

Characterization of the regulatory function of the 46-kDa isoform of Rubisco activase from *Arabidopsis*

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Abstract

Arabidopsis Rubisco activase was recently shown to be regulated by redox changes in the larger (46-kDa) isoform specifically mediated by thioredoxin-f [Zhang and Portis (1999) Proc Natl Acad Sci USA 96: 9438–9443]. Reduction greatly increases the activity of the 46-kDa isoform and the native protein at physiological ATP/ADP ratios. In this study we conducted additional experiments to characterize the regulation of Rubisco activase by thioredoxin-f. The K_m for both ATP hydrolysis and Rubisco activation by the 46-kDa isoform was lowered by 4 to 5-fold after reduction, but the maximum activity was increased by only 10%. Only 0.35 μ M thioredoxin-f was required for a half-maximal activity change after a 10 min preincubation and activation with 1 μ M was complete after 10 min. Equal amounts of 46-kDa and 43-kDa isoforms were required for a complete inhibition of the Rubisco activation activity after a reduction-oxidation cycle and assay at an ATP/ADP ratio of 3:1, whereas activity was only inhibited by 50% at a 2:1 ratio (43-/46-kDa) of the isoforms. This requirement is consistent with the fact that *Arabidopsis* normally contains about a 1:1 ratio of the two isoforms at both the mRNA and protein levels. Redox titrations indicated a midpoint potential of -344 mV for the 46-kDa isoform as compared to -342 mV for spinach fructose 1,6-bisphosphatase at pH 7.9, consistent with previous reports indicating that these proteins are co-regulated by light intensity in a similar manner.

Abbreviations: FBPase – fructose 1,6-bisphosphatase; RuBP – ribulose 1,5-bisphosphate

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (Rubisco) initiates photosynthetic carbon acquisition and is regulated by the enzyme, Rubisco activase (Portis 1992). By hydrolyzing ATP, Rubisco activase regulates the activity of Rubisco by removing the inhibitory sugar phosphates, RuBP and carboxyarabinitol 1-phosphate (Wang and Portis 1992; Robinson and Portis 1988). RuBP can bind to the inactive, uncarbamylated form of Rubisco and thus prevents carbamylation that is necessary for the activation of

the enzyme (Jordan and Chollet 1983). In some plants, carboxyarabinitol 1-phosphate can bind to the carbamylated form of Rubisco, blocking the access by substrate, RuBP (Seemann et al. 1990). Changes in the ATP/ADP ratio corresponding to the light/dark change in stroma can regulate the activity of Rubisco activase (Robinson and Portis 1989). However, an explicit mechanism to account for the light modulation of activity was only discovered recently (Zhang and Portis 1999) when it was found that the activity of Rubisco activase activity is also modulated by redox changes, which are specifically mediated

by thioredoxin-f. The activity of Rubisco activase *in vivo* probably reflects the integrated control by both systems.

In most plants examined to date, two isoforms of Rubisco activase co-exist in stroma and are produced by alternative splicing of a single gene transcript (Werneke et al. 1989). The alternative splice sites are known in *Arabidopsis*, spinach, barley and rice (Werneke et al. 1989; Rundle and Zielinski 1991; To et al. 1999). There is only a single nuclear gene encoding Rubisco activase in *Arabidopsis*, spinach and rice. There are two closely linked genes in barley with one encoding the shorter isoform and another one producing a pre-mRNA that is alternatively spliced to generate one shorter isoform mRNA and one larger isoform mRNA. The redox regulation of Rubisco activase depends on the larger isoform with two conserved cysteine residues (C392 and C411 in *Arabidopsis*) located near the C-terminus. The larger isoform can regulate the activity of both isoforms when both isoforms go through a reduction-oxidation cycle mediated by thioredoxin-f (Zhang and Portis 1999).

