Differential Effects of Chilling-Induced Photooxidation on the Redox Regulation of Photosynthetic Enzymes†

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ABSTRACT: Photosynthesis in plant species that are evolutionarily adapted for growth in warm climates is highly sensitive to illumination under cool conditions. Although it is well documented that illumination of these sensitive species under cool conditions results in the photosynthetic production of reactive oxygen molecules, the underlying mechanism for the inhibition of photosynthesis remains uncertain. Determinations of chloroplast fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase activity showed that the light-dependent, reductive activation of these key carbon reduction cycle enzymes was substantially inhibited in tomato (*Lycopersicon esculentum*) following illumination at 4 °C. However, other chloroplast enzymes also dependent on thioredoxin-mediated reductive activation were largely unaffected. We performed equilibrium redox titrations to investigate the thermodynamics of the thiol/disulfide exchange between thioredoxin *f* and the regulatory sulfhydryl groups of fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, NADP−glyceraldehyde phosphate dehydrogenase, and the chloroplast ATPsynthase. We determined that the redox midpoint potentials for the regulatory sulfhydryl groups of the various enzymes spanned a broad range (∼50 mV at pH 7.9). The electron-sharing equilibria among thioredoxin *f* and its target enzymes largely explained the differential effects of photooxidation induced at low temperature on thioredoxin-mediated activation of chloroplast enzymes in tomato. These results not only provide a plausible mechanism for the low-temperature-induced inhibition of photosynthesis in this important group of plants, but also provide a quantitative basis to evaluate the influence of thioredoxin/target enzyme electron-sharing equilibria on the differential activation and deactivation kinetics of thioredoxin-regulated chloroplast enzymes.

Most warm-climate plant species are sensitive to brief exposures to low, nonfreezing temperatures. Low-temperature exposure in combination with even moderate irradiance levels causes rapid, often very severe, inhibition of photosynthesis in a broad range of warm-climate plants including maize (*1*), cucumber (*2*), and tomato (*3*). Numerous potentially contributing elements to the inhibition have been identified, and all may ultimately arise from the photosynthetic production of active oxygen species (*4*).

The photosynthetic generation of biologically reactive molecules including reduced and excited species of oxygen, peroxides, radicals, and triplet state excited pigments is commonplace because plants frequently encounter light intensities that exceed their photosynthetic capacity (*5*). Higher plants are surfeited with diverse and sophisticated mechanisms both to minimize the generation of potentially damaging reactive molecules as well as to rapidly detoxify those that are produced. Strategic leaf and chloroplast movements can greatly attenuate the amount of incident light that is absorbed (*6*). Dynamic processes that operate within the chlorophyll antennae array of photosystem II can rapidly dissipate as heat a large portion of the light that is absorbed, thereby greatly diminishing the threat of photosyntheticly generated reactive molecules (*7*, *8*). Chloroplasts are further protected by the capability to employ numerous alternative electron acceptors when the availability of CO_2 is inadequate to accommodate the electron flux potential. Oxygen can sustain significant levels of photosynthetic electron flux either by operating as a substrate for rubisco in the photorespiratory pathway (*9*) or by interacting directly with photosystem I and/or ferredoxin in a Mehler reaction (*10*−*12*). The reduction of sulfite (*13*) and nitrite (*14*) can sometimes function as significant electron sinks within the chloroplast. Cyclic electron transport involving photosystem I and the cytochrome b_6f complex (*15*) and also a cryptic photosystem II cycle (*16*, *17*) have been implicated as potentially important mechanisms in avoiding the consequences of overexcitation under conditions of inadequate CO_2 availability.

While the reduction of oxygen is a highly effective strategy to sustain electron flow and thereby avoid dangerous pigment states and overreduction of NADP, it nevertheless carries

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the hazards inherent in formation of reduced oxygen and hydrogen peroxide (*5*). Superoxide radicals generated by the one-electron reduction of molecular oxygen by photosystem I/ferredoxin are rapidly converted within the chloroplast to hydrogen peroxide by CuZn-superoxide dismutase. Whereas hydrogen peroxide associated with the photorespiratory pathway is generated and detoxified by catalase in peroxisomes (*9*), detoxification of hydrogen peroxide produced in the chloroplast relies almost exclusively on the activity of ascorbate peroxidase bound to thylakoid membranes and localized in the vicinity of photosystem I (*18*). Because monodehydroascorbate reductase catalyzes the regeneration of ascorbate in the chloroplast at the expense of NAD(P)H, monodehydroascorbate reduction must be stoichiometric with oxygen reduction as a photosynthetic electron sink in order to ensure sustained capacity to detoxify reactive oxygen species.

The triplet ground state of molecular oxygen allows it to participate in unusual chemistry that generates an additional, potentially hazardous, form of reactive oxygen within chloroplasts. The triplet excited state of the primary donor of photosystem II, $^3P_{680}$*, can form during charge recombination reactions within the photosystem II reaction center (*19*). Although reaction center carotenoids generally efficiently quench chlorophyll triplets, when the rate of triplet formation is increased by reduced flux of electrons from the reaction center (e.g., at cool temperatures), molecular oxygen can interact with the excited triplet state of the pigment, generating strongly oxidizing and highly reactive singlet oxygen, $^1O_2$.

Under most circumstances, the aggregate effect of these protective and detoxification processes, coupled with considerable capacity for repair, effectively prevents the inhibition of photosynthesis by excessive irradiance. However, our evidence indicates that the capacity of these processes can be suppressed and exceeded in warm-climate plants illuminated at cool temperatures. We have shown that the light-dependent, reductive activation of chloroplast bisphosphatases was substantially inhibited following illumination at low temperature in tomato, whereas other chloroplast enzymes also dependent on thioredoxin-mediated reductive activation were largely unaffected (*20*). We report here on equilibrium redox titrations performed to determine the electron-sharing equilibria among thioredoxin *f* and its target enzymes, which reveal that the redox midpoint potentials for the regulatory sulfhydryl groups of the various enzymes span nearly 50 mV. We show that these results can largely explain the differential effects of photooxidation induced at low temperature on thioredoxin-mediated activation of chloroplast enzymes in tomato. In addition to providing a plausible mechanism for the low-temperature-induced inhibition of photosynthesis in this important group of chilling sensitive plants, these data also form a quantitative basis to help understand the differential activation and deactivation kinetics of the various thioredoxin-regulated chloroplast enzymes.

