Thiol Regulation of the Thylakoid Electron Transport Chain—A Missing Link in the Regulation of Photosynthesis?

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ABSTRACT: Avoidance of over-reduction of the chloroplast ferredoxin pool is of paramount importance for plants in avoiding oxidative stress. The redox state of this pool can be controlled through regulation of the thylakoid electron transport chain. A model is presented for regulation of this chain via a thiol reduction mechanism, possibly involving a thioredoxin. It is shown in isolated thylakoids that electron transport is inhibited by the thiol reducing agent dithiothreitol. The kinetics of this reduction are rapid and readily reversible. The midpoint redox potential is −365 mV at pH 7.7, with a pH dependency of about −90 mV/pH. At physiological pH values, this places the potential of the species titrated between that of ferredoxin and NADPH and thus in the right potential range to be regulating the redox poise of the ferredoxin pool. This is also close to the potential of NADPH-malate dehydrogenase, an enzyme known to be regulated by thioredoxin. Regulation of electron transport by thioredoxin provides a mechanistic link between the regulation of photosynthesis and gene expression by sugars and the redox regulation of gene expression mediated through the plastoquinone pool.

The absorption of light by chlorophyll and the subsequent light-driven transport of electrons are intrinsically hazardous in an oxygen-rich atmosphere (see ref *1* for a review). Oxygen, the product of photosynthesis, is readily reduced by species formed during electron transport (*2*, *3*). This reduction involves a single electron transfer, producing the superoxide radical anion, $O_2^{\bullet -}$ (*4*). A number of components of the thylakoid electron transport chain have a redox potential sufficiently negative to produce this species, including semiquinones associated with photosystem II (PSII),[1] plastoquinol, and reduced iron−sulfur (Fe−S) centers associated with photosystem I and ferredoxin. However, although there is evidence that in some circumstances reduction of O_2 can occur at PSII (*5*−*7*), under most conditions it is the Fe−S centers on the PSI acceptor side and ferredoxin that are believed to be the major source of superoxide (*8*−*10*). Photoreduction of oxygen by these centers results in superoxide that may be further reduced by ferredoxin to hydrogen peroxide (*10*). If a plant is to avoid the reduction of molecular oxygen, then it follows that the redox state of these centers must be closely regulated.

Charge separation by PSI reaction centers can be viewed as a committed step in the production of reduced Fe−S centers. The rate of PSI charge separation is a function of light absorption and of the rate at which the oxidized primary donor, $P700^+$, is rereduced. The major determinant of light absorption, the irradiance incident on the leaf, is largely outside the plant's control. Therefore, $P700^+$ rereduction rate appears to be the obvious route for regulating PSI acceptor side redox state. There is now considerable evidence that such regulation does indeed occur under physiological conditions. Harbinson (*11*) observed that the rate of $P700^+$ reduction declines when photosynthesis is inhibited by lowering CO_2 partial pressure. Ott et al. (*12*) demonstrated that such regulation also occurs in response to a range of physiological conditions. Golding and Johnson (*13*) recently presented evidence for such regulation during exposure of plants to drought.

Although there is clear evidence that the electron transport chain is regulated prior to PSI, the mechanism of this regulation remains to be elucidated (*12*). In vitro studies have shown that the slowest step in the thylakoid ETC is the oxidation of plastoquinol by the cytochrome b_6f complex (*14*). This step has also been shown to be sensitive to the pH of the thylakoid lumen (*15*). Specifically, the low lumen pH generated by proton translocation coupled to electron transport inhibits oxidation of plastoquinol. It might be postulated that lumen pH also regulates electron transport in vivo. How this would function, however (i.e., how the pH of the lumen would be controlled in response to the redox poise of the stroma), remains unclear. Furthermore, Ott et al. (*12*) compared the CO_2 and light dependence of P700 reduction kinetics and pH-dependent nonphotochemical quenching of chlorophyll fluorescence and demonstrated that the two processes varied in ways that are inconsistent with pH-dependent regulation of electron transport. An alternative mechanism for regulating electron transport must therefore be sought.

It is now well-known that the activity of enzymes in the Calvin cycle is regulated in response to the redox poise of the chloroplast (*16*). Under the oxidizing conditions prevailing in the dark, a number of enzymes are inactive. Following

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[1] Abbreviations. DTT, dithiothreitol; ETC, electron transport chain; FTR, ferredoxin thioredoxin reductase; MDH, NADPH-malate dehydrogenase; PSI, photosystem I; PSII, photosystem II; RDS, regulatory disulphide; Trx, thioredoxin.