Many enzymes in the chloroplast are regulated by the ferredoxin/thioredoxin system, a light signaling system (Buchanan 1991; Schürmann and Jacquot 2000). Light-driven photosynthetic electron transport reduces ferredoxin, which then can donate electrons to ferredoxin:thioredoxin reductase. This enzyme then reduces the thioredoxins, which reduce and thereby activate or inactivate their target metabolic enzymes. With the elucidation of the 3-dimensional structures of ferredoxin:thioredoxin reductase, thioredoxins, and two target enzymes, FBPase and NADP-malate dehydrogenase, the molecular details of the pathway are rapidly being understood (Schürmann and Jacquot 2000). Glucose 6-phosphate dehydrogenase is the only enzyme down-regulated by reduction through this system whereas all the other enzymes are up-regulated. Among the target enzymes, FBPase and NADP-malate dehydrogenase have been the two most thoroughly examined. Both enzymes can be activated at very low thioredoxin concentrations in very short time when examined *in vitro* (Geck et al. 1996; Schepens et al. 2000).

Since Rubisco activase is a newly found member regulated by the ferredoxin-thioredoxin system, understanding how it is regulated by thioredoxin as compared to the other enzymes is important. In this study we examined the time and concentration requirements for activation of Rubisco activase by thioredoxin-f. We

also show that reduction alters the affinity of the larger isoform for ATP and we examined the stoichiometry of the interaction between two isoforms for the activity change after a reduction-oxidation cycle. Based on this information, the composition of the C-terminal domain unique to the larger isoform, and a possible analogy with the redox control of malate dehydrogenase, we propose a potential mechanism for how the larger isoform regulates the activity of both isoforms.

Materials and methods

Cloning of thioredoxin-f cDNA from spinach

Total RNAs from 1–2-week-old spinach leaves were extracted as described (Qiagen plant RNA extraction kit). Two primers were designed based on the cDNA sequence of spinach thioredoxin-f (Aguilar et al. 1992). The upstream primer (corresponding to the amino acid sequence M-E-Q-A-L-G-T, which is the N-terminus of the mature peptide), contained a *NcoI* restriction site (underlined hexamer) and had the following sequence: 5'-CACA CCATGGAA CAA GCT TTG GGT ACC CAA-3'. The downstream primer (corresponding to the C-terminus of the mature peptide), contained a *BamHI* site (underlined hexamer) and had the following sequence: 5'-CCCC GGATCC TCA ACT ACT TCG AGC AGC TTG TAT-3'. RT-PCR was conducted as described (Zhang 2000). The RT-PCR products were digested with *NcoI* and *BamHI* and ligated to the expression vector, pET28a. The resulting plasmid was named pettrxf and was transformed into *Escherichia coli* (BL21DE3).

Purification of recombinant thioredoxin-f

E. coli (BL21DE3) cells transformed with the expression vector containing thioredoxin-f cDNA from spinach were grown and induced as described (Zhang 2000). Recombinant protein was isolated as reported (Hodges et al. 1994), but with the following modifications. After collecting the proteins precipitated between 35–90% $(\text{NH}_4)_2\text{SO}_4$ saturation, the sample was then eluted through a 100 ml Sephadex G-25 column with a buffer of 30 mM Tris-HCl (pH 7.9) and 0.2 M NaCl. After analysis of fractions with a 15% gel by SDS-PAGE, the fractions containing thioredoxin-f were collected and desalted into 30 mM Tris-HCl (pH 7.9) with a 25 ml Sephadex G-25 column. The sample was then loaded on a 20 ml Q-Sepharose column and a gradient (200 ml) from 0 to 23 mM NaCl

in 30 mM Tris-HCl (pH 7.9) was applied. Proteins eluting between 10 and 20 mM NaCl were collected, concentrated and analyzed with a 15% gel by SDS-PAGE. The preparation was divided into aliquots suitable for individual experiments and these were rapidly frozen and stored at -80°C until use. The protein was more than 95% pure and thus the amount of thioredoxin was determined by absorbance at 280 nm multiplied by a coefficient of 0.85 mg/ml (Hodges et al. 1994).

Assay of the ATPase activity of Rubisco activase

When ADP was initially present in the assay buffer, ATP hydrolysis was measured by the release of inorganic phosphate as previously described (Zhang and Portis 1999). Otherwise, ATP hydrolysis was measured by ADP production in a coupled spectrophotometric assay as reported (Wang and Portis 1992), but with the following modifications. The assay mixture contained 50 mM tricine pH 8.0, 20 mM KCl, 10 mM MgCl_2 , various concentrations of ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 40 U/ml pyruvate kinase and 40 U/ml lactate dehydrogenase in a total volume of 0.8 ml. The reaction was performed at 25°C and initiated by the addition of Rubisco activase to a final concentration of 25 $\mu\text{g/ml}$. The rate of ADP production was determined from the rate of NADH oxidation indicated by the decrease in absorbance at 340 nm.

