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Structural changes associated with the acute thermal instability of Rubisco activase

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ABSTRACT

Inhibition of photosynthesis by heat has been linked to the instability of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) chaperone, Rubisco activase. Examination of the recombinant enzyme showed that ADP and ATP protected against inactivation, whereas Mg^{2+} promoted inactivation. Heating caused aggregation of Rubisco activase characterized by disruption of secondary structure content and formation of insoluble protein. In contrast, incubation at room temperature without nucleotide caused the active ~660 kDa protein to form a soluble, but inactive aggregate of $>2 \times 10^6$ Da. Circular dichroism (CD) spectroscopy and fluorescence established that structural perturbations in the aggregate did not reduce alpha-helical content significantly. Differences in the thermal stability between wild type and mutant Rubisco activase were observed for the recombinant proteins and when the proteins were expressed in transgenic *Arabidopsis*. That the sensitivity of these plants to heat differs indicates that the thermal instability of Rubisco activase is a main determinant of the temperature-sensitivity of photosynthesis.

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Introduction

Photosynthesis is one of most heat-sensitive processes in plants [1]. This sensitivity can have enormous consequences for agricultural production, particularly in grain and oilseed crops that need a continuous source of recently fixed carbon to support the growing sink [2]. Exposure to severe heat stress, i.e., >10 °C above the thermal optimum, irreversibly damages the photosynthetic apparatus, increasing membrane leakiness [3–6] and adversely affecting the stability of the proteins of the photosynthetic electron transport chain, particularly the photosystem II (PSII[1]) complex [1,6,7]. In contrast, the effects of moderate heat stress, characterized by temperatures <10 °C above the thermal optimum, are reversible, affecting just a few, particularly heat labile reactions of photosynthesis [3,4].

Moderate heat stress does not appear to cause significant inhibition of photosynthetic electron transport [3,8,9], but does seem to affect membrane integrity with a consequent effect on the redox potential of the stroma [10]. In recent years, inhibition of net photosynthesis by moderate heat stress has been most closely linked with a decrease in the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) through inhibition of its chaperone, ribulose-1,5-bisphosphate carboxylase/oxygenase activase (Rubisco activase) [8,11–17]. Rubisco activase loosens the binding of inhibitors that either form from catalytic misfire at the active-site of Rubisco or that bind to inactive (i.e., decarbamylated) sites. Since both the formation of these inhibitors and the rate of decarbamylation of Rubisco sites increase with temperature, Rubisco activase activity must also increase with temperature to counteract processes that inactivate Rubisco [11,13]. Instead, Rubisco activase has a relatively low temperature optimum, approximately the same as the temperature optimum for net photosynthesis [13,14]. That Rubisco deactivates when plants are exposed to moderate heat stress indicates *a priori* that Rubisco activase activity cannot keep pace with the processes that inactivate Rubisco [11].

The poor performance of Rubisco activase at elevated temperatures *in vivo* has been attributed to the inherent structural instability of the enzyme [8,17,18]. Others have proposed that the inhibition of Rubisco activase under moderate heat stress is a secondary consequence of heat-induced changes in the chloroplast environment that affect the enzyme's activity and stability [10,12,19]. While there is no evidence for a decrease in energy charge under moderate heat stress [4,10], changes in membrane leakiness, stromal oxidation and the electrochromic shift at 515 nm occur at temperatures slightly above the thermal optimum and reflect alterations in the stromal environment [3,10,20]. In particular, changes in stromal Mg^{2+} would have a profound effect on Rubisco activation, since both the rate and extent of inhibition by

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[1] *Abbreviations used:* Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; Rubisco activase, ribulose-1,5-bisphosphate carboxylase/oxygenase activase; ATPγS, Adenosine-5′-*O*-(3-thio triphosphate); SEC, size exclusion chromatography; SDS, sodium dodecyl sulfate; PSII, photosystem II; CD, circular dichroism.

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catalytic misfire compounds, as well as the rate of decarbamylation are affected by the concentration of Mg^{2+} [21].

Reconstitution experiments have shown that activation of Rubisco by Rubisco activase decreases with increasing temperature even when pH, reductant and Mg^{2+} are constant and an ATP regenerating system is used to maintain a high ratio of ATP/ADP [11,13]. These results support other data showing that Rubisco activase has a relatively low temperature optimum for activity and poor structural stability both *in vivo* and *in vitro* [11,17,18].

In an early study, Robinson and Portis [22] showed that Rubisco activase was inactivated at temperatures below 43 °C and was unstable when incubated for several h at 25 °C in the absence of nucleotide. Both ATP and ADP protected against the loss of activity at 25 °C [22] and in a later study, adenosine-5′-*O*-(3-thio triphosphate) (ATPγS) was shown to protect the isolated enzyme from thermal aggregation [17]. However, the structural basis for inactivation of Rubisco activase activity and protection by nucleotides has not been determined. Consequently, we examined the inherent stability of Rubisco activase at elevated temperature in the presence and absence of nucleotide and related the loss of activity to changes in self-association. Of particular interest was the effect of Mg^{2+} concentration because (1) the concentration of Mg^{2+} affects both the binding affinity of Rubisco inhibitors and the rate of decarbamylation [21]; (2) stromal Mg^{2+} concentrations increase in the light [23] and would be affected by heat-induced increases in membrane leakiness [24] and (3) physiological concentrations of Mg^{2+} are generally much lower than those used experimentally for Rubisco assays or binding studies [21]. The results of experiments with a mutant of Rubisco activase that is structurally less stable were used to relate the *in vitro* findings to the *in planta* conditions.

