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A model for regulation of the light reactions based on *in vivo*, steady-state observations of both electron and proton fluxes in higher plants.

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Introduction

Methods have been available for some time to probe photosynthetic electron transfer reactions in intact plants. However, the proton fluxes that accompany these electron transfer reactions have not been studied. Because the ΔpH component of the proton motive force is the key intermediate in the down regulation of photosynthesis, probing the proton fluxes is essential for understanding the responses of plants to the environment. We have developed a series of instruments and techniques, with which to estimate in intact plants the fluxes of protons through the photosynthetic apparatus, the extent of *pmf* and its partitioning into ΔpH and Δψ components. This work is an overview of results obtained with the tools and techniques described in (Kramer and Sacksteder, 1998; Kramer, et al., 1999; Sacksteder, et al., 2000; Sacksteder, et al., 2000; Sacksteder and Kramer, 2000; Cruz, et al., 2001).

Materials and Methods.

Tobacco and *Arabidopsis* leaves or disc were prepared as described earlier (Kramer and Sacksteder, 1998; Sacksteder, et al., 2000). The non-focussing optics spectrophotometer was modified to allow the simultaneous collection of data from fours separate wavelengths, using an approach similar to that described in (Klughammer, et al., 1990). This instrument will be described elsewhere.

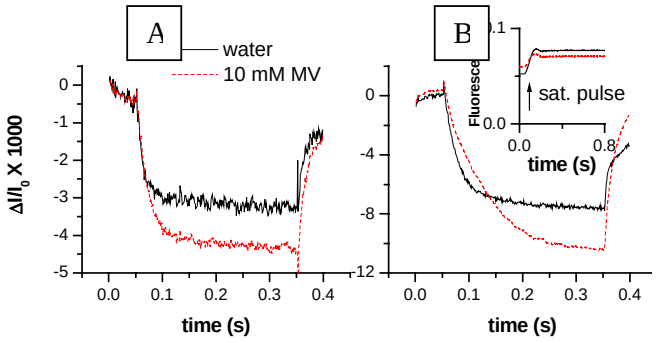


Figure 1. Absorbance changes at 820 nm (A) and 520 nm (B)during a 300 ms dark interval punctuating steady-state 550 μE m^{-2} s^{-1} red light in tobacco leaf discs infiltrated with water (solid lines) or 10 mM MV (dotted lines). Inset: saturation-pulse induced fluorescence yield changes under the same conditions.

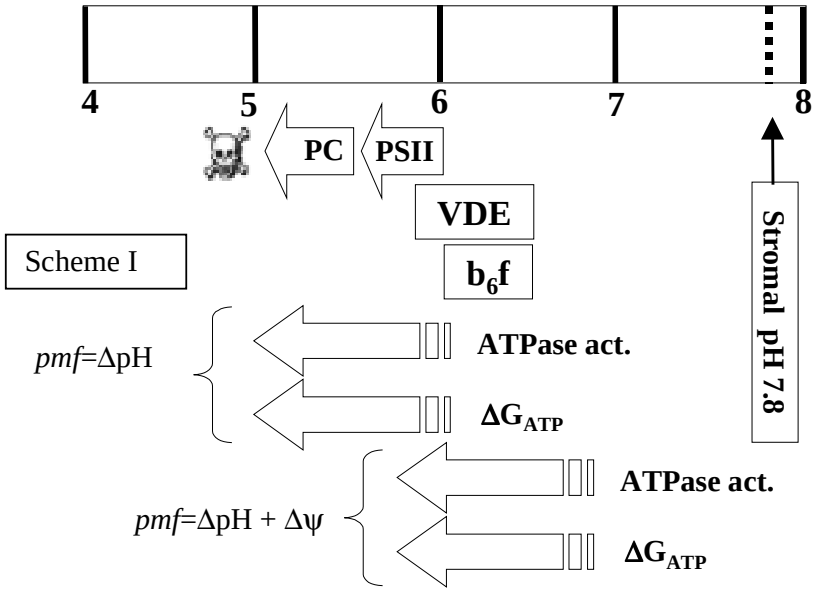
Results and Discussion.