Assay of the Rubisco activation activity of Rubisco activase

To determine the dependence of the activation activity on ATP concentration, activation of the inactive Rubisco-RuBP complex by Rubisco activase was assayed spectrophotometrically as described previously (Kallis et al. 2000) with the following modifications. The assay buffer contained 100 mM Tricine-KOH (pH 8.0), 10 mM MgCl_2 , 40 mM KCl, 0.3 mM NADH, 0.4 mM RuBP, 10 mM NaHCO_3 , 11.7 $\mu\text{g ml}^{-1}$ Rubisco-RuBP, 2 mM phosphoenolpyruvate, various concentrations of ATP and the coupling enzymes as described (Kallis et al. 2000). Recombinant Rubisco activase (20 $\mu\text{g ml}^{-1}$) was added to the mixture to initiate the reaction. The Rubisco activation activity is expressed as the increase in Rubisco activity with time. To determine the effects of reduction and oxidation on the activation of Rubisco with different ratios of the two isoforms, the activity of Rubisco was fol-

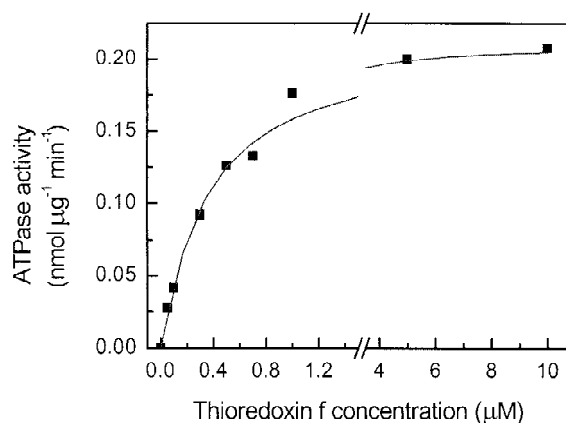


Figure 1. ATPase activity of the 46-kDa isoform after incubation with various concentrations of thioredoxin-f and 5 mM DTT for 10 min at room temperature. The ATPase activity was assayed by phosphate release at an ADP/ATP ratio of 0.33 (1 mM ADP/ 3 mM ATP). The data was fit to the Hill equation to estimate an $S_{0.5}$ of 0.35 μM .

lowed by the fixation of $[^{14}\text{C}]\text{NaHCO}_3$ as described previously (Zhang and Portis 1999).

Assay of FBPase activity

Approximately 0.27 unit recombinant FBPase (P. Schürmann, unpublished) was activated at room temperature in a 100 μl mixture containing 100 mM Tricine-NaOH (pH 7.9), 10 mM (total) reduced and oxidized DTT at various ratios, and 0.2 μg thioredoxin-f for at least 1.5 h (Hutchison et al. 2000). A 50 μl aliquot of the activation mixture was added to a 1 ml spectrophotometer cuvette containing 0.95 ml of the following reaction buffer: 100 mM Tris-HCl (pH 7.9), 10 mM MgSO_4 , 1.7 mM EDTA, 2.4 mM sodium fructose 1,6-bisphosphate, 1.75 units of phosphoglucose isomerase (EC 5.3.1.9, glucose-6-phosphate isomerase), 0.7 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 0.3 mM NADP. The FBPase activity was determined from the rate of NADP reduction as indicated by the increase in absorbance at 340 nm (Schürmann 1995).

Results

Thioredoxin-f concentration and time dependence for the redox regulation of the ATPase activity

The concentration of thioredoxin-m has been reported to be 100 to 160 μM (Scheibe 1981) in plants.