MATERIALS AND METHODS

Plant Growth and Low-Temperature Treatment Conditions. Tomato plants (*Lycopersicon esculentum* Mill. cv. Floramerica) were grown from seed in growth chambers under a 14 h (26 °C) light/10 h (21 °C) dark cycle at 400−500 μmol·m^{-2}·s^{-1} PPFD[1] and 75% relative humidity as detailed in Jones and Ort (*21*). Plants were fertilized twice weekly with Plant Marvel liquid formula (12-31-14) supplemented with 10 mM KNO_3. All samples were taken from young, fully expanded leaves of plants 21−28 days after planting. Potted plants were chilled for 6 h in a growth chamber at 3 °C and 80% relative humidity at a light intensity of 1000 μmol·m^{-2}·s^{-1} PPFD. During chilling, the temperature of fully illuminated leaves was 4−5 °C. Plants were rewarmed (23 °C) at 100% relative humidity to prevent wilting.

Determination of Photosynthetic Rates. Photosynthetic rates were measured at 30 °C by infrared gas analysis on single attached tomato leaves at 1500 μL·L^{-1} CO_2 under PPFD of 1000 μmol·m^{-2}·s^{-1}. The chlorophyll content of tomato leaves ground in 80% acetone was measured spectrophotometrically and calculated using the specific absorption coefficients for chlorophylls *a* and *b* of Ziegler and Egle (*22*).

Isolation and Activity Measurements of Thioredoxin Regulated Enzymes. The purification procedure for chloroplast fructose-1,6-bisphosphatase (FBPase) was adapted from Nishizawa et al. (*23*) as detailed in Hutchison and Ort (*24*). Sedoheptulose-1,7-bisphosphatase (SBPase) was isolated from tomato leaves as described by Cadet et al. (*25*) but omitting the initial step of chloroplast isolation. Analysis by SDS−PAGE indicated greater than 95% purity for both enzymes. FBPase and SBPase were concentrated by ultrafiltration and stored frozen at −20 °C. FBPase (fructose 1,6-bisphosphate ⇔ fructose 6-phosphate + P_i) and SBPase (sedoheptulose 1,7-bisphosphate ⇔ sedoheptulose 7-phosphate + P_i) activities were measured by monitoring the enzyme-dependent release of inorganic phosphate using a procedure (*24*) modified from Chifflet et al. (*26*).

Phosphoribulokinase (PRK) was isolated and purified to >90% homogeneity essentially as described by Porter et al. (*27*). The concentrated protein was dialyzed against 50 mM tricine−NaOH (pH 7.8), 1 mM EDTA, 10% (v/v) glycerol, and 10 mM DTT and stored frozen at −80 °C. PRK activity (ribulose 5-phosphate + ATP ⇔ ribulose 1,5-bisphosphate + ADP) was determined spectrophotometrically in a reaction coupling the PRK-dependent formation of ADP with pyruvate kinase/lactate dehydrogenase-dependent NADH oxidation (*27*).

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was isolated and purified to >90% homogeneity according to the published procedure of Anderson et al. (*28*). The GAPDH-dependent oxidation of NADPH was monitored spectrophotometrically at 340 nm in a 800 μL sample containing 100 mM tricine−NaOH (pH 7.9), 2.5 mM ATP,

[1] Abbreviations: $DTT^{red/ox}$, dithiol/disulfide redox forms of dithiothreitol; $\Delta\mu_{H^+}$, transthylakoid membrane electrochemical potential of protons; E_h, ambient oxidation−reduction potential referenced to hydrogen electrode; $E_{m,7.9}$, oxidation−reduction midpoint potential at pH 7.9; FBPase, chloroplast fructose-1,6-bisphosphatase (EC 3.1.3.11); FBP, fructose 1,6-bisphosphate; FTR, ferredoxin:thioredoxin reductase; GAPDH, chloroplast glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); PPFD, photosynthetic photon flux density; PRK, phosphoribulokinase (EC 2.7.1.19); rubisco, ribulose bisphosphate carboxylase/oxygenase (EC 4.1.1.39); SBPase, sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37); SBP, sedoheptulose 1,7-bisphosphate; tricine, *N*-tris(hydroxymethyl)methylglycine.

10 mM $MgCl_2$, 2 mM DTT, 0.1 mM NADPH, and 4 units of phosphoglycerate kinase. The reaction was initiated by the addition of 5 mM 3-P-glycerate.

Measurement of Chloroplast ATPsynthase Activation. Measurements of the relaxation kinetics of the flash-induced ΔA_{518} were used to monitor the catalytic turnover of the chloroplast ATPsynthase in thylakoids isolated from dark-adapted leaves. Instrumentation, data acquisition, and analysis were performed as detailed by Oxborough and Ort (*29*). The assay medium for in vitro experiments (Figure 5) contained thylakoids equivalent to 40 μM chlorophyll, 100 mM sorbitol, 5 mM tricine−KOH (pH 7.9), 20 mM KCl, 5 mM $MgCl_2$, 100 μM methyl viologen, 100 μM ADP, and 1 mM NaH_2PO_4. For experiments with intact tomato leaves (Table 2), 3 saturating single-turnover xenon flashes spaced 10 ms apart were used to overcome the diminishing effects of the chilling exposure on membrane energization (*29*).

Thioredoxin Isolation. The isolation procedure for spinach chloroplast thioredoxin was adapted from Crawford et al. (*30*) as given in detail in Hutchison and Ort (*24*). Following desalting, the thioredoxin samples were concentrated by ultrafiltration and stored at −20 °C. Analysis by SDS−PAGE showed that greater than 90% of the protein present was thioredoxin. We also used thioredoxin *f* provided by Professor Schürmann isolated from *E. coli* that had been transformed with the cloned spinach thioredoxin *f* gene (*31*).

Equilibrium Redox Titrations of Intramolecular Dithiol/Disulfide Regulatory Groups. The dependence of catalytic activity on the ambient redox potential was used to determine the electron-sharing equilibria for the reversible exchange of a protein intramolecular disulfide between thioredoxin *f* and chloroplast target enzymes (*24*).