Is the Q-cycle always engaged? In previous work (Sacksteder, et al., 2000), we have shown using analysis of dark-interval relaxation kinetics (DIRK) (Sacksteder and Kramer, 2000) that

the H^+/e^- ratio for non-stressed photosynthesis *in vivo* is constant from low to saturating light intensity, in apparent contradiction with earlier results using pH-indicators *in vitro* (Berry and Rumberg, 1999). The pH-probe assays require a complete abolition of the $\Delta\psi$ component of *pmf* (Cruz, et al., 2001). We suggest that the apparent decrease in H^+/e^- at high light intensities observed with the pH probe assays may be is due to a light intensity-dependent buildup of K^+ gradient, and subsequent buildup of $\Delta\psi$. The residual $\Delta\psi$ will lead to a slowing of the decay of ΔpH upon light off. We found that 1) addition of membrane-permeable buffers to thylakoid suspensions used to increase the pH-probe signals could increase lumen proton buffering capacity (β) to the point where $\Delta\psi$ is sustained even in the presence of valinomycin (not shown), suggesting a decreases in H^+/e^-.

Figure 1 shows that, at constant light intensity, ϕ_{II} (F_V/F_M') and initial decay slopes for the ECS ($DIRK_{ECS}$) were 50% lower in tobacco leaf discs infiltrated with 10 mM methyl viologen (MV), compared to those infiltrated with water. The ratio of $\phi_{II}/DIRK_{ECS}$ remained constant, despite a 2-fold difference in electron transfer rates (see below), indicating a constant H^+/e^- ratio, even under these extreme conditions, as in (Sacksteder, et al., 2000).

How acidic is the lumen? As discussed in detail in (Kramer, et al., 1999), lumen pH plays a central role in feedback regulation of the photosynthetic apparatus. However, to date quantitative relationships between lumen pH and downregulatory processes, between electron transfer and lumen pH, or between proton motive force and lumen pH have yet to emerge. A recent review by Kramer et al. (1999) attempted to address these issues by comparing the pH and *pmf*-dependence of photosynthetic processes and enzymes, as illustrated in Scheme I. The pH-dependence of the oxygen evolving complex on PS II and plastocyanin suggested that the pH of the lumen remained above about 6. In agreement with this, *in vivo* steady-state turnover rates of the quinol oxidase site of the cyt b_6f complex are only attained *in vivo* when the pH is above about 6. Also, violaxanthin deepoxidase (VDE), which controls the conversion of violaxanthin to antheraxanthin and zeaxanthin, and is thus important for down-regulation of photosynthesis, is essentially inactive at pH above about 6.5 and fully active at pH below about 5.8. The xanthophyll cycle is modulated over the entire photosynthetic light-saturation curve in vivo, suggesting that lumen pH remains above about 5.8.



Scheme I

Lumen pH estimated by *in situ* probes

If certain assumptions are made, lumen pH can also be estimated by the thermodynamics and kinetics of the ATP synthase reaction (Kramer, et al., 1999). If all *pmf* is held in the form of ΔpH, as suggested by some *in vitro* experiments (see review in (Cruz, et al., 2001), and if one sets the pH of the stroma at 7.8, and assumes a stoichiometry of 4 H^+/ATP (see review in Kramer, et al., 1999), then the upper lumen pH required to support observed ΔG_{ATP} levels

(also assuming near equilibrium between *pmf* and ΔG_{ATP}) would be about 5.9. Because the chloroplast system in the steady-state is likely far from equilibrium, the required lumen pH would likely be considerably more acidic. If recent structural-based predictions for H^+/ATP of 3.3 (e.g. Jiang, et al., 2001) hold true, then the lumen pH would have to be more acidic than 5.4. A similar set of predictions can be derived from the energetic requirements for the activation of the ATP synthase (reviewed in Kramer, et al., 1999), see Scheme I.

Does $\Delta\psi$ contribute to pmf in vivo? With the assumption that all *pmf* is in the form of ΔpH, the predictions based on ATP synthase energetics are apparently at odds with those based on lumenal enzyme stability and function. It was suggested (Kramer, et al., 1999) that the two estimates could be reconciled if as little as 30 mV of *pmf* were sorted as $\Delta\psi$. Measurements of the electrochromic shifting of carotenoids in response to $\Delta\psi$ have shown that *pmf* can be stored as $\Delta\psi$ for extended periods of time in both thylakoids and intact leaves (Cruz, et al., 2001) as well as in whole cells of *Chlamydomonas reinhardtii* (Cruz, et al., 2001). Recent data on this topic are presented in Cruz et al. (this Proceedings). Figure 2 shows the light-induced kinetics of ECS in the NPQ-4 mutant of Arabidopsis (Björkman and Niyogi, 1998) which has a normal xanthophyll cycle but decreased NPQ. Importantly, it does not show the 535 nm "light-scattering signals" that interfere with observations of ECS in intact leaves. The data in Figure 2 shows a significant steady-state $\Delta\psi$, validating our previous observations.