Although there is no similar report about thioredoxin-f, the concentration of thioredoxin-f in the stroma is likely to be similar because of the need to service a high concentration of the target enzymes (Harris and Koniger 1997). The redox regulated chloroplast enzymes examined to date have high affinities for thioredoxin. For example, FBPase was reported to reach half of its maximum activity with about $0.2 \mu\text{M}$ thioredoxin-f (Hutchison et al. 2000) and $4 \mu\text{M}$ was reported for the plastidic acetyl-CoA carboxylase (Sasaki et al. 1997) under similar experimental conditions. Hence the affinity of the 46-kDa isoform for thioredoxin-f was examined. In contrast to most of the other enzymes, almost no activation of the 46-kDa isoform by DTT is observed in the absence of thioredoxin-f (Zhang and Portis 1999). ATPase activity was measured at an ADP/ATP ratio of 1:3 after preincubation of 46-kDa isoform with various amounts of thioredoxin-f and DTT at room temperature for 10 min. As shown in Figure 1, the 46-kDa isoform only needed $0.35 \mu\text{M}$ thioredoxin-f to reach half of its maximum activity. A related experiment was conducted to examine the time required for thioredoxin-f to activate the 46-kDa isoform. The 46-kDa isoform reached its maximum activity in less than 10 min when preincubated with $1 \mu\text{M}$ thioredoxin-f and when incubated with $3 \mu\text{M}$ thioredoxin-f, activation was complete within 1 min (data not shown). This indicates that the 46-kDa isoform can react with low thioredoxin-f concentrations in a very efficient way.

Comparisons of ATP response with the reduced 46-kDa isoform, the oxidized 46-kDa isoform and the 43-kDa isoform

When reduced thioredoxin interacts with FBPase or NADP-MDH, it changes the conformation of the target enzyme and exposes the catalytic site to the substrate (Chiadmi et al. 1999; Johanson et al. 1999). There is no three-dimensional structure available for activase, but an alteration in the binding of ATP to activase can be detected by assaying the ATPase and Rubisco activation activities. As shown in Figure 2A, the amount of ATP needed for the 43-kDa isoform, the oxidized 46-kDa isoform and the reduced 46-kDa isoform to reach half of their maximum ATPase activities were 15, 164 and $32 \mu\text{M}$, respectively, and the corresponding V_{max} were 1.03 , 0.67 and $0.73 \mu\text{mol} \mu\text{g}^{-1} \text{min}^{-1}$. As shown in Figure 2B, similar results were obtained by assaying the Rubisco activation activity. In this case the amount of ATP needed for the half

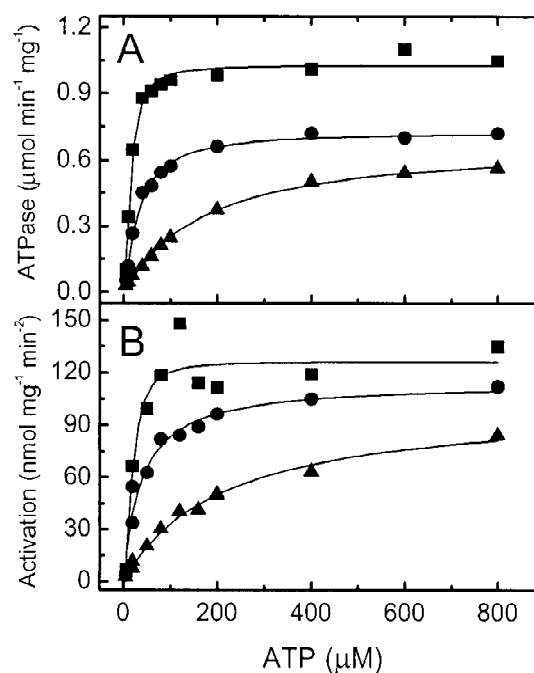


Figure 2. Dependence of the ATPase (A) and Rubisco activation (B) activities of the 43-kDa isoform (■), the reduced 46-kDa isoform (●) and oxidized 46-kDa isoform (▲) on ATP concentration in the absence of ADP. For the ATPase activity, the V_{max} ($\mu\text{mol} \mu\text{g}^{-1} \text{min}^{-1}$) and $S_{0.5}$ (μM) estimated by fitting the data to the Hill equation are: 1.03 and 15 for the 43-kDa isoform; 0.73 and 32 for the reduced 46-kDa; and 0.67 and 164 for the oxidized 46-kDa isoform. For the Rubisco activation activity, the estimated V_{max} ($\text{nmol} \text{mg}^{-1} \text{min}^{-2}$) and $S_{0.5}$ (μM) are 138 and 21 for the 43-kDa isoform; 115 and 38 for the reduced 46-kDa isoform; and 103 and 212 for the oxidized 46-kDa isoform.