Materials and methods

Expression and purification of recombinant proteins

Escherichia coli BL21*(DE3) pLysS cells transformed with the pET23d plasmid (Novagen) harboring the cDNA encoding for the β-isoform of *Arabidopsis thaliana* Rubisco activase or an affinity tagged version of this isoform with an 8 amino acid Strep (S) tag peptide (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) on the C-terminus were used for the expression of recombinant activase [16]. Recombinant activase was purified from cells as described previously [14]. Though carrying an affinity tag, the C-terminal S-tagged Rubisco activase was also purified by the same method used for untagged protein [14] to maintain identical conditions for comparison of the resulting recombinant proteins.

Plant material

Transgenic *Arabidopsis thaliana* plants expressing either the wild-type β-isoform of Rubisco activase, rwt43 plants [15], or the S-tagged version, Δ43 plants [16], were grown as described previously [18]. Heat stress was imposed on intact plants by increasing the temperature of the growth chamber to 39 °C for 60 min. The relative humidity in the chamber was maintained at >85% during the entire time-course. Two 0.5 cm^2 leaf disks were excised and immediately frozen in liquid N_2 at the times indicated in the text. Leaf discs were stored at −80 °C until extraction for Rubisco activase protein determination (below).

Light scattering measurements

Thermal unfolding and aggregation was measured by light scattering at 500 nm as described previously [17]. Reactions were conducted in a quartz microcuvette in a total volume of 500 μL at the temperatures indicated in the text. Reactions containing 50 mM Tris–HCl (pH 7.8), Rubisco activase at 3 mg ml^{-1} concentration and the concentrations of $MgCl_2$ and ADP indicated in the text were stirred continuously before and during the measurement. The cuvette was maintained at the indicated temperatures by circulating water through a water-jacketed cuvette holder.

Enzyme assays and protein content following heat treatment

To determine the stability of activity, Rubisco activase at 3 or 10 mg ml^{-1} was incubated in Tris–HCl (pH 7.8) at 30, 35 and 40 °C in the presence of the indicated amounts of $MgCl_2$ and/or ADP or ATP, Prior to incubation, the protein solutions were equilibrated at room temperature for 5 min and then centrifuged at 13,000*g* at 4 °C for 5 min to remove protein that precipitated during storage and freeze/thaw.

To determine activity, aliquots corresponding to 10 μg Rubisco activase were transferred to a reaction mix at 25 °C to measure ATP hydrolysis. ATP hydrolysis was determined by measuring the amount of Pi release [25]. To account for the inhibitory effect of ADP and the differences in the Mg^{2+} and ADP amounts in the heat-treated samples, reaction mixtures were adjusted accordingly to a final concentration of 50 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 1 mM ADP and 10 mM ATP in a total volume of 25 μL. Reactions were generally conducted for 15 min, except for the size-exclusion (SEC)-HPLC column fractions, which were assayed for 60 min. Reactions were terminated by the addition of 25 μL 12% (w/v) sodium dodecyl sulfate (SDS) and Pi was determined from the absorbance at 850 nm [25] using a microtiter plate reader. Residual activity was calculated as the percent of maximum Rubisco activase activity determined prior to heat exposure.

To determine the amount of Rubisco activase protein that was soluble after incubation, separate 25 μL aliquots were removed and centrifuged at 13,000*g* for 5 min at 4 °C. Protein content in the supernatant was determined using the method of Bradford [26] with bovine serum albumin as a standard. Activity assays and soluble protein content were conducted in triplicate and the values presented are the means ± SE.

Size-exclusion-HPLC

Control and heat-treated Rubisco activase was centrifuged at 13,000*g* for 5 min at 4 °C and 100 μL aliquots of the supernatant were chromatographed at 0.5 ml min^{-1} on a Biosep-SEC-4000 PEEK 300 × 7.5 mm gel filtration column containing a 75 × 7.5 mm guard column (Phenomenex, Torrance, CA) and a 2 μm 4 mm pre-filter (Alltech, Deerfield, IL). Aliquots of the sample were assayed for ATP hydrolysis and soluble protein content prior to chromatography. The column was equilibrated and run with 50 mM Hepes–KOH (pH 7.2) and 1 mM $MgCl_2$. For some experiments, the column buffer was supplemented with 0.1 mM ADP. The eluted protein was detected by its absorbance at 280 nm. Peak fractions from the column were collected and analyzed for activity and intrinsic fluorescence. For measurements of circular dichroism (CD), 50 mM potassium phosphate (pH 7.2), 1 mM $MgSO_4$ and 0.1 mM ADP was used as the column buffer. For analysis of the polypeptide composition, aliquots of the fractions were treated with 80% acetone and incubated overnight at −20 °C. Following centrifugation at 13,000*g* for 5 min at 4 °C, the precipitated protein was suspended in SDS and bromophenol-blue containing sample loading buffer and electrophoresed on 12% SDS–PAGE gels [18].

The molecular mass of Rubisco activase was calculated based on calibration of the SEC-HPLC column with blue dextran and protein standards of known molecular weight. The calibration curve was fitted with a 4 parameter logistic dose–response curve.