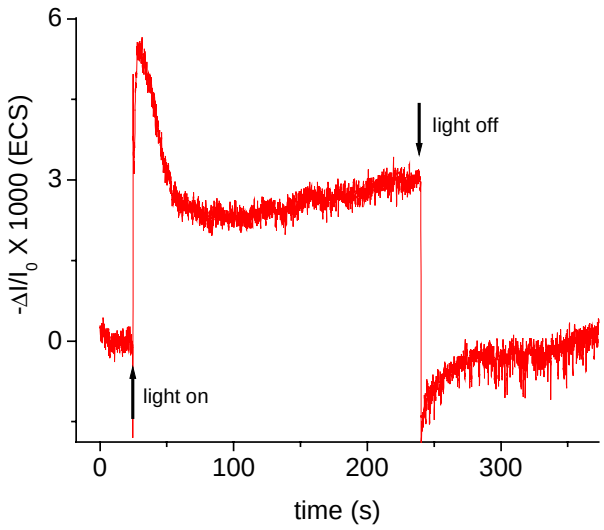


Figure 2. Absorbance changes attributable to ECS in an intact, npq4 mutant *Arabidopsis* leaf. The actinic light (1200 µE m^{-2} s^{-1} red light) was activated at the up arrow and deactivated at the down arrow. Absorbance data was collected at 4 wavelengths simultaneously (505, 525 nm, 535 nm and 545 nm) at intervals of 4 ms and the trace was deconvoluted as described in (Cruz et al, 2000)

Estimates based on the analysis of Cruz et al. (2001) from this data suggests $\Delta\psi$ contribution 25-60% of *pmf* under non-stressed steady-state conditions, sufficient to produce observed levels of ΔG_{ATP}, while maintaining a moderate lumen pH, as illustrated in Scheme I.

What modulates feed-back regulation of the antenna? In Kanazawa et al. (these Proceedings), we demonstrated that alterations in the conductivity of the ATP synthase (g_{H^+}) can modulate the sensitivity of antenna downregulation, or non-photochemical quenching of antenna excitons (NPQ) by changing the relationship between electron transfer and lumen acidification. This is also clearly illustrated in the data in Figures 1A and B, which show that electron transfer (estimated as $i*F_V/F_M'$ or $i*\phi_{II}$) in tobacco leaf discs infiltrated with 10 mM MV was only 50% of that observed for controls infiltrated with water. At first glance, this appears in contradiction with the expected effect of MV as a strong PS I electron acceptor. These and previous (Varadi, et al., 2000) results can be explained by the essentially complete inhibition of CO_2 fixation by MV, which is caused by the depletion of NADPH. Since ADP

and Pi regeneration is strongly dependent on the operation of the Calvin cycle, we expect the addition of MV to increase ATP/(ADP+Pi) and eventually slow the dissipation of *pmf* through the ATP synthase. Indeed, the H^+ conductivity of the ATP synthase, as estimated by the half time for decay of the ECS, was observed to decrease by about 50% upon addition of MV (Figure 1B). This decrease in g_{H^+} should increase steady-state *pmf* at a given electron transfer rate. This is reflected in as an increase in NPQ, (as reflected in a decrease in F_M', Figure 1B), which was most likely the result of increased lumen acidification. Previously it was shown that infiltration of leaf discs with MV increased the rate of violaxanthin deepoxidation (Büch, et al., 1994), consistent with this interpretation. This is also reflected in a decrease in the cyt b_6f turnover time, as indicated by an increase in the halftime for reduction of P_{700}^+ upon light-dark (Figure 1B), as well as an increase in steady-state Q_A reduction level (compare F_s levels just before saturating pulses in Figure 1B), again in apparent contradiction with the accepted view of MV action. A comparison with *in vitro* data on the pH-dependence of the cyt b_6f turnover rate (reviewed in Kramer, et al., 1999), yields an estimate for lumen pH in the presence of 10 mM MV about 5.5, lower than that expected for the control, but still (by our standards) moderate.