maximum activities for the 43-kDa isoform, the oxidized 46-kDa isoform and the reduced 46-kDa isoform were 21, 212 and $38 \mu\text{M}$ respectively and the corresponding V_{max} with $20 \mu\text{g} \text{ml}^{-1}$ of activase were 138, 103, and $115 \text{nmol} \text{mg}^{-1} \text{min}^{-2}$. Therefore, when reduced, the affinity of the 46-kDa isoform for ATP increased by 4–5 fold, but was still lower than that of the 43-kDa isoform. Earlier, we observed that reduction of the 46-kDa isoform stimulated the ATPase activity by more than 30% when assayed in the absence of ADP (Zhang and Portis 1999). However the assay used in that experiment did not contain an ATP regenerating system to prevent the accumulation of ADP, which is more inhibitory for the oxidized isoform. Therefore, the fact that the V_{max} of the 46-kDa isoform after reduction increased by only about 10% and thus remained lower than that of 43-kDa isoform indicates that the additional amino acids at the C-terminus of the 46-kDa isoform have a negative effect

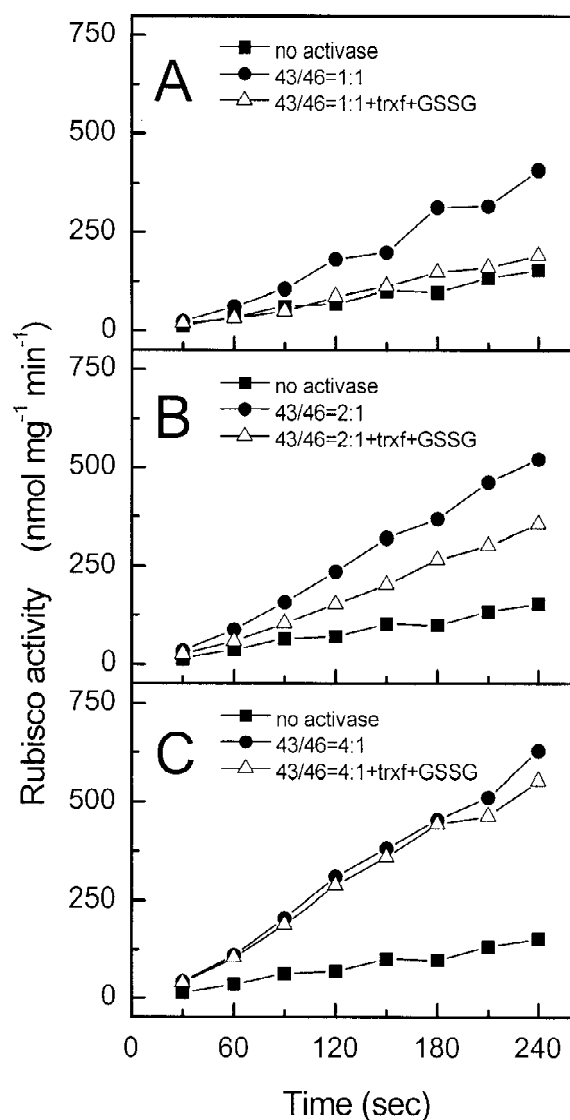


Figure 3. Effects of reduction and oxidation on the activation of Rubisco with (A) 1:1, (B) 2:1, (C) 4:1 ratios of the 43- and 46-kDa isoforms. The time course for the increase in Rubisco activity at an ADP/ATP ratio of 0.33 (1 mM ADP/ 3 mM ATP) in the absence of activase (■), the activase mixtures (●) and reduced/oxidized mixtures (Δ) are shown.

on the turnover of the enzyme. A similar conclusion with respect to the activation activity was reached in an earlier study in which a C-terminal deletion of the smaller spinach isoform was made (Esau et al. 1996).

Stoichiometric requirements for the inhibition of the activity of both isoforms by the 46-kDa isoform

When the 46-kDa isoform was combined at an equal

ratio with 43-kDa isoform, after a reduction-oxidation cycle the ATPase and Rubisco activation activities of both isoforms were almost completely eliminated at an ADP/ATP ratio of 1:3 (Zhang and Portis 1999). In order to determine how much of the 46-kDa isoform is needed to inhibit the activities of both isoforms at an ADP/ATP ratio of 1:3, the Rubisco activation activities of mixtures at 1:1, 2:1 and 4:1 ratios of the 43-kDa and 46-kDa isoforms were determined. As shown in Figure 3, only at a 1:1 ratio (A) of the two isoforms was the activity of both isoforms inhibited by almost 100% after a reduction-oxidation cycle. At a ratio of 2:1 (B), the activity of 43-kDa isoform was only inhibited by 50% and at a ratio of 4:1 (C) little inhibition of the activity after oxidation occurred. These results are consistent with the fact that in *Arabidopsis*, the mRNA and protein level of both isoforms are about equal.