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a dark to light transition, these enzymes are activated via a number of thioredoxins present in the chloroplast stroma. The redox state, and so activity, of these is determined by the redox state of ferredoxin, the terminal acceptor of the electron transport chain. As the ferredoxin pool becomes reduced in the light, Calvin cycle enzymes are activated by thioredoxin, so increasing the rate of oxidation of that pool and imposing a limit on the extent of reduction of ferredoxin.

Under conditions where the capacity of the Calvin cycle matches the rate of electron transport, such a system works well to ensure a moderate degree of reduction of the ferredoxin pool. However, under many physiological conditions (e.g., during exposure to drought), the Calvin cycle is limited by the supply of CO_2. Could a similar mechanism operate to feedback to the electron transport chain and so lower the rate of electron transport, preventing over-reduction of ferredoxin when CO_2 fixation is limited? Here, data are presented that show that the capacity of the electron transport chain is sensitive to thiol redox state, demonstrating that the potential for such regulation of electron transport does indeed exist.

EXPERIMENTAL PROCEDURES

Chloroplasts were isolated from market-bought spinach using the method of ref *17*. Thylakoid membranes were produced by osmotically shocking chloroplasts in a solution containing 5 mM $MgCl_2$, the osmotic potential of the solution being restored by addition of an equal volume of 660 mM sorbitol, 50 mM HEPES (or, for redox titrations, tricine; pH as stated in text), 5 mM $MgCl_2$, and 10 mM NaCl. All measurements presented were made in the presence of 20 mM potassium ferricyanide as an electron acceptor. Other details of measurements were as stated in the figure legends.

Measurements of the relaxation kinetics of $P700^+$ were made following application of a 100 ms flash of white light (intensity 3000 μmol m^{-2} s^{-1} at entry to the cuvette). Measurements were made in a Walz KS-101 cuvette (Heinz Walz, Effeltrich, Germany) regulated to 20 °C using a circulating water bath (Grant, Royston, UK). The redox state of P700 was monitored using changes in the absorbance at 830 nm, using a Walz ED-P700DW-E detector/emitter unit connected to a PAM 101 fluorometer. Absorbance changes were detected in reflective mode and recorded as described previously (*7*). Transient absorbance changes from up to 50 flashes, spaced 900 ms apart, were accumulated. The pseudo-first-order rate constant for P700 rereduction, *k*, was determined by fitting a monoexponential curve to the data as described previously (*12*).

Measurements of oxygen evolution were made using a Clarke-type oxygen electrode (Hansatech, King's Lynn, UK). Saturating illumination was provided by a Schott KL1500 illuminator.

Redox titrations of the midpoint potential for inhibition of electron transport were made using a method based on that described by (*18*). Samples were incubated in tricine buffer, as above, in the presence of either 10 or 20 mM dithiothreitol, with different potentials being produced by varying the ratio of oxidized to reduced DTT. Samples in a given titration were incubated simultaneously on ice for a minimum of 1 h (maximum 2 h) prior to measurement, to allow samples to reach redox equilibrium. Successive samples were assayed in order of descending and then ascending potential, enabling the absence of hysteresis because of variation in incubation time to be confirmed. Samples were then transferred to an oxygen electrode cuvette. Once the sample had reached cuvette temperature (20 °C), potassium ferricyanide, and where appropriate, ammonium chloride were added, and oxygen evolution under saturating light was measured. To test for reaction between DTT and ferricyanide, a mixture of these chemicals was incubated, and the absorbance at the λ_{max} for ferricyanide followed. No measurable change in absorbance was observed within 10 min, although over a time scale of hours there was a loss of absorbance. In the experimental protocol used, the ferricyanide was added to the sample at the end of the DTT incubation, and the measurement, lasting about 1 min, started within 10 s. Ferricyanide was always present in a large excess. Fitting of theoretical curves to data was performed using the Grafit software package (v. 3; Erithacus Software).

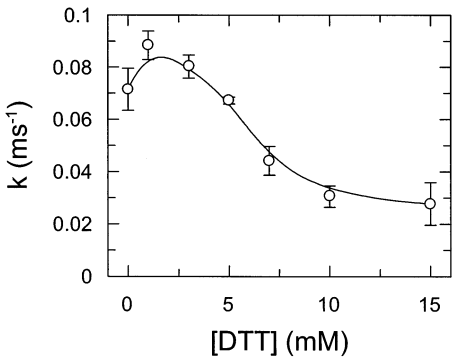

FIGURE 1: Relationship between concentration of dithiothreitol and the pseudo-first-order rate constant for rereduction of P700 (*k*) in isolated spinach thylakoids in the presence of ammonium chloride as an uncoupler, pH 7.6.

