Inhibition of net photosynthesis (Pn) by moderate heat stress has been attributed to an inability of Rubisco activase to maintain Rubisco in an active form. To examine this proposal, the temperature response of Pn, Rubisco activation, chlorophyll fluorescence, and the activities of Rubisco and Rubisco activase were examined in species from contrasting environments. The temperature optimum of Rubisco activation was 10°C higher in the creosote bush (Larrea tridentata) compared with the Antarctic hairgrass (Deschampsia antarctica), resembling the temperature response of Pn. Pn increased markedly with increasing internal CO₂ concentration in Antarctic hairgrass and creosote bush plants subjected to moderate heat stress even under nonphotorespiratory conditions. Nonphotochemical quenching of chlorophyll fluorescence, the effective quantum yield of photochemical energy conversion (ΔF/Fₘₛ) and the maximum yield of PSII (Fᵥ/Fₘₙ) were more sensitive to temperature in Antarctic hairgrass and two other species endemic to cold regions (i.e. Lysipomia pumila and spinach [Spinacea oleracea]) compared with creosote bush and three species (i.e. jojoba [Simmondsia chinensis], tobacco [Nicotiana tabacum], and cotton [Gossypium hirsutum]) from warm regions. The temperature response of activity and the rate of catalytic inactivation of Rubisco from creosote bush and Antarctic hairgrass were similar, whereas the optimum for ATP hydrolysis and Rubisco activation by recombinant creosote bush, cotton, and tobacco activase was 8°C to 10°C higher than for Antarctic hairgrass and spinach activase. These results support a role for activase in limiting photosynthesis at high temperature.

The temperature optimum for higher plant photosynthesis is usually rather broad and generally matches the average daytime temperature encountered in the natural environment (Berry and Björkman, 1980; Larcher, 1995). Significant inhibition of photosynthesis occurs at temperatures above the optimum, resulting in considerable loss of potential productivity. Inhibition of photosynthesis by heat stress has long been attributed to an impairment of electron transport activity, caused in part by changes in membrane fluidity (Raison et al., 1982; Havaux, 1993; Murakami et al., 2000). However, others (Bilger et al., 1987; Jones et al., 1998; Bukhov et al., 1999; Bukhov and Dzhibladze, 2002) support the idea that the initial site of inhibition is associated with a Calvin cycle reaction, specifically the inactivation of Rubisco (Weis, 1981a, 1981b; Kobza and Edwards, 1987). This idea has been revitalized recently by newer data from our laboratory (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000) coupled with a more thorough understanding of the biochemistry of the activation process (Andrews, 1996; Spreitzer and Salvucci, 2002; Portis, 2003). The activation state of Rubisco in leaves reflects a balance between sequestration of Rubisco active sites in a closed, inactive conformation and the reactivation of these sites by conformational changes induced by Rubisco activase (Andrews, 1996; Spreitzer and Salvucci, 2002; Portis, 2003). Previous studies provided a biochemical basis for inactivation of Rubisco under heat stress by showing that this balance shifts to a lower activation state at higher temperature because of faster rates of Rubisco inactivation and slower rates of activase activity (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004). In cotton (Gossypium hirsutum), wheat (Triticum aestivum), tobacco (Nicotiana tabacum), and maize (Zea mays), a decrease in Rubisco activation under moderate heat stress correlated with reduced rates of net photosynthesis (Pn) and was accompanied by increased levels of RuBP and decreased levels of 3-phosphoglycerate (Kobza and Edwards, 1987; Law and Crafts-Brandner, 1999; Crafts-Brandner and Law, 2000; Sharkey et al., 2001; Crafts-Brandner and Salvucci, 2002). Analysis of the response of Pn to temperature and CO₂ concentration in cotton under both photorespiratory and nonphotorespiratory conditions showed that the temperature response of Pn could be predicted from the

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kinetic properties of Rubisco if the measured changes in Rubisco activation were included in the calculation (Crafts-Brandner and Salvucci, 2000).

When taken together, the accumulated data from gas exchange and biochemical analyses suggest that deactivation of Rubisco, caused at least in part by thermal inactivation of activase (Salvucci et al., 2001), is the primary cause of inhibition of photosynthesis under moderate heat stress (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004). To determine if this concept is broadly applicable and to further document the role of activase, we compared the temperature response of Pn, Rubisco activation, chlorophyll fluorescence, and Rubisco and activase activities in plants from contrasting thermal environments. The results suggest that the upper temperature limit of photosynthesis is determined by the thermal properties of activase.