Enzyme samples were incubated at 25 °C in 0.1 mL of solution containing 100 mM tricine−NaOH (pH 7.9), 0.2 μg of thioredoxin *f*, and 10 mM DTT in various dithiol/disulfide ratios. The incubation solution had been purged with argon, and samples were kept under a constant stream of humidified argon throughout the incubations. The dipolar ionic buffer tricine is an effective chelator of trace metals that might otherwise catalyze the oxidation of thiols by oxygen. The stability of the redox poise under these conditions for incubations of 5 h was confirmed using Ellman's reagent (*32*) to monitor the concentration of DTT^{red}. For phosphoribulokinase, in which the dithiol/disulfide midpoint potential was the most oxidizing of the proteins that were titrated, we used 80 mM total DTT to ensure stable redox poise at the most oxidizing potentials where the proportion of the DTT in the dithiol form was very low.

The titration data were fit to the Nernst equation in the form:

$$E_h = E_{m,7.9} + \frac{59}{n} \log \frac{[\text{disulfide}]}{[\text{dithiol}]}$$

E_h denotes that the redox potential values are referenced to the standard hydrogen half-cell. $E_{m,7.9}$ specifies the midpoint potential under conditions when the components of the redox reaction are present at less than unit activities and the ambient pH value was 7.9, mimicking the stromal pH in the light (*33*). There are two electrons involved in the thioredoxin-mediated dithiol/disulfide interconversions, so we evaluated this equation for $n = 2$. Alternative analyses in which the best fit for the value of n was evaluated never produced values that deviated significantly from 2. The [disulfide] was calculated as 100% − % of maximum activity at any given E_h, and the [dithiol] was then the % of maximum activity at that E_h. The data points were computer fit to the Nernst equation using an iterative nonlinear least-squares analysis.

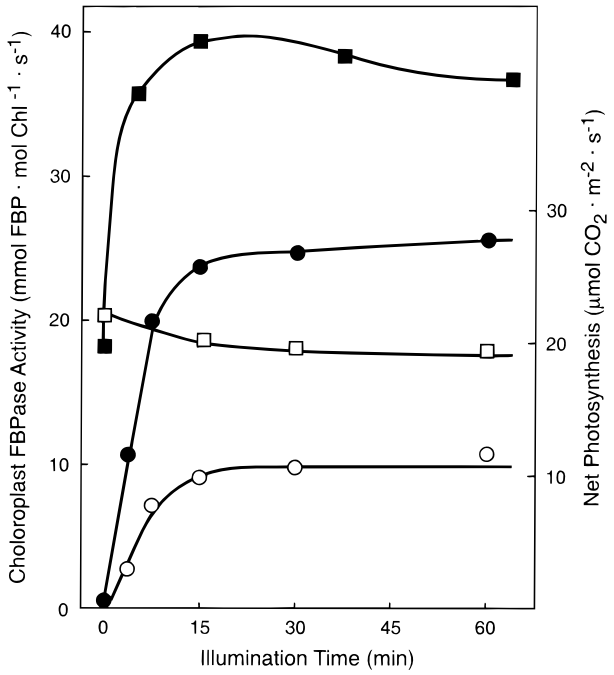

FIGURE 1: Light-dependent induction of photosynthesis and FBPase activity in tomato before (■, ●) and after (□, ○) light and low-temperature treatment. The induction of net photosynthesis (●, ○) as a function of illumination time was measured at 30 °C by infrared gas analysis on single attached tomato leaves at 1500 $\mu L \cdot L^{-1}$ CO_2 under PPFD of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Leaf disks were isolated at different times from separate plants under identical conditions to determine FBPase activity (■, □). The means of three independent FBPase determinations are plotted.

The absolute values determined for the midpoint potential of the regulatory sulfhydryls are dependent upon the E_h values assigned to various ratios of DTT^{red}/DTT^{ox} at pH 7.9. The initial value for the midpoint potential of DTT at pH 7 reported by Cleland (*34*) of −332 mV is well supported by more recent measurements employing diverse techniques (*34*, *35*). Since the redox reaction of DTT involves protons, the E_m of this redox buffer will have a substantial pH dependence that must be taken into account in E_h calculations. The reduced form of DTT is a very weak acid with the pK_a values of the thiols reported to be 9.2 for the first ionization and 10.1 for the second (*36*). Since DTT^{ox} is a very strong acid, the redox chemistry of DTT will involve one proton per electron at pH values below ∼8.2 (at higher pH values, ionization of the first thiol on DTT^{red} will lower the proton-to-electron ratio below 1). We have used the value of Lees and Whitesides (*37*) for the $E_{m,7}$ for DTT of −327 mV at 25 °C, giving a value for $E_{m,7.9}$ of −380 mV.

RESULTS

Chilling Inhibits the Light Activation of FBPase and SBPase but Not Other Reductively Activated Carbon Reduction Cycle Enzymes. Figure 1 illustrates that 6 h of illumination at 3 °C caused more than a 50% inhibition of light- and

Table 1: Effects of Light and Chilling Treatment on the Light Activation of Photosynthetic Carbon Reduction Cycle Enzymes

enzyme	treatment	dark act.[a] [mmol·(mol of chl)$^{-1}$·s^{-1}]	light act.[a] [mmol·(mol of chl)$^{-1}$·s^{-1}]
FBPase	control	17 ± 2	41 ± 3
	light and chill	19 ± 2	17 ± 2
SBPase	control	12 ± 3	37 ± 5
	light and chill	13 ± 4	15 ± 4
PRK	control	18 ± 2	39 ± 2
	light and chill	16 ± 2	34 ± 3
GAPDH	control	70 ± 8	106 ± 12
	light and chill	51 ± 11	118 ± 22

[a] Mean values and standard errors calculated for a minimum of three separate determinations.

CO_2-saturated photosynthesis measured after rewarming to 30 °C. Following this light and chill treatment, tomato plants had largely lost the ability to reactivate FBPase in the light (Figure 1, cf. ■, □) although quantitative western blot analysis revealed that there was no loss in the amount of this enzyme present in the leaf (data not shown). The light and chilling treatment also caused more than a 2-fold increase in the accumulation of radioactivity in FBP following a 2 min pulse-labeling with $^{14}CO_2$ (*20*), consistent with the loss of light-induced FBPase activation. We also observed a substantial increase in SBP labeling following the light and chilling episode (*20*), inferring an inability of the chilled plants to light-activate SBPase as well.