RESULTS

Incubation of isolated thylakoid membranes with the thiol reducing agent dithiothreitol was found to inhibit the rereduction of oxidized P700 following a flash of light (Figure 1). At pH 7.6, the pseudo-first-order rate constant for $P700^+$ rereduction, *k*, fell from a maximum around 0.08 ms^{-1} to around 0.02 ms^{-1} at a DTT concentration of 10−15 mM. In some preparations, low concentrations of DTT (∼1 mM) increased the rate constant for P700 reduction.

Inhibition of P700 rereduction kinetics was also reflected in the rate of oxygen evolution (Figure 2). DTT inhibited the rate of potassium ferricyanide-dependent oxygen evolution. (Potassium ferricyanide was found to react only very slowly with DTT, see Experimental Procedures.) As with P700 rereduction kinetics, at pH 7.6, a small activation of oxygen evolution was noticed in the presence of low concentrations of DTT. Increasing the pH to 8.0 considerably enhanced this effect. The inhibition of oxygen evolution seen in uncoupled thylakoids at high pH could be fully reversed by addition of DTT. The concentration of DTT required to inhibit oxygen evolution also increased at high pH.

The inhibition of oxygen evolution (and of P700 rereduction) was found to be fully reversible (Figure 3). Inhibition of electron transport by DTT was rapid (<5 min; not shown) but could be fully reversed by washing out the DTT, even after incubation for 1 h.

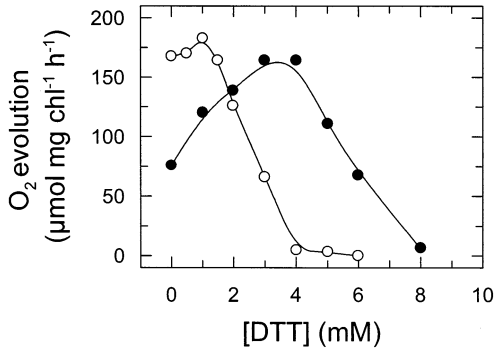

FIGURE 2: Effect of dithiothreitol on the rate of potassium ferricyanide dependent oxygen evolution in uncoupled thylakoids at pH 7.6 (open symbols) and pH 8.0 (closed symbols).

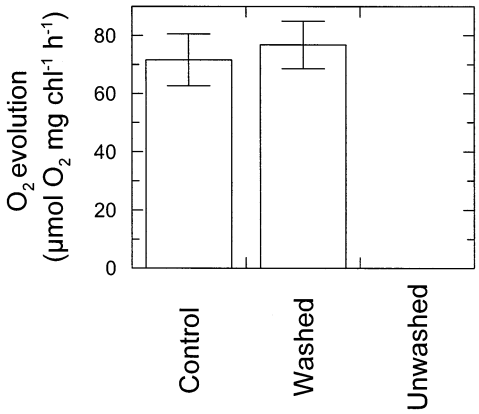

FIGURE 3: Effect of washing with dithiothreitol on the rate of potassium ferricyanide dependent oxygen evolution in uncoupled thylakoids. Thylakoids were incubated at a concentration of 35 μg chl mL^{-1} for 1 h in the presence or absence of 10 mM DTT. Samples were then centrifuged and washed twice in buffer either containing (unwashed) or lacking (control/washed) DTT.

This rapid induction and reversibility of the effect of DTT make it possible to perform equilibrium redox titrations of the midpoint potential of the component being reduced by DTT (*18*). When attempts were made to titrate this component in thylakoid membranes in the presence of the uncoupler ammonium chloride, a non-Nernstian response was observed (Figure 4A). As before, moderate potentials activated oxygen evolution. Low potentials inhibited oxygen evolution; however, the curve was steeper than expected for a two-electron Nernst curve. Fitting with higher electron numbers also did not give a satisfactory fit. When titrations were performed in the absence of uncoupler, however, response curves fitted well to theoretical two-electron Nernst curves (Figure 4B).

The dependence of oxygen evolution on thiol redox potential was titrated across the range of pH values that might be expected to occur in the chloroplast stroma. At all pH values measured, data fitted well to theoretical Nernst curves (Figure 5A). The midpoint redox potential for the inhibition of electron transport was, however, found to vary by −99 mV/pH, not the 59 mV expected for a thiol reduction (Figure 5B).