RESULTS

Pn and Rubisco activation were measured in two C3 plant species from contrasting thermal environments (Fig. 1). The Antarctic hairgrass (Deschampsia antarctica) is one of two angiosperms endemic to maritime Antarctica (Xiong et al., 1999; Alberdi et al., 2002). Creosote bush (Larrea tridentate) is a desert shrub adapted to hot, arid regions of the U.S. southwest (Mooney et al., 1978; Hamerlynck et al., 2000). Gas exchange measurements showed that the temperature optimum of Pn for Antarctic hairgrass and creosote bush differed by about 10°C. For Antarctic hairgrass, Pn was inhibited by temperatures higher than about 20°C, whereas inhibition of creosote bush photosynthesis occurred at temperatures higher than 30°C. Rubisco activation (i.e. the fraction of active Rubisco in the leaf) decreased at precisely the same temperatures that inhibited Pn, and the extent of inhibition of Pn and Rubisco activation was similar at a given high temperature. A similar close relationship between the responses of Pn and Rubisco activation to elevated temperature has been reported in isolated spinach (Spinacia oleracea) chloroplasts (Weis, 1981b) and in intact leaves of maize (Crafts-Brandner and Salvucci, 2002), wheat, and cotton (Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000).

The responsiveness of Pn to increasing internal CO2 concentration (Ci) was determined for Antarctic hairgrass and creosote bush at leaf temperatures that caused moderate heat stress. These measurements were conducted in low O2 (i.e. 10 mbar) to eliminate the complications associated with photorespiration (Monson et al., 1982; Kobza and Edwards, 1987). At air levels of CO2 under both atmospheric and low O2, Pn in creosote bush was inhibited by approximately 28% at 42.5°C compared with 28°C (Table I). When Ci was increased progressively at a leaf temperature of 42.5°C, the rate of Pn at 10 mbar O2 increased markedly. Similar results were observed with Antarctic hairgrass, even though the temperature required for inhibition of Pn was lower (Table II). For example, Pn at air levels of CO2 in Antarctic hairgrass was inhibited by approximately 38% at 36°C compared with 16°C under both atmospheric and low O2, and the rate of Pn at 36°C increased markedly when Ci was increased in low O2.

Measurements of the activation state of Rubisco in Antarctic hairgrass and creosote bush under the conditions described above revealed that Rubisco deactivated when leaf temperature and Ci were increased or when the O2 concentration was decreased (Tables I and II), consistent with previous observations with other plant species (Schnyder et al., 1984; Sharkey et al., 1986; Kobza and Edwards, 1987; Sage et al., 1989; Crafts-Brandner and Salvucci, 2000). These data were used to determine if the kinetic properties of Rubisco could account for the measured rates of Pn after adjustment for changes in Rubisco activation. For both creosote bush and Antarctic hairgrass, rates of Pn predicted from Rubisco kinetics without adjustment for changes in Rubisco activation generally deviated from the measured rates, with the most drastic differences occurring at high temperatures and Ci, i.e. conditions that promote considerable deactivation of Rubisco (Tables I and II). By contrast, the rates of Pn predicted from the kinetic properties of Rubisco were similar to the measured rate under all conditions when adjusted for changes in Rubisco activation (Fig. 2). The slight deviation between the measured rates of Pn and the rates predicted after adjustment for changes in Rubisco activation was probably caused by mitochondrial respiration that was not considered in the calculations because of uncertainty about its magnitude in the light.
The effect of temperature on chlorophyll fluorescence was determined for attached leaves of Antarctic hairgrass and creosote bush (Fig. 3A). The values of Fv/Fm for the two plant species were relatively constant with temperature below about 35°C. At temperatures higher than 35°C, Fv/Fm decreased much more abruptly with temperature for Antarctic hairgrass compared with creosote bush. The temperature response of nonphotochemical quenching (NPQ) and ΔF/Fm', the effective quantum yield of photochemical energy conversion (Genty et al., 1989), were markedly different for Antarctic hairgrass and creosote bush (Figs. 3A and 4A). NPQ increased and ΔF/Fm' decreased when leaf temperatures exceeded about 28°C for Antarctic hairgrass and 38°C for creosote bush.