Table 1 shows that the loss of the light-dependent reductive activation of FBPase and SBPase by the light chilling treatment is in contrast to the behavior of other thioredoxin-mediated, reductively activated enzymes in the chloroplast. The light activation of PRK and GAPDH was largely unaffected by illumination at cool temperatures.

Low-Temperature Illumination Treatment Does Not Alter the Redox Characteristics of Tomato Chloroplast FBPase. FBPase isolated from tomato leaves illuminated at growth (26 °C) and chilling (4 °C) temperatures for 6 h was incubated for 10 min with various amounts of thioredoxin *f* in order to reveal any effects that chilling might have had on the affinity of FBPase for thioredoxin and thereby interfere with activation. Figure 2 shows, in the presence of 10 mM DTT^{red}, the effect of thioredoxin *f* on the rate of FBPase activation saturated at about 1 μM thioredoxin for enzyme isolated from either control (■) or chilled (□) leaves. This dependence on thioredoxin concentration is nearly identical to that reported by Prado et al. (*38*) and López-Jaramillo et al. (*39*) but differs from the unsaturable, first-order dependence of FBPase reduction rate on thioredoxin *f* concentration reported by Clancey and Gilbert (*40*).

Figure 3 shows the dependence of FBPase activity on the ambient redox potential at pH 7.9. Although the dithiol−disulfide exchange rates between thioredoxin and the target enzymes must be relatively rapid under physiological conditions in order to explain observed rates of light activation, this situation cannot be assumed for in vitro titrations. While the pH sensitivity of dithiol/disulfide exchange is well-known, more cryptic factors associated with enzyme substrates and cofactors may play important roles in determining the equilibration kinetics. Changes in the protein that affect the local electrostatic environment around the regulatory

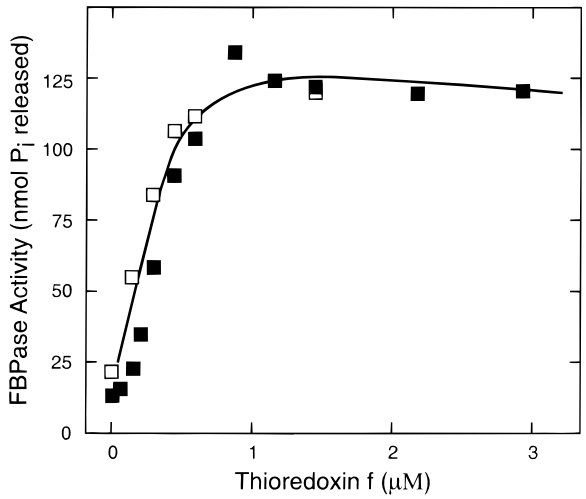

FIGURE 2: Dependence of FBPase activation on thioredoxin *f* concentration. FBPase isolated from tomato leaves before (■) or after (□) light and low-temperature treatment was incubated for 10 min in 100 mM tricine−NaOH (pH 8.0), 10 mM DTT^{red}, and the indicated concentration of thioredoxin *f*.

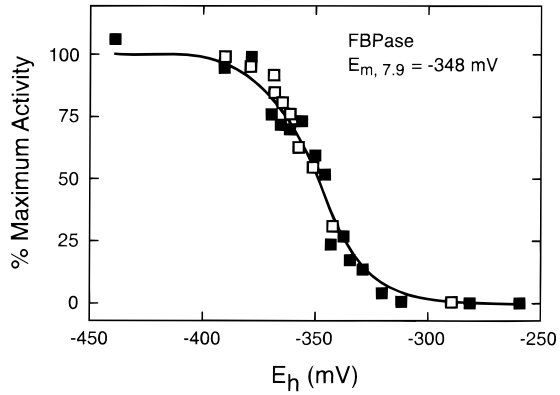

FIGURE 3: Equilibrium redox titration of thiol/disulfide regulatory groups on tomato chloroplast FBPase before (■) or after (□) light and low-temperature treatment. Purified FBPase was incubated at pH 7.9 for 1.5 h in the presence of 0.2 μM thioredoxin *f* and 10 mM DTT in various thiol/disulfide ratios. The dependence of FBPase activity was measured as phosphate release from fructose 1,6-bisphosphate as a function of the ambient redox potential (E_h) at 25 °C. The solid curve shows the nonlinear least-squares fit of the data to the Nernst equation with the value of *n* fixed at 2.

sulfhydryl groups or changes in the physical accessibility due to rearrangements within the protein could have large and unforeseen effects on the dithiol/disulfide exchange rate (*41*). In our experiments, we monitored the time course of enzyme activation in order to ensure that redox equilibrium had been attained. Under the conditions cited for the FBPase titration reported in Figure 3, maximum activity was achieved within 1.5 h, and we observed no loss of activity after 3 h of incubation at 25 °C (data not shown). Without thioredoxin *f*, the rate of activation of FBPase by direct thiol exchange with DTT was exceedingly slow, and equilibrium was not attained during the lifetime of the protein under these incubation conditions. In the presence of 0.2 μM thioredoxin *f*, we observed no dependence of the rate of FBPase activation on the total concentration of DTT present during the incubation (data not shown).

The solid line in Figure 3 depicts the nonlinear least-squares fit of the redox titration data to the Nernst equation

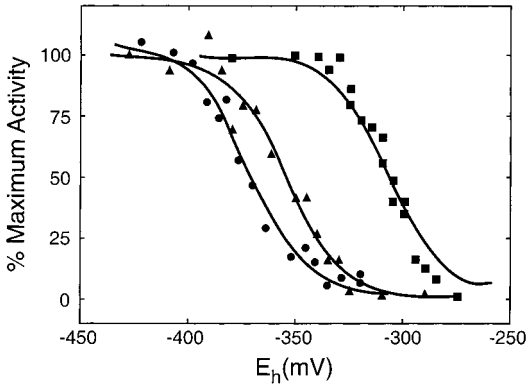

FIGURE 4: Equilibrium redox titrations of the reductive activation of tomato PRK (■), SBPase (▲), and GAPDH (●). Purified enzyme was incubated at pH 7.9 for 1.5 h in the presence of 0.2 μM thioredoxin *f* and 10 (SBPase and GAPD) or 80 (PRK) mM DTT in various thiol/disulfide ratios. The dependence of activity on the ambient redox potential (E_h) was measured at 25 °C. The solid curves depict the theoretical relationships for each protein calculated by fitting the data to the Nernst equation with the value of *n* fixed at 2.

with the value of *n* fixed at 2. This analysis shows that with the ambient potential poised at −350 ± 10 mV at pH 7.9, half the FBPase population will be in the dithiol form and half in the disulfide form. FBPase isolated from tomato leaves following low-temperature illumination (Figure 3, □) had the same E_h dependence for activation as the enzyme isolated from control leaves (Figure 3, ■).