Comparison of the equilibrium redox midpoint potentials for the activity changes in Rubisco activase and FBPase

In order to model the kinetics of activation and deactivation of the various thioredoxin regulated enzymes, accurate knowledge of the oxidation-reduction midpoint potentials (E_m) of the thiol/disulfide couples between thioredoxin and target enzymes is required. In turn, the midpoint potentials of the redox-regulated enzymes can be compared with the differential responses of these enzymes to light intensity (Kramer et al. 1990; Hutchison et al. 2000). As shown in Figure 4, redox titration of both enzymes at pH 7.9 gave excellent fits to the Nernst equation for the reduction of a single two-electron component. The midpoint potential of FBPase (-342 mV) agrees, within the experimental uncertainties of the measurements, with the value obtained from earlier studies (Hutchison et al. 2000). The behavior ($n = 2$ for the Nernst equation) of Rubisco activase is consistent with the involvement of one disulfide in the activation of the enzyme and supports the results of the site-directed mutagenesis studies. The similar values of the midpoint potentials for FBPase and Rubisco activase may account for the similar response of FBPase and Rubisco activation to the light intensity (Sassenrath-Cole et al. 1994).

Discussion

In this study, we show that the larger isoform of Rubisco activase in *Arabidopsis* can respond to a very

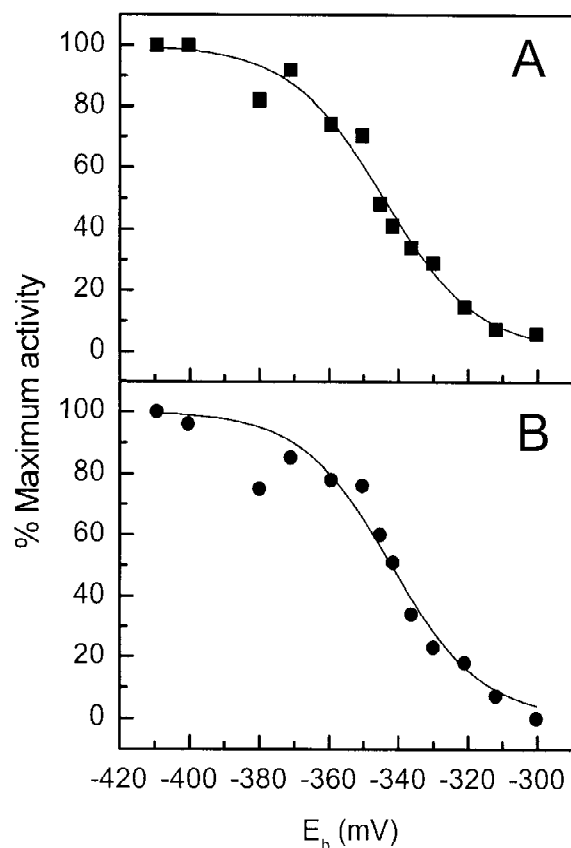


Figure 4. Equilibrium redox titration of thiol/disulfide regulatory groups on 46-kDa isoform (A) and spinach chloroplast FBPase (B). (A) Purified recombinant 46-kDa isoform was incubated at pH 7.9 for 10 min in the presence of 1 μ g thioredoxin-f and 10 mM DTT in various thiol/disulfide ratios. The dependence of ATPase activity was measured as phosphate release from ATP at an ADP/ATP ratio of 0.33 (1 mM ADP/ 3 mM ATP) as a function of the ambient redox potential (E_h) at 25 °C. (B) Purified FBPase was incubated at pH 7.9 for 1.5 h in the presence of 0.2 μ g thioredoxin-f and 10 mM DTT in various thiol/disulfide ratios. The dependence of FBPase activity was measured as the rate of NADP⁺ reduction as a function of the ambient redox potential (E_h) at 25 °C. The curves show the nonlinear least squares fit of the data to the Nernst equation with the value of n fixed at 2.

low thioredoxin-f concentration in a short time. This behavior is similar to that of the FBPase (Hutchison et al. 2000) and the midpoint potential of the larger isoform is also very similar to that of FBPase. These similarities may account for the similar response to light intensity of the activation states of Rubisco and FBPase observed *in vivo* (Sassenrath-Cole et al. 1994).