To determine if other cold and warm climate-adapted species also exhibited a differential effect of temperature on Fv/Fm, NPQ, and ΔF/Fm', these parameters were measured in jojoba (Simmondsia chinensis), another shrub from the arid deserts of the U.S. southwest, and Lysipomia pumila, an Andean monocot adapted to the cool wet climate of the Altiplano region of South America (Figs. 3B and 4B), as well as in cultivated plants of temperate (i.e. spinach) and subtropical (i.e. cotton and tobacco) origin (Figs. 3C and 4C). The values of Fv/Fm in jojoba, L. pumila, and the three cultivated species were similar and relatively constant at temperatures lower than 37°C. At temperatures higher than about 37°C, Fv/Fm decreased more abruptly in L. pumila and spinach compared with jojoba, tobacco, and cotton. For all species, a marked increase in NPQ and a decrease in ΔF/Fm' with temperature preceded the decrease in Fv/Fm. These changes in NPQ and ΔF/Fm' occurred when temperatures exceeded 28°C to 35°C for L. pumila and spinach and 35°C to 38°C for tobacco, cotton, and jojoba.

Since the activation state of Rubisco is determined by the balance between inactivation of Rubisco and its reactivation by activase (Spreitzer and Salvucci, 2002; Portis, 2003), we examined the thermal properties of both Rubisco and activase from Antarctic hairgrass and creosote bush.

Table I. Effect of temperature, Ci, and O2 on Pn and Rubisco activation in intact creosote bush leaves

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>O2 mbar</th>
<th>Ci μbar</th>
<th>Pn μmol m⁻² s⁻¹</th>
<th>Rubisco activation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>210</td>
<td>261</td>
<td>29.0 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>28</td>
<td>10</td>
<td>243</td>
<td>43.9 ± 1.1</td>
<td>79.4</td>
</tr>
<tr>
<td>40</td>
<td>210</td>
<td>258</td>
<td>23.3 ± 1.6</td>
<td>84.7</td>
</tr>
<tr>
<td>42.5</td>
<td>210</td>
<td>262</td>
<td>19.3 ± 1.6</td>
<td>60</td>
</tr>
<tr>
<td>42.5</td>
<td>10</td>
<td>241</td>
<td>32.8 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>42.5</td>
<td>10</td>
<td>333</td>
<td>39.8 ± 1.4</td>
<td>ND</td>
</tr>
<tr>
<td>42.5</td>
<td>10</td>
<td>489</td>
<td>49.5 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>42.5</td>
<td>10</td>
<td>687</td>
<td>56.4 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>42.5</td>
<td>10</td>
<td>844</td>
<td>59.0 ± 0.3</td>
<td>39.1</td>
</tr>
</tbody>
</table>

Table II. Effect of temperature, Ci, and O2 on Pn and Rubisco activation in intact Antarctic hairgrass leaves

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>O2 mbar</th>
<th>Ci μbar</th>
<th>Pn μmol m⁻² s⁻¹</th>
<th>Rubisco activation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>210</td>
<td>290</td>
<td>19.4 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>270</td>
<td>25.6 ± 2.3</td>
<td>77.8</td>
</tr>
<tr>
<td>32</td>
<td>210</td>
<td>234</td>
<td>13.4 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>36</td>
<td>210</td>
<td>241</td>
<td>11.4 ± 0.7</td>
<td>66.4</td>
</tr>
<tr>
<td>36</td>
<td>10</td>
<td>213</td>
<td>16.8 ± 1.0</td>
<td>50.3</td>
</tr>
<tr>
<td>36</td>
<td>10</td>
<td>284</td>
<td>22.1 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>36</td>
<td>10</td>
<td>395</td>
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<td>36</td>
<td>10</td>
<td>549</td>
<td>36.1 ± 2.4</td>
<td>ND</td>
</tr>
<tr>
<td>36</td>
<td>10</td>
<td>702</td>
<td>40.8 ± 2.5</td>
<td>37.1</td>
</tr>
</tbody>
</table>

*ND, Not determined.*
grass and creosote bush. The temperature response of the carboxylase activities of fully activated (i.e. carbamylated) Rubisco isolated from Antarctic hairgrass and creosote bush were nearly identical; activity increased with increasing temperature to at least 50 °C (Fig. 5). The rates of carboxylation and the response of activity to temperature were similar to the results reported previously for purified Rubisco from tobacco and cotton (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004). Rubisco from Antarctic hairgrass and creosote bush also showed similar rates of inactivation when incubated at 40 °C under catalytic conditions (Fig. 5, inset).