The redox midpoint potentials for the regulatory sulfhydryl groups of thioredoxin-activated chloroplast enzymes span a broad range. Like FBPase, light activation of SBPase was inhibited in tomato by the light and chill treatment (Table 1). The E_h dependence of SBPase activation (Figure 4, ▲) was experimentally indistinguishable from FBPase. Light activation of PRK was not detectably altered by the light and chilling treatment, and the E_h dependence of PRK activity (Figure 4, ■) reveals the $E_{m,7.9}$ (−315 ± 10 mV) for reductive activation of this enzyme is 35 mV more oxidizing than for the bisphosphatases. Because the equilibrium constant between DTT and PRK is large in comparison to FBPase and SBPase, the accuracy of the PRK titration is more sensitive to impurities (including DTT^{ox}) in the DTT^{red} used to poise the ambient E_h (*35*, *42*). Thus, the actual experimental uncertainty of the $E_{m,7.9}$ for PRK may be somewhat larger than ±10 mV calculated from the experimental reproducibility.

The chloroplast ATPsynthase differs from other thioredoxin-regulated enzymes in that reduction of the regulatory sulfhydryl group is not required for catalytic activation of the enzyme. The light-dependent activation of the chloroplast ATPsynthase requires the formation of a threshold $\Delta\mu_{H^+}$, which in turn causes a conformational rearrangement that permits thioredoxin to interact with the regulatory sulfhydryl group on the γ subunit (*43*). The in situ activity of the ATPsynthase can be monitored in intact leaves by taking advantage of the contribution that the electric potential of the $\Delta\mu_{H^+}$ makes to flash-driven ATP formation (e.g., see refs *44*−*46*). In photosynthetic membranes, the fate of the electric potential can be conveniently monitored through its effect on the absorption spectrum of a specialized group of pigments within the membrane. Membrane depolarizing

Table 2: Effect of Light and Chilling Treatment on the Light-Dependent Reduction of the Chloroplast ATPsynthase

	ΔA_{518} relaxation half-time (ms)		
treatment	dark-adapted[a]	light-adapted[b]	% reduction[c]
control	50	15	100
light and chill	49	31	51

[a] Tomato plants were dark-adapted for 12 h prior to excitation with 3 closely spaced saturating single-turnover xenon flashes. [b] Light adaptation was performed by illumination of dark-adapted plants for 30 s with 300 μmol·m^{-2}·s^{-1} of red light followed by a 2 min dark interval to permit relaxation of the $\Delta\mu H^+$ formed during the light adaptation (*45*). [c] % reduction (i.e., dithiol/disulfide ratio) was determined as $[(t_{1/2}\text{ dark-adapted} - t_{1/2}\text{ light-adapted})^{\text{chilled}}/(t_{1/2}\text{ dark-adapted} - t_{1/2}\text{ light-adapted})^{\text{control}}] \times 100$. The light-adapted control was considered to have 100% of the ATPsynthase in the dithiol form.

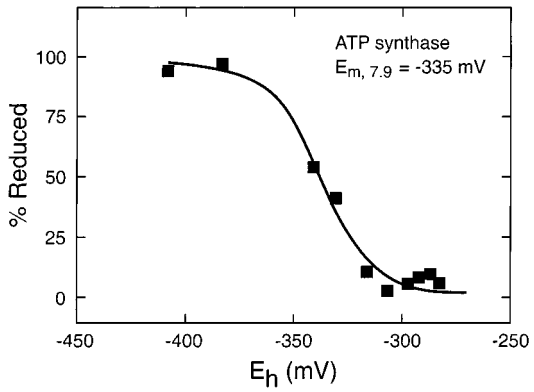

FIGURE 5: Determination of the oxidation−reduction midpoint potential of the regulatory dithiol/disulfide couple located on the chloroplast ATPsynthase γ subunit. Purified thylakoid membranes equivalent to 0.4 mM chlorophyll were incubated in darkness for 1.5 h at 4 °C in 50 mM tricine−NaOH (pH 7.9), 5 mM $MgCl_2$, in the presence of 0.2 μM thioredoxin *f* and 10 mM DTT in various thiol/disulfide ratios. The solid curve shows the nonlinear least-squares fit of the data to the Nernst equation with the value of *n* fixed at 2.

proton efflux through the ATPsynthase associated with ATP synthesis results in an accelerated relaxation of the electric field-associated absorption change (*47*). This approach can be extended to monitor the reduction of the ATPsynthase since reduction of the γ subunit significantly lowers the energetic threshold, and therefore the number of actinic flashes, necessary to activate the ATPsynthase and initiate ATP formation (*48*). The wavelength dependence of the flash-induced absorbance change in leaves has been published elsewhere (*45*) and is dominated by the electrochromic bandshift showing an absorption change maximum at 518 nm. The effect of the light and chilling treatment on the reduction of the chloroplast ATPsynthase [Table 2 and (*29*)] was intermediate (i.e., ∼50%) between the complete inhibition of light activation of the bisphosphatases and the complete insensitivity of PRK (Table 1). Figure 5 shows that the $E_{m,7.9}$ for reduction of this enzyme (−330 ± 15 mV) measured on isolated thylakoid membranes is also intermediate between the bisphosphatases and PRK.