Earlier results with the recombinant spinach Rubisco activase showed that the larger isoform was more easily inhibited by ADP (Shen et al. 1991) and it was suggested that the larger isoform might have a regulat-

ory function. The recent discovery that the activity of the larger isoform is redox regulated (Zhang and Portis 1999) provided new support for this hypothesis. In our experiments, even after reduction, the maximum activity of the larger isoform only increased by about 10%, remaining about 20 to 30% lower than that of the shorter isoform. This result further indicates that the main function of the larger isoform is to regulate the activities of both isoforms. The regulatory role of the larger isoform was also revealed in our mixing experiments where an equimolar ratio of the larger isoform was able to completely inhibit the activity of the 43-kDa isoform at an ADP/ATP ratio of 1:3, typical of light conditions in the stroma. Furthermore, the 43-kDa isoform has been shown to have substantial ATPase activity under ADP/ATP ratio of 1:1 (Zhang and Portis 1999), the typical dark condition in stroma. With no need to activate Rubisco in the dark, 46-kDa isoform may be needed to shut down the activity of the 43-kDa isoform in order to conserve energy and allow optimal growth.

The necessity for an equimolar ratio of the two *Arabidopsis* isoforms in order to observe effective regulation *in vitro* is consistent with the observation that the two isoforms are present in equal amounts at both the protein (Salvucci et al. 1987; Eckardt et al. 1997) and mRNA level (Zhang 2000). However, this does not seem to be true for all species (Salvucci et al. 1987). For example, in spinach there is about twice as much of the shorter isoform at both the protein (Salvucci et al. 1987) and mRNA level (Werneke 1989). Furthermore, tobacco only appears to have the shorter isoform, but redox regulation of the activase is perhaps the best explanation for the observed Rubisco activation characteristics in this species (Ruuska et al. 2000). Clearly, the role of redox regulation in these other species and its relationship to the relative expression of the two isoforms will require further study.

The structures of NADP-MDH from sorghum (Johansson et al. 1999) and *Flaveria* (Carr et al. 1999) were determined recently. Incorporating previous site-directed mutagenesis studies of this enzyme, it was proposed that the C-terminal extension (381-388aa) is docked into the active site through a negatively charged residue (E387) (Ruelland et al. 1998) after the enzyme is oxidized. Several C-terminal mutants of the 46-kDa isoform (T410A, V412A, Y413A) did not interfere with the redox regulation of the 46-kDa isoform as might be expected if the C-terminus of activase also docks in the active site (N. Zhang, unpublished). However the K_m for ATP of the reduced