To facilitate purification and to obviate problems with proteolysis, activase cDNAs from creosote bush and Antarctic hairgrass were cloned, and the recombinant enzyme was analyzed after expression in *Escherichia coli*. Immunoblots of creosote bush and Antarctic hairgrass leaves showed that the α and β forms of activase (Salvucci et al., 2003) were present in approximately equal abundance in both of these species (data not shown). Preliminary experiments revealed that the ATPase activity of the α and β forms of activase within a species exhibited a similar response to temperature (data not shown). Consequently, a mixture of equal amounts of both forms of the enzyme was used for subsequent analysis. Activase from creosote bush had a broad temperature optimum for ATP hydrolysis centered at 35°C, whereas the enzyme from Antarctic hairgrass exhibited maximal rates at 27.5°C (Fig. 6A). At the optimum for creosote bush activase, activase from Antarctic hairgrass was inactive.

Differences in the temperature response of ATPase activity between creosote bush and Antarctic hairgrass activases were also striking at temperatures between 5°C and 20°C. For example, at 15°C the rate of ATP hydrolysis by creosote bush activase was less than 5% of maximum compared to about 50% for Antarctic hairgrass activase. Despite the relatively poor performance of creosote bush activase at low temperatures, the activation state of Rubisco in creosote bush leaves was nearly 100% when leaf temperatures were less than 20°C (Fig. 1).

The temperature response of activity was also examined for recombinant activase from the three cultivated species used above for the chlorophyll

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**Figure 2.**, Relationship between the measured and predicted rates of Pn in nonstressed and heat stressed leaves of creosote bush and Antarctic hairgrass at ambient and elevated Ci. The predicted rates of Pn for creosote bush (A) and Antarctic hairgrass (B) were calculated from the kinetic properties of Rubisco with (●) and without (■) adjustment for the measured changes in Rubisco activation. The two dashed lines show the linear regression of the relationship between measured and predicted rates of Pn with (●) and without (■) adjustment for changes in Rubisco activation. Values for Pn and Rubisco activation, as well as the conditions of temperature, Ci, and O₂, are from Tables I (A) and II (B). The solid line denotes a 1:1 relationship between measured and predicted rates.

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**Figure 3.** Effect of temperature on NPQ of chlorophyll fluorescence and the maximum yield of PSII (Fv/Fm) in plants native to warm and cold regions. The Fv/Fm (●, ▲) and NPQ (□, ▼) were determined at the indicated temperatures for attached leaves of the following plant species: A, the Antarctic hairgrass (●, ▲) and the creosote bush, (●, ▼); B, the Andean monocot *L. pumila* (●, ▲) and the desert shrub jojoba (●, ▼); and C, spinach (●, ▲), cotton (●, ▼), and tobacco (▲, ▼).
fluorescence analysis. Since spinach and cotton express both forms of activase, equal amounts of the α and β forms were used for the experiments and compared to the results with the β form of tobacco activase, the only form expressed by this species (Portis, 2003). The temperature optimum for ATP hydrolysis by cotton and tobacco activase was almost 10°C higher than the optimum for the spinach enzyme (Fig. 6B). For example, a temperature of 35°C was nearly optimal for cotton and tobacco activase yet almost completely inhibited the enzyme from spinach. Differences in the temperature response of ATPase activity were apparent at temperatures between 5°C and 20°C. For example, at 15°C the rate of ATP hydrolysis by cotton activase was less than 5% of maximum compared to about 50% for spinach activase.

The effect of temperature on activation of Rubisco by activase was compared in an in vitro assay using purified activase and Rubisco (Fig. 7). Activation of inactive Antarctic hairgrass Rubisco by recombinant Antarctic hairgrass activase was optimum at 20°C and decreased progressively with increasing temperature. Activation was completely inhibited at temperatures of 35°C and higher. By contrast, the temperature optimum for activation of creosote bush Rubisco by recombinant creosote bush activase was 30°C, and considerable (i.e. approximately 70%) activation activity was evident at 35°C. At 12°C, Antarctic hairgrass activase promoted a high level of activation, whereas activase from creosote bush was ineffective in activating Rubisco at this temperature. Together, these differences in the temperature response of Rubisco activation between recombinant Antarctic hairgrass and creosote bush activases were consistent with the differences observed for ATPase activity.