Seemingly breaking the emerging pattern between the sensitivity of catalytic activity to illumination at low temperature and the redox midpoint point potential of the regulatory sulfhydryl groups, light activation of GAPDH is unaffected by the light and chilling treatment (Table 1) whereas the $E_{m,7.9}$ for the reductive activation of this enzyme

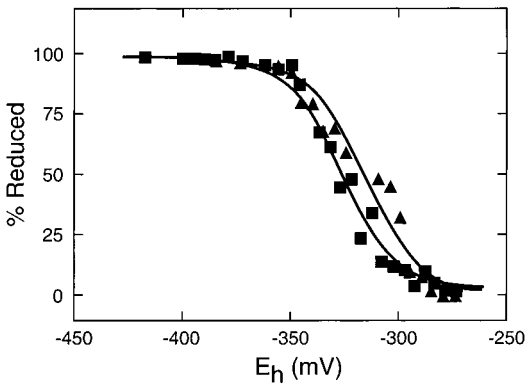

FIGURE 6: Oxidation−reduction titrations of *E. coli* thioredoxin (▲) and spinach chloroplast thioredoxin *f* (■) at pH 7.9. The solid curves depict the theoretical relationships for each protein calculated by fitting the data to the Nernst equation with the value of *n* fixed at 2.

(−360 ± 10 mV) is as at least as reducing as the bisphosphatases (Figure 4, ●).

The Midpoint Potential of Thioredoxin f Is Nearly Equipotential with the Chloroplast ATPsynthase. Samples of chloroplast thioredoxin f or *E. coli* thioredoxin were equilibrated with various ratios of $DTT^{red/ox}$ redox buffer as described for FBPase titrations. The thioredoxins were then reacted with the sulfhydryl reagent *N*-ethylmaleimide according to the procedure of Rebeille and Hatch (*49*) to inactivate the dithiol form of the protein. The disulfide form of thioredoxin that remained unlabeled after *N*-ethylmaleimide treatment was then reduced with 10 mM DTT and assayed for its ability to activate oxidized FBPase.

The $E_{m,7.0}$ for *E. coli* thioredoxin of −260 mV is well established in the literature (*50*, *51*). This is equivalent to an $E_{m,7.9}$ of −315 mV providing that *E. coli* thioredoxin thiols are very weak acids (i.e., pK_a > pH 9.5) and that the disulfide of oxidized thioredoxin is a strong acid, thus causing the E_m to have a −59 mV/pH dependence at pH values below 8. This extrapolated value for *E. coli* thioredoxin agrees well with our measured value of $E_{m,7.9} = -320 \pm 10$ mV. Our measurements (Figure 6) indicate that spinach chloroplast thioredoxin *f* is essentially equipotential ($E_{m,7.9} = -325 \pm 10$ mV) with the *E. coli* protein.

Redox Poising of Intact Leaves Confirms the Broad E_m Range for Thioredoxin-Regulated Enzymes Identified by in Vitro Titrations. Figure 7 depicts the energetic positioning at pH 7.9 of thioredoxin *f* to five of its target enzymes for which dithiol/disulfide exchange titrations were performed in this study. This pH was selected for the redox determinations because the pH of the stroma is believed to increase from about pH 7.0 to about pH 7.9 as a result of illumination (*33*). The vertical bars depict the redox range over which the dithiol/disulfide ratio changes from 0.1 to 0.9 for each protein. This illustration shows that the $E_{m,7.9}$ of thioredoxin *f* is positioned energetically below most of its target enzymes while still maintaining a substantial energetic overlap with each reaction partner.

To validate the physiological significance of the electron-sharing equilibria depicted in Figure 7, dark-adapted tomato leaves were vacuum-infiltrated with 30 mM DTT in 20 mM HEPES buffer at pH 7.9. The dithiol/disulfide ratio of the DTT mixture was selected to poise the redox potential of

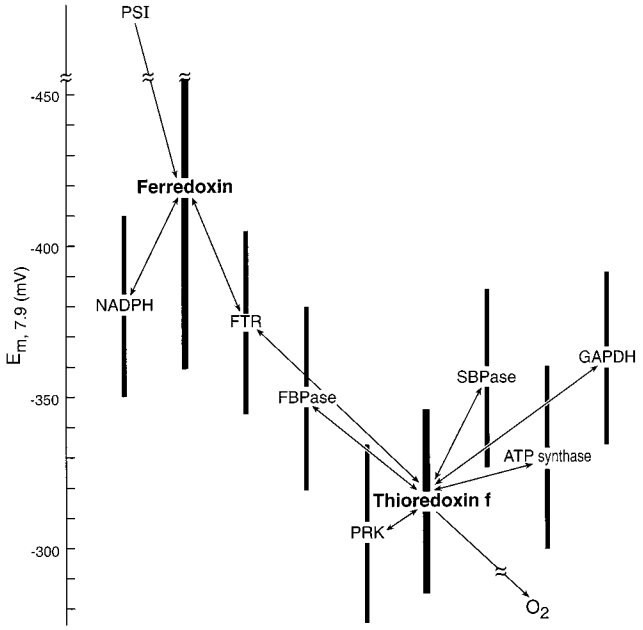

FIGURE 7: Scheme showing the energetic relationship of the thiol/disulfide exchange between thioredoxin *f* and its various reaction partners within illuminated tomato chloroplasts (i.e., pH 7.9). The vertical bars represent the redox potential range of the species from 10% oxidized to 10% reduced (i.e., 59 mV for an $n = 2$ reaction).

Table 3: Reduction/Activation of PRK, ATPsynthase, and FBPase upon Poising Intact Tomato Leaves at E_m of Thioredoxin *f*

enzyme	E_h[a] (mV)	% reduction/activation[b]
PRK	−325	61 ± 12
ATPsynthase[c]	−325	25 ± 8
FBPase	−325	10 ± 7

[a] Dark-adapted tomato leaves were vacuum-infiltrated with 30 mM DTT in 20 mM HEPES buffer at pH 7.9. [b] Mean values and standard errors calculated for a minimum of five separate determinations. [c] Conducted as for dark-adapted conditions in Table 2.

the leaf at −325 mV, the midpoint potential determined for thioredoxin *f* (Figure 6). The in vitro titrations depicted in Figure 7 predict that under this redox poise, FBPase would be <10% activated whereas PRK activity would be 75% of maximum. Table 3 illustrates good agreement with these predictions although PRK was only 60% activated, indicative of a slightly more negative E_m under in situ conditions. The 25% reduction level of the ATPsynthase agrees closely with expectation from the in vitro titration (Figure 5).