The possibility of variable pmf parsing. Recent work in our laboratory has suggested that the extent of $\Delta\psi$ contributions to steady-state *pmf* may change depending upon physiological status, and that the change in the 'parsing' of *pmf* into $\Delta\psi$ and ΔpH may alter the sensitivity of pH-sensitive down-regulatory processes to electron and proton transfer fluxes, as first suggested in (Cruz, et al., 2001). For reasons that are not yet clear, plants transferred from our greenhouse facility to our laboratory and immediately assayed show low storage of *pmf* as $\Delta\psi$, whereas plants adapted for 12 hours to the laboratory show about 50% $\Delta\psi$ storage. We took advantage of this behavior to explore the effects of *pmf* parsing on NPQ. Figure 3 shows parameters derived from the dark-interval kinetics of a rapidly-transferred tobacco leaf under room air (about 400 ppm, closed squares) and air supplemented with 5% CO_2 (open circles). The fraction of *pmf* stored as $\Delta\psi$ was considerably higher under 5% CO_2 than under room air (about 400 ppm). The half-times for ECS decay upon the light-dark transitions were constant, indicating that changes any changes in the sensitivity of NPQ to electron transfer cannot be explained by alterations in g_{H^+}. Figure 3A shows that increasing [CO_2] increased the maximal photosynthetic rate as well as the half-saturating light intensity. Figure 3B and C are plots of the apparent $\Delta\psi$ and ΔpH contributions to *pmf*, estimated by the ECS_{ss} and ECS_{inv} parameters described in (Cruz, et al., 2001). In room air, it rises with light intensity, reaching a maximum at about half saturating electron transfer rates. In high CO_2, maximal ECS_{ss} rose by about 50%. The steady-state ΔpH component of *pmf* showed a sigmoidal relationship with electron transfer rates, with steep rises occurring during the decrease in the $\Delta\psi$ component. This corresponded well with the increases in light scattering measured at 535 nm (Figure 3E) and with the appearance of NPQ (Figure 3D). Importantly, NPQ followed the appearance of the ΔpH, but not the $\Delta\psi$ component of *pmf*, as illustrated by the positive relationship between NPQ and ECS_{inv} (Figure 3F). Moreover, the relationship between ECS-estimated ΔpH and NPQ did not change between the two treatments. This suggests that, 1) under certain conditions, alterations in the parsing of *pmf* into $\Delta\psi$ and ΔpH can modulate the sensitivity of NPQ to electron transfer; 2) ECS_{inv} may be used as an indicator of ΔpH *in vivo*, and 3) the relationship between ΔpH and NPQ is nearly constant over the time scale of hours.

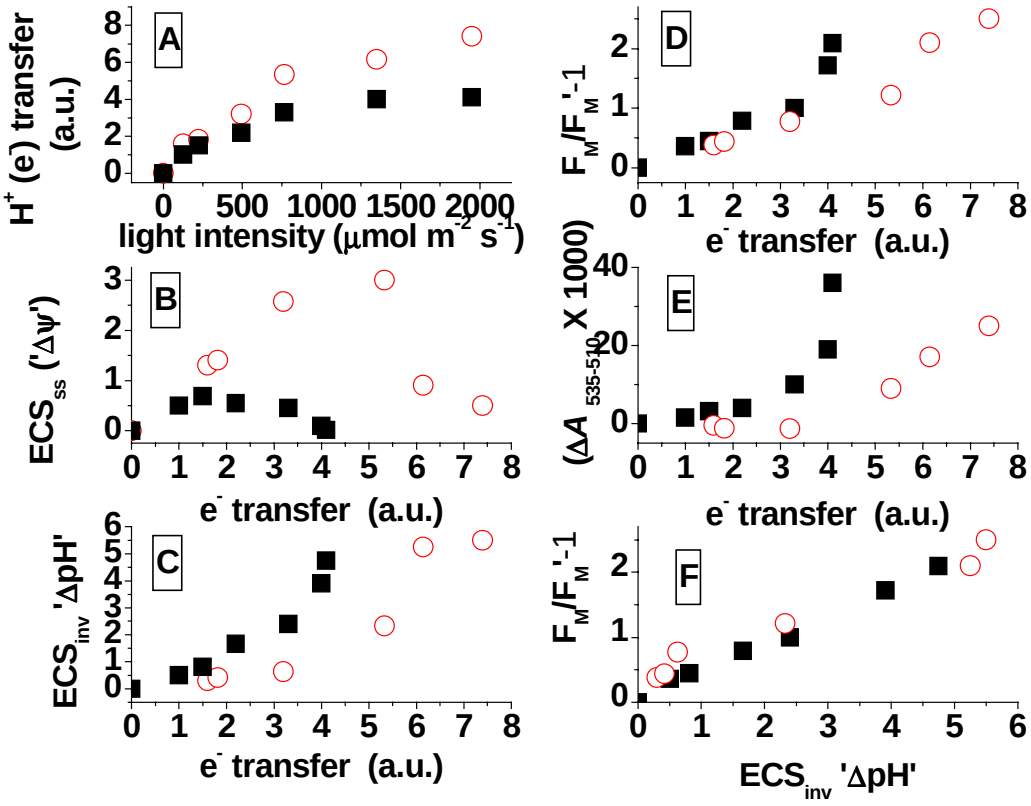


Figure 3. Dependence of photosynthetic parameters on light intensity and $[CO_2]$ in leaves of freshly-transferred tobacco plants. Data was collected as described in the text using the diffused-optics flash spectrophotometer (Kramer and Sacksteder, 1998).