Loss of activase activity above the temperature optimum is probably caused by a structural breakdown of the protein. Consequently, the thermal stabilities of Antarctic hairgrass and creosote bush activase were compared by incubating the two enzymes at various temperatures in the presence of the substrate analog, ATPγS, and then measuring residual ATPase activity at a constant assay temperature (Fig. 8A). Since ATPγS is known to protect the enzyme against denaturation (Crafts-Brandner et al., 1997), the activase measured in these experiments was in its most stable form. Under these conditions, the temperature required to inactivate ATPase activity by 50% (i.e. T50) was 38.5°C and 43°C for Antarctic hairgrass and creosote bush activase, respectively. Similarly, measurements of light scattering during continuous heating in the presence of ATPγS showed that thermal denaturation and aggregation occurred at 39°C and 44°C for Antarctic hairgrass and creosote bush activase, respectively, (Fig. 8B). These temperatures were identical to the temperatures that completely

![Figure 4](image-url)  
**Figure 4.** Effect of temperature on the effective quantum yield of photochemical energy conversion (ΔF/Fm') in plants native to warm and cold regions. The ΔF/Fm' were determined at the indicated temperatures for attached leaves of the following plant species: A, the Antarctic hairgrass (○) and the creosote bush (●); B, the Andean monocot *L. pumila* (○) and the desert shrub *jojoba* (●); and C, spinach (○), cotton (●), and tobacco (△).

![Figure 5](image-url)  
**Figure 5.** Effect of temperature on the carboxylase activity of Rubisco isolated from Antarctic hairgrass and creosote bush. The carboxylase activity of Rubisco from Antarctic hairgrass (○) and creosote bush (●) was determined at the indicated temperatures. The enzyme was incubated with 30 mM NaHCO3 and 10 mM MgCl2 to fully carbamylated the enzyme prior to assay. Inset, Time course of inactivation of Rubisco under catalytic conditions. Fully carbamylated Rubisco from Antarctic hairgrass (○) and creosote bush (●) was incubated at 40°C in the presence of RuBP, and residual activity was determined at 30°C at the indicated times.
inhibited enzyme activity (Fig. 8A), an indication that differences in the temperature response of activity between Antarctic hairgrass and creosote bush activase reflect differences in the inherent thermal stability of the protein.

DISCUSSION

Relationship between Pn and Rubisco Activation under Moderate Heat Stress

The temperature response of Pn is complex, reflecting the temperature dependencies of several interacting physical and biochemical processes. That the rate of Pn does not continue to increase with temperature has been attributed, in part, to reduced CO₂ uptake and increased CO₂ evolution caused by higher photorespiratory and respiratory activities and lower gas solubility at higher temperatures (Berry and Björkman, 1980; Monson et al., 1982; Jordan and Ogren, 1984). These factors are partially offset by increased carboxylation from the faster rates of Rubisco turnover at higher temperatures (Salvucci and Crafts-Brandner, 2004; Fig. 5). As a result, the response of Pn to temperature predicted from the kinetics of Rubisco is relatively flat above the optimum (Crafts-Brandner and Salvucci, 2000), indicating that another factor(s) must be responsible for the pronounced decrease in Pn at supraoptimal temperatures.

Recent studies with cotton, wheat, tobacco, and maize have confirmed earlier observations (Weis, 1981a, 1981b; Kobza and Edwards, 1987) that Rubisco deactivates markedly in response to moderate heat stress (Law and Crafts-Brandner, 1999; Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000, 2002). Significant decreases in the activation state of Rubisco under moderate heat stress, when superimposed on the relatively flat temperature response predicted for Pn, exert a major inhibitory effect on Pn. As a result, we found that the progressive decrease in Rubisco activation that accompanies increasing leaf temperatures closely correlates with the extent of photosynthetic inhibition both here (Fig. 1) and in previous studies (Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000, 2002).