DISCUSSION

The chloroplast thioredoxin regulatory pathway begins with the ferredoxin-dependent reduction of a cystine disulfide at the active site of ferredoxin:thioredoxin reductase (FTR), forming two cysteine thiols. This enzyme is a ∼26 kDa heterodimer located in the chloroplast stroma (*52*) and contains a unique (4Fe-4S) cluster that couples the single-electron redox chemistry of ferredoxin with the two-electron dithiol/disulfide exchange reaction at the active site (*53*). FTR functions as a protein disulfide reductase in catalyzing an intermolecular dithiol transfer to either form of chloroplast thioredoxin (i.e., *f* or *m*). Equilibrium redox titrations of spinach FTR by Hirasawa et al. (*54*) show an $E_{m,7.0}$ of −320 mV, and, protein instability problems at alkaline pH notwithstanding, their work implies an $E_{m,7.9}$ near −375 mV.

Higher plant chloroplast thioredoxins, like thioredoxins from all photosynthetic organisms, have an identical active site sequence to that of the analogous protein of *E. coli*, Trp-Cys-Gly-Pro-Cys; the two cysteines of the active site form a disulfide bond in the oxidized state of the protein (*55*). Thioredoxins *f* and *m* have differential but overlapping reactivities with the ensemble of redox-regulated target enzymes (*53, 56*). As a protein disulfide oxidoreductase, thioredoxins are exceptionally reactive within the physiological pH range, perhaps 4 orders of magnitude more reactive than the best chemical redox reagents in disulfide exchange reactions involving certain protein thiols (*57*). The $E_{m,7.9}$ of -325 ± 10 mV that we measured for thioredoxin *f* is, when corrected for the small difference in the E_m of DTT used in the calculations, about 20 mV more oxidizing than the value extrapolated from the pH dependence of E_m recently determined for spinach thioredoxin *f* by Hirasawa et al. (*54*). Our value for the equilibrium midpoint potential of *E. coli* thioredoxin is again somewhat more positive ($\sim$15 mV) than the value of Knaff and co-workers (*54*) but is nearly identical ($\sim$5 mV more positive) with other values in the literature (*50, 51*) assuming a -59 mV/pH dependence.

We determined that the tomato chloroplast bisphosphatases, FBPase and SBPase, have experimentally indistinguishable $E_{m,7.9}$ values of about -350 ± 10 mV. Since these enzymes work in tandem in the photosynthetic carbon reduction pathway, it is sensible that their activity be coherently regulated. Assuming a -59 mV/pH unit dependence of the FBPase E_m (*54*), our $E_{m,7.9}$ determination (Figure 3) is about 15 mV more oxidizing than the $E_{m,x}$ recently reported for pea (*54*). We also performed titrations of FBPase provided by Peter Schürmann isolated from *E. coli* that had been transformed with the cloned spinach gene. We obtained an $E_{m,7.9}$ for recombinant spinach FBPase essentially identical to the tomato enzyme of -350 ± 10 mV (data not shown). Both Schürmann's and Knaff's laboratories independently corroborated this value on identical samples of recombinant spinach FBPase (personal communications), thus supplanting the 30 mV more oxidizing value earlier reported for the spinach enzyme (*54*). Our E_m determination for the regulatory disulfide/dithiol couple of tomato SBPase (Figure 4) is the first published value for this enzyme.

Recent investigations of the redox regulation of FBPase portend unexpected complexity. Site-directed mutagenesis studies with the pea enzyme suggest a potential regulatory role for three different cysteines, which has led to the proposal that the redox regulation of FBPase involves alternative partnering of Cys-153 with either Cys-173 or Cys-178 (*58*−*60*). But, redox titrations of FBPase activity at pH values of 7.0 and above provide the best fit in tomato (Figure 2), pea (*54*), and spinach (*40, 54, 61*) when the Nernst equation is evaluated for a single $n = 2$ component consistent with the involvement of a single disulfide/dithiol couple in the activation. These data do not necessarily rule out a mixed partnering in disulfide formation if the $E_{m,x}$ values were within about 10 mV, and, at pH values below 7, there are indications for the presence of two separate redox components in the activation process of spinach FBPase (*54*). Nevertheless, recently refined structural information on the pea enzyme indicates that a C153/C178 disulfide could not form in the native protein (*62*) even though it almost certainly does in the C173S mutant. Thus, although the equivalent of C178 in pea is rigorously conserved in all thioredoxin-regulated FBPase enzymes, the involvement of more than a single disulfide/dithiol couple in FBPase activation under physiological conditions remains to be validated. With the exception of the recent work of Dunford et al. (*63*), the molecular basis for redox control of SBPase activity has been relatively little studied.

The proton-translocating F_0F_1-ATPsynthase is a ubiquitous, multisubunit enzyme present in the plasma membrane of eubacteria, the inner mitochondrial membrane, and the photosynthetic membranes of higher plants, algae, and cyanobacteria. While the activities of all ATPsynthases are intricately regulated, redox regulation is a unique feature of the chloroplast ATPsynthase from algae and higher plants. The CF_1 γ subunit contains an intrapeptide disulfide bond between Cys-199 and Cys-205, which is located in an extra domain of the chloroplast subunit that is not present in the mitochondrial or bacterial γ subunit sequence (*64*). This cystine disulfide is the target of thioredoxin in the physiological reduction pathway (*65*−*68*). Although there was an initial suggestion of no differential selectivity between thioredoxin *f* and thioredoxin *m* in γ subunit reduction (*69*), later work showed clear specificity for thioredoxin *f* (*70*). The redox titration depicted in Figure 5 is the first reported for γ subunit reduction.

