This fits adequately with a proposed model where gₜ⁺ is modulated by stromal Pᵢ levels (Kanazawa and Kramer, 2002). Pᵢ levels in chloroplasts are typically 20 mM and may drop to 10 mM during photosynthesis (Furbank *et al.*, 1987; Usuda, 1988). Under stress conditions Pᵢ concentrations may dip as low as 2 mM (Sharkey and Vanderveer, 1989). Since 1–2 mM of stromal Pᵢ is inactive (Furbank *et al.*, 1987; Robinson and Giersch, 1987), and not available for photophosphorylation, active Pᵢ concentration could be close to the reported Kᵢm, 0.6 mM (Selman and Selman-Reimer, 1981). Indeed, decreases in the gₜ⁺ of spinach and *Arabidopsis* leaf discs have been observed when pretreated with mannose, which has been
shown to act in vivo as a phosphate sink. Commensurate increases in pmf and Ψp were also observed, and all effects were reversed with phosphate replenishment (K Takizawa, unpublished data). An alternative possibility is that ATP synthase activity is modulated allosterically. Interestingly, evidence has been presented that a 14-3-3 protein can bind a phosphorylated chloroplast ATP synthase and that binding partially inhibits turnover (Bunney et al., 2001).

References