Differential Effects of Temperature on Pn, Rubisco Activation, and Chlorophyll Fluorescence in Plants from Contrasting Thermal Environments

Photosynthetic rates are generally optimal at temperatures that are prevalent during the day in a species’ native environment (Berry and Björkman, 1980). Consequently, the response of Rubisco activation to temperature should differ in plants from different
The biochemical basis for inactivation of Rubisco under heat stress

The biochemical basis for inactivation of Rubisco under heat stress is an imbalance between the rates of Rubisco inactivation and reactivation by activase (Crafts-Brandner and Salvucci, 2000). As temperature increases, mechanism-based inactivation of Rubisco
proceeds more rapidly, mainly because of a faster rate of substrate misprotonation, which increases the production of the closed, inactive form of the enzyme (Salvucci and Crafts-Brandner, 2004). Since conversion to the open conformation is extremely slow without activase (Andrews, 1996; Duff et al., 2000), maintenance of Rubisco in an active state at high temperatures requires faster rates of activase activity to offset the faster rates of Rubisco inactivation. However, in vitro assays using purified activase and Rubisco and saturating levels of ATP and RuBP have shown that activase activity, which is sufficient for Rubisco activation at optimal temperatures, is insufficient to keep pace with the faster rates of Rubisco inactivation at high temperatures (Crafts-Brandner and Salvucci, 2000). Thus, the activation state of Rubisco decreases under heat stress because activase activity cannot overcome the faster rates of Rubisco inactivation. The poor performance of activase at high temperature has been attributed to its relatively low temperature optimum for catalysis (Salvucci and Crafts Brandner, 2004), caused in part by thermal instability (Fig. 8; Salvucci et al., 2001; Rokka et al., 2001), as well as to other unspecified causes (Sharkey, 2000).

It is well known that the thermal properties of homologous enzymes from different species often match the temperature of the environments to which the organisms are adapted (Somero, 1995). This axiom did not hold true for Rubisco since the temperature response of Rubisco activity from creosote bush and Antarctic hairgrass was similar and nearly identical to the responses reported for cotton and tobacco Rubisco. Similar measurements by Sage (2002) showed that Rubisco activity was actually higher at elevated temperatures in species adapted to cool environments compared with species from warm environments.

In contrast to Rubisco, the thermal properties of activase in plant species from contrasting thermal environments differed, corresponding to the differential response of Pn and Rubisco activation to temperature. Specifically, the temperature response and thermal stability of activase from the desert shrub was skewed toward higher temperatures, while the activase from the Antarctic hairgrass performed optimally at much lower temperature. Similarly, activase from cotton and tobacco, two cultivated species of subtropical origin, was more thermostolerant than activase from the temperate species, spinach.

**Limitations to Pn Imposed by the Thermal Properties of Activase**

Since the activation state of Rubisco is determined by the balance between inactivation and subsequent reactivation by activase, and the rate of inactivation was similar in Rubisco from creosote bush and Antarctic hairgrass (Fig. 5, inset), then it follows that the temperature limits for Rubisco activation and probably photosynthesis are determined to a large extent by the thermal properties of activase. The stimulation of Pn by increasing Ci at low O2 for both creosote bush and Antarctic hairgrass (Tables I and II) supports this view by establishing that the supply of ATP for RuBP regeneration is not limiting at air levels of CO2 when Pn is inhibited by moderate heat stress (see also Monson et al., 1982; Crafts-Brandner and Law, 2000).

The increase in NPQ that accompanies moderate heat stress, while not a direct measure of ΔpH, nevertheless suggests that the proton gradient is increased or at least maintained under moderate heat stress to provide ample ATP for RuBP regeneration. A similar conclusion about the status of the transthylakoid pH gradient under moderate heat stress has been drawn from measurements of 9-aminoacridine fluorescence and light scattering (Bukhov et al., 1999). These and other measurements suggest that the transthylakoid pH gradient proton is maintained under heat stress by increased electron flow through PSI (Bukhov et al., 1999, 2000; Bukhov and Dzhibladze, 2002).

**CONCLUSIONS**

The ability of activase to maintain Rubisco in an active conformation appears to place a limit on the temperatures at which higher plants can photosynthesize. Heat shock proteins, changes in the chloroplast milieu, and probably other factors affect the properties of activase and other components of the photosynthetic apparatus. In turn, these factors undoubtedly modulate the precise response of photosynthesis and Rubisco activation to temperature, including the degree to which a given plant species can acclimate to temperatures outside its normal range (Law and Crafts-Brandner, 1999). While it will be interesting in the future to determine if the properties of activase change when plants acclimate to temperature, the focus of this study was to compare plants grown at temperatures similar to those encountered in their natural environments. The results showed that under natural growth temperatures the temperature response of Pn and Rubisco activation was consistent with the thermal properties of activase. Thus, the thermal stability of activase represents a major biochemical factor limiting the ability of plants to photosynthesize at high temperature. By limiting photosynthetic activity, activase may ultimately affect the geographic distribution of higher plants, their productivity in a particular thermal environment, and their ability to respond to changes in climate.