A model for variable regulation and balancing of the light reactions. To avoid photoinhibition under fluctuating demands for ATP and NADPH, the downregulatory system for the light reactions must have variable sensitivity to electron flux. In this work and in Kanazawa et al. (this Proceedings), we have identified two potential "missing links" between electron transfer and lumen pH-responsive downregulation. The first of these is g_H^+, which is smaller under conditions of restricted $[CO_2]$ (Kanazawa et al., this Proceedings) or alteration in the demand for NADPH:ATP (Figure 1). It was previously shown (Kramer, et al., 1990) that regulation of the redox-control of the ATP synthase via thiol reduction is saturated at very low light levels and thus likely does not contribute to the observed modulation of g_H^+. Other likely candidates for the g_H^+ modulation signal include [ATP], [ADP] and [Pi]. Previous measurements showed only small (ca. 2-fold) changes in *in vivo* chloroplast [ATP]/[ADP] upon altering $[CO_2]$ (Dietz and Heber, 1984), too small to account for the observed differences in g_H^+. Furthermore, at constant $[CO_2]$, light intensity changes has an equally large effect on [ATP]/[ADP] (Siebke, et al., 1990). Since g_H^+ was strongly affected by $[CO_2]$, but not by light intensity above about 50 µmol photons m^{-2} s^{-1} (Kanazawa et al., this Proceedings), we conclude that neither [ATP] nor [ADP] is likely to determine g_H^+. As a working model, we suggest that [Pi] or another (yet unidentified) regulatory signal alters g_H^+ in response to the concentration of an unidentified Calvin cycle intermediate pool.

The data in Figure 3 suggests that NPQ may also be modulated by altering the parsing of *pmf* into ΔpH and Δψ components, though we have not thoroughly explored the conditions under which this may occur. At present we suggest that alteration in *pmf* parsing is affected by alterations in the activities of thylakoid membrane-permeable counterions, as described in (Cruz, et al., 2001).

The data presented here and in Kanazawa et al. (these Proceedings) lead us to propose that, at least over the time range of hours, alteration in the relationship between electron transfer and NPQ can be accounted for by changes primarily in g_H^+, and secondarily in *pmf* parsing. Under our conditions, i.e. short-term changes, it was not necessary to invoke any modification of the sensitivity of the antenna phenomena to lumen pH, nor did we observe any evidence that alternate cycles significantly contributed to the onset of NPQ. We propose that these two modulatory mechanisms allow NPQ to protect PS II from photodamage while maintaining a moderate lumen pH.

The data in Figure 1 also suggests a simple mechanism for balancing the output of ATP and NADPH without requiring a change in H^+/e^-. The longest decay halftime for the ECS obtained in both inhibitor- and heat/water stress-treated leaves was about 150 ms, i.e. about 10-fold slower than the most rapid, obtained under permissive conditions. Thus, proton flux remained fairly rapid, considering that treatment with MV resulted in complete abolition of CO_2 fixation. We suggest that the residual proton flux occurs via a slippage reaction, which acts to dissipate excess *pmf* or ATP. In principle slippage could occur at the levels of the ATP synthase (e.g. by allowing uncoupled proton flux), or at the level of the ATP pool (e.g. by activation of an energy-dissipating ATPase). ECS decay kinetics in isolated thylakoids (not shown) show a similar (150 ms) halftime, which is inhibited by tentoxin and DCCD, suggesting that the slippage occurs at the level of the ATP synthase. Slippage reactions within the ATP synthase were postulated previously based on *in vitro* assays (e.g. Evron and Avron, 1990), and can readily be incorporated in the rotational catalysis models, e.g. by allowing the F_1 portion to rotate freely with respect to F_O. Junge and coworkers (Fritsche and Junge, 1996) pointed out that the majority of the observed *in vitro* slippage reactions could be eliminated by additions of physiological concentrations of nucleotides. On the other hand, there is yet no conclusive test of this phenomenon *in vivo*. We thus propose a model where excess NADPH demand is balanced by slippage. Excess ATP demand, on the other hand, could be balanced by alternate electron acceptors and pathways, as recently reviewed in (Badger, et al., 2000). This work was supported by US Department of Energy Grant DE-FG03-98ER20299. We thank Profs. U. Heber and A. Portis for important discussions

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