Whereas our titrations for spinach thioredoxin *f*, spinach and pea FBPase, and *E. coli* thioredoxin agree reasonably well with the determinations by Knaff and collaborators, the $E_{m,7.9}$ that we obtained for tomato PRK is about 35 mV more oxidizing than their determinations for this enzyme from spinach (*54, 70, 71*). This difference seems convincingly outside the $\pm$20 mV uncertainty for the difference between two E_m values, each with an experimental uncertainty of $\pm$10 mV. These very different E_m values likely represent true differences between species. PRK is unique among thioredoxin-regulated chloroplast enzymes in that one of the regulatory cysteines is also involved in facilitating catalysis (*72, 73*). PRK may also be unique in having pK_a values of the regulatory cysteines within the physiologically relevant pH range. Hirasawa et al. (*54*) estimated pK_a values of 8.1 and 8.6 for the regulatory cysteines of spinach PRK from E_m vs pH data fit in three different regions to slopes of -60 mV/pH, -30 mV/pH, and in the most alkaline range, 0 mV/pH. These data corroborate an earlier pK_a value of 7.9 for the more acidic pK_a of spinach PRK estimated from the pH dependence of the rate of alkylation for Cys-16 (*72*). The more oxidizing $E_{m,7.9}$ that we observed for tomato PRK could, within $\pm$20 mV experimental uncertainty, be accounted for if the corresponding cysteine on the tomato enzyme were about one-half pH unit more acidic. Since thioredoxin *f* has an -59 mV/pH unit dependence throughout the physiological range (*54*), the acidic regulatory cysteine of PRK introduces a pH-dependent (i.e., light intensity dependent) ΔE for the reductive activation of PRK. Adjustments to the pK_a of this cysteine may compensate for differences in the light intensity dependence of the stromal pH among different species.

We set out to explain the differential effect that chilling and light treatment has on the light-dependent, reductive activation on thioredoxin-regulated chloroplast enzymes in tomato. There is plentiful evidence that illumination of chilling-sensitive plants at low temperature causes an enhanced production of photosynthetically and photochemically

generated reactive oxygen species that can outpace the protective and detoxification process of the chloroplast (*4*, *5*), thereby expending a substantial portion of the chloroplast's reductant reserves. Figure 7 provides the quantitative basis for explaining how the resultant lowered redox poise of the stroma could be translated into the differential effect of chilling that we observed on the reductive activation of tomato chloroplast enzymes. For example, were the chilling and light treatment to cause a lowering of the redox poise of thioredoxin *f* in the light to its midpoint potential, Figure 7 illustrates that, while ~75% of PRK could be (i.e., at equilibrium) in the reduced state at pH 7.9, FBPase and SBPase would be only minimally reduced. When we mimicked this situation by poising intact tomato leaves in the dark at −325 mV (Table 3), this prediction was largely borne out although PRK activation reached only 60%. The intermediate midpoint potential and the intermediate effect of the chilling and light treatment on ATPsynthase reduction (Tables 2 and 3) further support this interpretation. However, the $E_{m,7.9}$ for the regulatory disulfide/dithiol couple of GAPDH was the most negative of any that we measured, yet we did not observe any effect of the chilling and light treatment on the reductive activation of this enzyme (Table 1). Although we do not have an explanation of this seeming discrepancy, an important caveat in the interpretation of these in vitro E_m determinations is that there is only very limited information available about the influence that physiological conditions may have on individual and therefore on the hierarchy of values presented in Figure 7. It is well established that the catalytic activity of each of the thioredoxin-modulated chloroplast enzymes depends on factors in addition to the redox state (e.g., see refs *52*, *74*).

Evidence of the intricacy and interdependence of different modes of regulation is perhaps nowhere more evident than for GAPDH. For example, there are numerous indications for the involvement of GAPDH in multienzyme complexes (e.g., see refs *28*, *75*), including recent evidence for a redox-sensitive complex involving GAPDH, PRK, and the small nuclear-encoded chloroplast protein CP12 (*76*, *77*). Thus, while the electron-sharing equilibria among thioredoxin *f* and its target enzymes can, and we believe largely does, explain the differential effects of chilling-induced photooxidation on the thioredoxin-mediated activation of chloroplast enzymes that we observed in tomato, GAPDH may be an example of a situation in which the complexity of the regulation defies straightforward interpretation of the in vitro redox titrations.

Our results summarized in Figure 7 also contribute to an emergent data set forming a quantitative basis to evaluate the influence of thioredoxin/target enzyme redox equilibria on the differential kinetics and light intensity profile for the activation and deactivation of thioredoxin-regulated chloroplast enzymes. Whereas the reduction of the chloroplast ATPsynthase γ subunit disulfide occurs rapidly even at exceedingly low irradiance levels (i.e., 20 μmol·m^{-2}·s^{-1}) and clearly is not rate-limiting in the induction of photosynthesis (*78*), the activation of other of the chloroplast enzymes regulated by thioredoxin requires much more light and may play a central role in the slow induction of photosynthetic carbon reduction. FBPase and NADPH−malate dehydrogenase are widely reported to activate slowly even under bright illumination (*79*−*82*). Figure 1 shows that more than 10 min of illumination at 1000 μmol·m^{-2}·s^{-1} is required for full activation of FBPase and full induction of net photosynthesis in tomato. In contrast to the carbon reduction cycle enzymes, activity of glucose-6-phosphate dehydrogenase is inhibited by thioredoxin-dependent reduction. Reductive inhibition of glucose-6-phosphate dehydrogenase saturates at very low irradiance levels, ensuring that the oxidative and reductive pentose phosphate pathways do not operate antagonistically. Additionally, Farr et al. (*83*) showed that the oxidative pentose phosphate pathway can be activated and the carbon reduction cycle inactivated in N-limited *Chlamydomonas* cells in the light by the addition of nitrate, illustrating a sophisticated role for redox control in coordinating enzyme activity to meet alternative physiological needs for photosynthetic reductive power. Recently, it has become evident that dithiol/disulfide redox regulation is the basis for light induction of translation of the *psbA* message in *Chlamydomonas* (*84*), light activation of rubisco by rubisco activase (*85*) and acetyl-coenzyme A carboxylase (*86*), and light-dependent control of the aggregation of carbon reduction cycle enzymes (*77*), and there is every expectation that others will soon be discovered. Earlier we produced a model that sought to simulate the regulation of three thioredoxin target enzymes based on the redox properties of the proteins that were known at the time (*78*). Although this computer simulation was based on very limited redox data and did not take into account complexities in the regulation of these enzymes that have since become evident (e.g., see refs *54*, *58*−*61*), the model nevertheless successfully simulated many facets of the behavior of enzyme regulation and highlighted the central role that electron-sharing equilibria likely play. Substantially more sophisticated and far-reaching models should now be possible that would make testable predictions about the mechanism and coordination of chloroplast processes by redox modulation.

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