DISCUSSION

Data presented in this paper clearly indicate that the potential exists for regulation of thylakoid electron transport by a thiol-linked mechanism, possibly involving a thioredoxin. This regulation is reflected both in the inhibition of

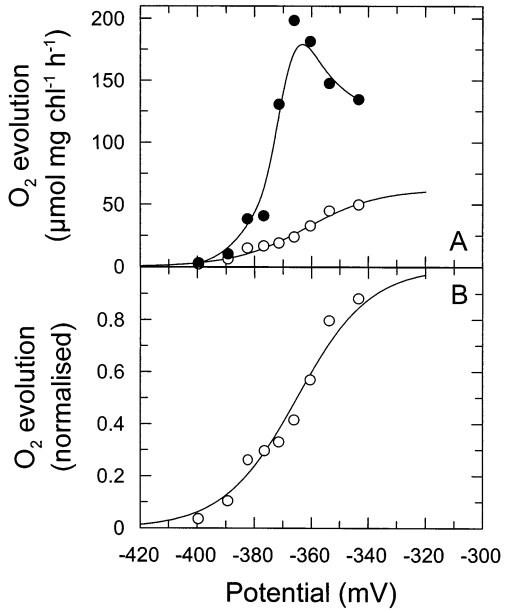

FIGURE 4: (A) Redox titrations of potassium ferricyanide dependent oxygen evolution at pH 7.7 in the presence (closed symbols) or absence (open symbols) of ammonium chloride as an uncoupler. (B) Data measured in the absence of uncoupler, normalized to the rate of oxygen evolution achieved in the presence of 10 mM oxidized DTT, fitted with a Nernst curve ($n = 2$; $E_m = -365$ mV).

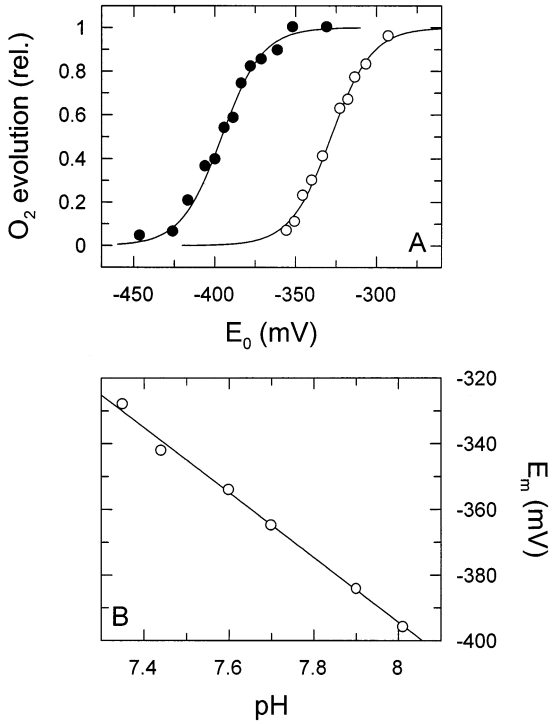

FIGURE 5: (A) Redox titrations of potassium ferricyanide dependent oxygen evolution in coupled thylakoids at pH 7.35 (open symbols) and pH 8.01 (closed symbols) fitted with Nernst curves ($n = 2$; $E_m = -328$ and -384, respectively). Rates of oxygen evolution were normalized to the maximum rate, observed in the presence of 10 mM oxidized DTT. (B) pH dependence of the midpoint redox potential of ferricyanide dependent oxygen evolution in coupled thylakoids, measured as in panel A. Line represents a linear regression, slope = −98.7, $r = -0.9978$.

P700 rereduction and in the inhibition of potassium ferricyanide-dependent oxygen evolution. In the chloroplast, the redox state of the thioredoxin pool is linked to the redox state of ferredoxin (*19*). Hence, regulation of electron