**MATERIALS AND METHODS**

**Plant Material**

Antarctic hairgrass (*Deschampsia antarctica*) plants were collected from the Stepping Stone Islands along the west coast of the Antarctic Peninsula by Dr.
Gene Cloning, Protein Expression, and Purification

Active cDNAs were cloned by reverse transcription-PCR from mRNA isolated from leaves of creosote bush and Antarctic hairgrass (Salvucci et al., 2003). The clones were engineered for expression of the mature a and b forms of the protein in Escherichia coli by replacing nucleotides that encode for the transit peptide and an ATP-binding cassette with coding sequences using PCR. The cDNAs for creosote bush (accession no. AY312575, a form; accession no. AY312576, b form) and Antarctic hairgrass (accession no. AY312573, a form; accession no. AY12574, b form) actives were inserted into the PET23d and 23a vectors, respectively, for expression in E. coli strain BL21(DE3) pLysS after induction with isopropyl-β-D-thiogalactopyranoside (van de Loo and Salvucci, 1996). Clones of the spinach, cotton, and tobacco activase have been described previously (van de Loo and Salvucci, 1996; Crafts-Brandner et al., 1997; Salvucci et al., 2003). Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining permission will be the responsibility of the requestor.

Active protein was purified from E. coli cells as described previously (Salvucci and Klein, 1994; van de Loo and Sal. et al., 1996), with the following changes. After precipitation with 37% (w/v) ammonium sulfate, the suspended protein was centrifuged at 210,000 g for 30 min in a Beckman SW55 rotor. The clarified supernatant was fractionated at 1 mL min⁻¹ on a 26 × 60-cm Toyopearl HW-55S column in 50 mM HEPES-KOH, pH 7.2, 10 mM MgCl₂, and 2 mM dithiothreitol. This chromatography step replaced separation by rate zonal centrifugation on Suc gradients (Salvucci and Klein, 1994). Fractions containing ATPase activity were further fractionated by anion-exchange chromatography (Salvucci and Klein, 1994) on a 15-mL Q-Sepharose HiTrap column (Amersham Biosciences, Piscataway, NJ). Rubisco was purified from leaves of creosote bush and Antarctic hairgrass as described previously (Crafts-Brandner and Salvucci, 2000) with the following changes. For isolation of creosote bush Rubisco, protein was precipitated with 70% (v/v) ammonium sulfate. For both creosote bush and Antarctic hairgrass, rate zonal centrifugation was replaced by gel-filtration chromatography through Toyopearl HW-55S as described above in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM NaHCO₃, and 10 mM 2-mercaptoethanol. Fractions containing Rubisco activity were further fractionated by anion-exchange chromatography (Salvucci and Klein, 1994) on a 1 × 10-cm Mono-Q column (Amersham Biosciences).

Net Photosynthesis and Rubisco Activation

Pn of intact leaves was determined in an atmosphere of 350 μbar CO₂ as indicated, 210 or 10 mbar O₂ and a saturating irradiance of 1,800 μmol photons m⁻² s⁻¹ using a Li-COR 6400 portable photosynthesis system (Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000). The leaf temperature was increased or decreased after reaching steady-state photosynthesis at the starting temperature. After completion of each run, the leaf temperature was increased or decreased after reaching steady-state photosynthesis at the starting temperature. After completion of each run, the leaf temperature was increased or decreased after reaching steady-state photosynthesis at the starting temperature. After completion of each run, the leaf temperature was increased or decreased after reaching steady-state photosynthesis at the starting temperature. After completion of each run, the leaf temperature was increased or decreased after reaching steady-state photosynthesis at the starting temperature.