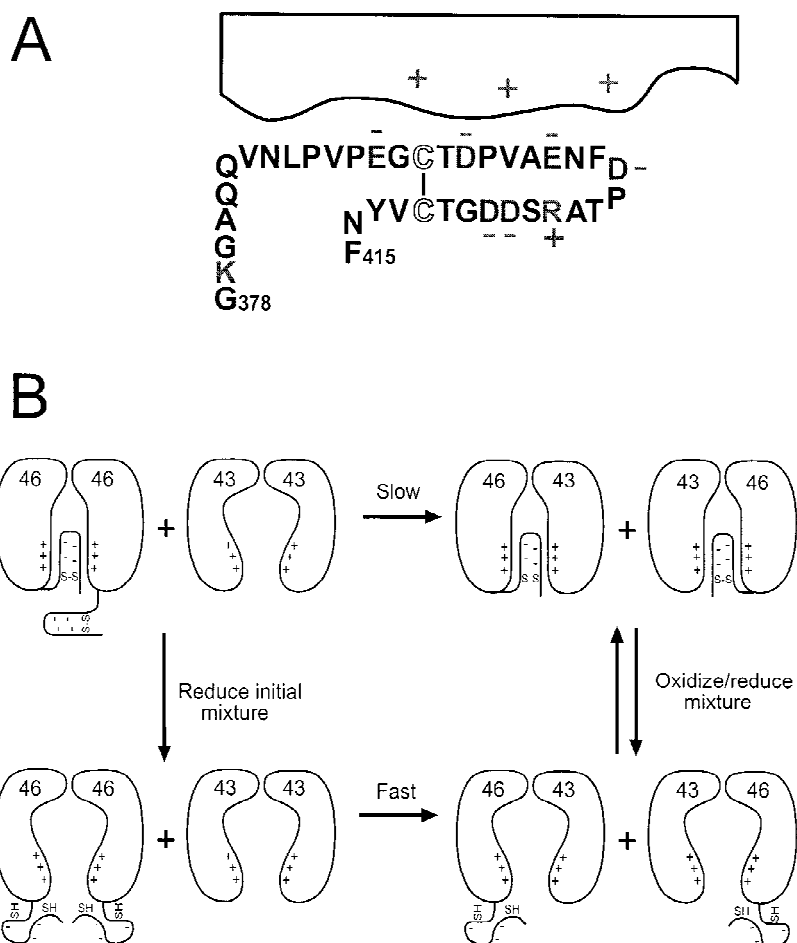


Figure 5. A schematic representation of a hypothesis for the redox regulation of both isoforms of Rubisco activase by the C-terminus of the larger isoform. (A) High negative charge density and formation of a disulfide bridge by oxidation allows docking of the C-terminus to a positively charged site in or adjacent to the nucleotide binding site of activase, altering its nucleotide binding properties and possibly causing other conformational changes. (B) Scheme showing how a reduction/oxidation cycle is required for *in vitro* mixing experiments in order to observe inhibition of the activity of both isoforms. Reduction of the larger isoform allows rapid monomer-dimer exchange after *in vitro* mixing and the conformation changes associated with disulfide formation in the C-terminal domain of the larger isoform could then alter the nucleotide binding properties of both isoforms. It should be noted that while the activase appears to function as an oligomer much larger than a dimer, the active site in each monomer could be formed of residues from both monomers and therefore could be affected by the docking of a single C-terminal domain as illustrated. Alternatively, a conformational change in the larger isoform caused by the redox changes in the C-terminal domain may be sufficient to alter the activity of the other isoform through allosteric interactions alone.

46-kDa isoform was decreased 4-5 fold compared to that of the oxidized 46-kDa isoform. Reduction also diminishes the ability of ADP to inhibit the activity of this isoform so that the inhibition is similar to that of the smaller isoform (Zhang and Portis 1999). These changes would be consistent with the active site of 46-kDa isoform becoming more exposed and suitable for ATP binding after reduction. In the absence of a structure for Rubisco activase, the following model is proposed (Figure 5). There are five negatively charged residues (E390, E398, D401, D407, D408) located in

the same region as C392 and C411. There are several positively charged residues located in the P-loop, which is known to be located in the ATP binding site (Salvucci et al. 1993) and there may be other positively charged areas in the active site. When the 46-kDa isoform is oxidized, the increased stability and altered conformation of the C-terminus caused by the formation of a disulfide, allows the domain to be docked into part of the ATP binding site where it is further stabilized by electrostatic interactions between one or more of these charged residues (Figure 5A). When

the 43-kDa isoform and the oxidized 46-kDa isoforms were mixed together *in vitro*, 46-kDa had little effect on the 43-kDa isoform, which may be due to slow monomer exchange and thus the active site of the 43-kDa isoform was unaffected (Figure 5B). When DTT and thioredoxin-f were subsequently added to this mixture, the disulfide bond at the C-terminus of the 46-kDa isoform is cleaved, its active site becomes more suitable for ATP binding as the C-terminal domain is released from the active site, and monomer exchange is rapid. Then after oxidized glutathione was added to this mixture to reform the disulfide bond, the C-terminal domain may become docked into the active site of both isoforms, making both less accessible to the substrate. Alternatively, the larger isoform may influence the activity of the smaller isoform indirectly through allosteric interactions. The model is consistent with the observation that in *Arabidopsis* only an equal amount of 46-kDa isoform can completely inhibit the activity of both isoforms. The model provides a basis for the design of further mutagenesis experiments with the *Arabidopsis* isoforms to examine this hypothesis.

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