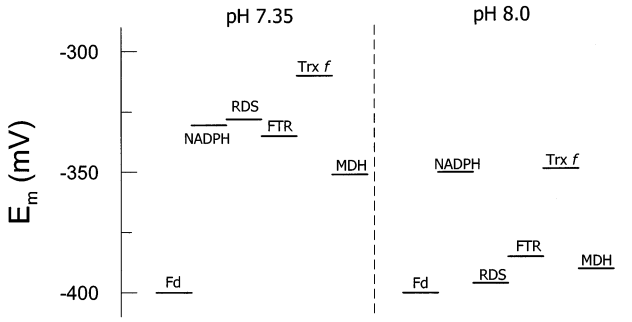

FIGURE 6: Redox midpoint potentials for chloroplast components at pH 7.35 and pH 8.0. Values given for ferredoxin (Fd), NADPH, the regulatory disulfide titrated here (RDS), ferredoxin thioredoxin reductase (FTR), thioredoxin *f* (Trx *f*), and NADPH-malate dehydrogenase (MDH). Data for FTR and Trx *f* from ref *19* and for MDH from ref *27*.

transport by thioredoxin would provide an optimal mechanism for the regulation of the ferredoxin redox state. Photoreduction of oxygen is thought to be a spontaneous reaction, primarily between reduced ferredoxin and molecular oxygen. Oxygen is always present in the chloroplast at high concentrations, so the rate of this reaction will be largely determined by the redox state of the ferredoxin pool. Hence, regulation of the redox poise of this pool provides a mechanism for regulating electron flow to oxygen.

The midpoint redox potential of the component titrated here was found to be in the range of −340 to −400 mV across the pH range of 7.3−8.0. It is difficult to be certain of the pH of the stroma of an illuminated chloroplast, as this will be affected not only by the buffering capacity of the cell but also by the extent of the pH gradient across the thylakoid membrane. We can, however, determine the plausibility of this component being reduced by thioredoxin in vivo by comparing this potential range with the midpoint potentials of other redox-active components in the chloroplast, including ferredoxin, NADPH, and thioredoxin (Figure 6).

The component titrated here has a midpoint redox potential that lies close to but slightly more positive than that of ferredoxin across the pH range titrated here. As such, it will always tend to be reduced by ferredoxin. At the lowest pH measured, pH 7.35, the potential of the regulatory disulfide is close to that of NADPH, however, because of the difference in pH sensitivity (NADPH $\approx$ −30 mV/pH) at the highest pH measured, the regulatory component is markedly more negative in potential, close to the potential of ferredoxin.

Thiol-regulated enzymes involved in the Calvin cycle are typically of more positive potential than the regulatory component titrated here (see e.g., ref *19*). This is as would be expected, as the enzymes involved in using reducing equivalents generated by electron transport should become activated at a more positive potential than that at which the electron transport chain is inhibited. Thioredoxin itself has a midpoint redox potential of −324 mV at pH 7.6, with a 59 mV/pH dependence typical of a reduction involving two electrons and two protons ($1H^+/e^-$) (*19*). This is again more positive than the midpoint redox potential of the component titrated here. Hence, thioredoxin would have to be largely reduced before the putative regulatory component became reduced. This might suggest that this component is of too

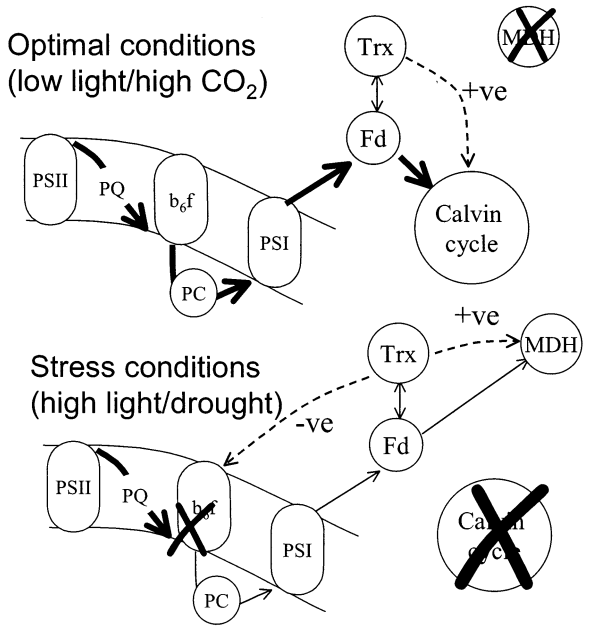

FIGURE 7: Model for the regulation of thylakoid electron transport chain and photosynthetic carbon assimilation by thioredoxin. Under optimal conditions for photosynthesis (low light and saturating CO_2), electron transport will occur through the electron transport chain with reductant being used in carbon fixation by the Calvin cycle. Under such conditions, the thioredoxin (Trx) pool will be moderately reduced and will feed forward to activate Calvin cycle enzymes. Under stressful conditions (e.g., at high light or under drought), the thioredoxin pool becomes more reduced. Under such conditions, Trx inactivates electron transport by reducing a regulatory disulfide, probably associated with the cytochrome b_6f complex. At the same time, malate dehydrogenase is activated, promoting export of reducing potential from the chloroplast. PC, plastocyanin.

negative a potential to be regulated by thioredoxin. The midpoint titrated here is, however, close to that of an enzyme known to be regulated by thioredoxin—NADPH-malate dehydrogenase (MDH) (*20*).