Rubisco activation was determined by rapid extraction and assay of leaf tissue sampled by freeze-clamping immediately following measurement of gas exchange (Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000). The activation state was determined by comparing the activity of Rubisco immediately following extraction with the activity after incubation with 10 mM MgCl₂ and NaHCO₃. The values presented are the means ± se of the three different samples. The predicted rates of Pn were calculated from the gas solubilities and the kinetic properties of Rubisco without adjustment for dark respiration and assuming that RuBP was saturating (Crafts-Brandner and Salvucci, 2000). Predicted rates were normalized to the measured rate of Pn at the temperature where Rubisco activation was 100%, i.e. 28°C and 16°C for creosote bush or Antarctic hairgrass, respectively. The activation energy for Rubisco was determined experimentally using purified creosote bush or Antarctic hairgrass Rubisco (Fig. 5).

Enzyme Assays

ATPase activity was measured spectrophotometrically as described previously (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001). The temperature response of activity was determined by incubating 50 μg of activase in assays without ATP for 5 min at the indicated temperatures and then initiating the reaction with ATP. The pH of the assay varied from 8.11 to 7.4°C to 7.84°C at 50°C, a range that coincides with the broad pH optimum of activase (Robinson and Portis, 1989). Preliminary experiments showed that the pH optima of the linkers were similar to those determined previously (van de Loo and Salvucci, 1996; Crafts-Brandner et al., 1997; Salvucci et al., 2003). Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining permission will be the responsibility of the requestor.

Active protein was purified from E. coli cells as described previously (Salvucci and Klein, 1994; van de Loo and Sal. et al., 1996), with the following changes. After precipitation with 37% (v/v) ammonium sulfate, the suspended protein was centrifuged at 210,000 g for 30 min in a Beckman SW55 rotor. The clarified supernatant was fractionated at 1 mL min⁻¹ on a 26 × 60-cm Toyopearl HW-55S column in 50 mM HEPES-KOH, pH 7.2, 10 mM MgCl₂, and 2 mM dithiothreitol. This chromatography step replaced separation by rate zonal centrifugation on Suc gradients (Salvucci and Klein, 1994). Fractions containing ATPase activity were further fractionated by anion-exchange chromatography (Salvucci and Klein, 1994) on a 15-mL Q-Sepharose HiTrap column (Amersham Biosciences, Piscataway, NJ). Rubisco was purified from leaves of creosote bush and Antarctic hairgrass as described previously (Crafts-Brandner and Salvucci, 2000) with the following changes. For isolation of creosote bush Rubisco, protein was precipitated with 70% (v/v) ammonium sulfate. For both creosote bush and Antarctic hairgrass, rate zonal centrifugation was replaced by gel-filtration chromatography through Toyopearl HW-55S as described above in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM NaHCO₃, and 10 mM 2-mercaptoethanol. Fractions containing Rubisco activity were further fractionated by anion-exchange chromatography (Salvucci and Klein, 1994) on a 1 × 10-cm Mono-Q column (Amersham Biosciences).

Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in air using a PAM 2000 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Measurements were conducted with intact plants placed inside a humidified plant growth chamber (Law and Crafts-Brandner, 1999). The fiber optic probe was positioned above the leaf at a constant distance using an open leaf clip holder with the light block removed. Light-scattering measurements were performed after 5 min by measuring Rubisco activity at 30°C in assays containing 100 mM Tricine-NaOH, pH 8, 10 mM MgCl₂, 10 mM NaHCO₃, (1 mM NaCl), and 0.4 mM RuBP. Time course experiments showed that 5 min was sufficient time to reach full activation (see also van de Loo and Salvucci, 1996).

Rubisco activation and deactivation under catalytic conditions were determined for the purified enzyme in the absence of activase, as described previously (Crafts-Brandner and Salvucci, 2000). The pH of the assay varied from 8.10 to 4°C to 7.84°C at 50°C, a range that coincides with the broad pH optimum of Rubisco (Andrews et al., 1975). Preliminary experiments established that the Mg²⁺ concentration was saturating for activity throughout the temperature range. Light-scattering measurements were conducted in the presence of 0.75 mM ATP; and as described previously (Salvucci et al., 2001). Assays of the isolated enzymes were conducted in at least duplicate and the values presented are the means ± se.
terms \( \frac{F_{m} - F_{o}}{F_{m}} \) and \( \frac{F_{m} - F_{o}}{F_{m}} \), respectively. (Roháček, 2002) and were determined after 4.5 min of continuous actinic light.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ312573–AJ312576.

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