MDH functions in the chloroplast to synthesize malate from oxaloacetate, using NADPH as a reductant. Export of malate and its subsequent oxidation allow the export of reducing equivalents from the chloroplast (the so-called malate shunt). This pathway has been suggested to operate as a safety valve for electron transport, exporting excess reductant from the chloroplast (*21*). That this enzyme is activated at around the same potential as the down-regulation of electron transport, speaks for this potential range being the maximum extent of reduction tolerated by the plant—the point at which regulatory mechanisms act to prevent oxidative stress.

So, we can envisage a hierarchy of response to redox potential (Figure 7). In the dark, the potential in the chloroplast is high, as no reductant is being generated by the electron transport chain. As the plant is transferred to light, the potential drops, and enzymes of the Calvin cycle become progressively activated. This activation acts to counter the drop in potential because of electron transport. With increasing light and/or the imposition of stress, the potential will fall further. At this point defense mechanisms start to operate—reducing equivalents will begin to be exported from the chloroplast, and the production of reducing equivalents, through the electron transport chain, will be down-regulated.

The observation that, in uncoupled thylakoids, down regulation of electron transport does not fit to a theoretical Nernst curve may be of functional significance. The non-Nernstian behavior can be explained if it is assumed that, in the uncoupled state, the limiting step in the electron transport chain is at a different site to the redox sensitive step. Kirchhoff and co-workers (*22*) observed that in stacked uncoupled thylakoids, control of electron transport lies mostly with PSII. In the coupled state, in isolated thylakoids, the limiting step in electron transport is the oxidation of plastoquinol by the cytochrome b_6f complex (*15*). That data in coupled thylakoids fit to a theoretical Nernst curve indicates that the thiol-regulated component is probably at the same site (i.e., that it is the cytochrome b_6f complex that is being inactivated by thiol reduction). It is not clear what level the pH gradient across the thylakoid membrane reaches in vivo; however, circumstantial evidence would suggest that it does not normally attain a level that would inhibit electron transport directly (see discussion in ref *12*). If this is the case, we might expect thiol regulation to produce a very sharp down-regulation of electron transport, the potential of which may be slightly more negative than that observed for MDH activation.

The pH sensitivity of the regulatory component titrated here is unusual. The value of approximately −90 mV/pH implies the requirement of three protons per thiol reduced, as compared to the two required for the reduction of a disulfide bond. This would suggest that the reduction is accompanied by the obligatory protonation of an acidic residue in the protein. Such a pH dependence could have subtle effects on the response of the chloroplast to stress. As a ΔpH accumulates across the thylakoid membrane, the thylakoid stroma becomes more alkaline. Thus, prior to the development of a ΔpH, the regulatory component will be rather sensitive to reduction, placing a tight control on electron transport. As the ΔpH develops, the control over ferredoxin redox state will be somewhat relaxed, allowing a higher rate of electron transport to be maintained once a ΔpH has developed. The net result of this might be to produce a slow start to photosynthesis, following a dark-light transition, as is indeed observed in intact leaves.

The observation that, at high pH, low concentrations of DTT activate electron transport implies that a second, high potential, thiol group is involved in regulating electron transport. Presumably this group only becomes oxidized at high pH, when its potential is shifted below the ambient potential. That this effect is not observed in coupled thylakoids implies that this thiol group is located at a site other than the cytochrome b_6f complex or that the regulation operates in a way that it is not co-limiting with the ΔpH effect on the b_6f complex. Whether this effect is of physiological relevance is unclear.

The regulation of photosynthesis is already recognized to be complex (*23*). It can occur over both short time scales, involving adjustments in the operating efficiency of individual processes, or over long time scales, with changes occurring in gene expression (*24*). It is now well-established that the products of photosynthesis (i.e., sugars) both regulate gene expression and inhibit photosynthesis (*25*). Recently, it has been shown that the redox state of the plastoquinone pool also plays a role in regulating gene expression, helping to balance the activity of PSII and PSI (*26*). Previously, no explicit link between these two mechanisms has been identified. The regulatory pathway described here provides such a link.

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