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The Two Forms of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase Differ in Sensitivity to Elevated Temperature

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Ribulose-1,5-bisphosphate carboxylase/oxygenase activase often consists of two polypeptides that arise from alternative splicing of pre-mRNA. In this study recombinant versions of the spinach (*Spinacea oleracea* L.) 45- and 41-kD forms of activase were analyzed for their response to temperature. The temperature optimum for ATP hydrolysis by the 45-kD form was 45°C, approximately 13°C higher than the 41-kD form. When the two forms were mixed, the temperature response of the hybrid enzyme was similar to the 45-kD form. In the absence of adenine nucleotide, preincubation of either activase form at temperatures above 25°C inactivated ATPase activity. Adenosine 5′-(γ-thio)triphosphate, but not ADP, significantly enhanced the thermostability of the 45-kD form but was much less effective for the 41-kD form. Intrinsic fluorescence showed that the adenosine 5′-(γ-thio)triphosphate-induced subunit aggregation was lost at a much lower temperature for the 41-kD than for the 45-kD form. However, the two activase forms were equally susceptible to limited proteolysis after heat treatment. The results indicate that (a) the 45-kD form is more thermostable than, and confers increased thermal stability to, the 41-kD form, and (b) a loss of subunit interactions, rather than enzyme denaturation, appears to be the initial cause of temperature inactivation of activase.

Rubisco activase is a chloroplastic enzyme that regulates the activity of Rubisco in a light-dependent manner (Salvucci et al., 1985). Current evidence indicates that activase physically interacts with Rubisco, catalyzing ATP hydrolysis and facilitating the release of ribulose-1,5-bisphosphate and other tight-binding sugar phosphates from the active site of decarbamylated Rubisco (Wang and Portis, 1992; Andrews et al., 1995; Salvucci and Ogren, 1996). Once freed of these compounds, Rubisco can be activated by spontaneous carbamylation with CO_2 (Lorimer and Miziorko, 1980).

In most of the plant species that have been examined, Rubisco activase is composed of two different-sized polypeptides (Salvucci et al., 1987). The two activase polypeptides in spinach, Arabidopsis, and barley have been shown to arise from alternative splicing of pre-mRNA species (Werneke et al., 1988, 1989; Rundle and Zielinski, 1991a). In spinach the 45- and 41-kD activase polypeptides are identical except for 37 additional amino acids on the C-terminal end of the 45-kD form. Both the 45- and the 41-kD forms of spinach activase catalyze ATP hydrolysis, as well as activate Rubisco (Shen et al., 1991). However, aside from relatively minor differences in ATP binding (Shen et al., 1991), there is no information about other functional differences between the two forms of activase.

In this paper we report the effect of elevated temperature on the 45- and 41-kD forms of recombinant spinach activase. Our results show that the two forms differ significantly in thermal stability and, when mixed, the thermal stability of the hybrid enzyme is similar to the 45-kD form. The greater thermal stability of the 45-kD form appears to be related to tighter subunit association.

MATERIALS AND METHODS

Cloning and Isolation of the Long and Short Forms of Rubisco Activase

Clones encoding the 45- and 41-kD forms of spinach (*Spinacea oleracea* L.) Rubisco activase (pPLEX1.9 and pPLEX1.6), described by Werneke et al. (1989) and Shen et al. (1991), were obtained from W.L. Ogren and R. Kallis (U.S. Department of Agriculture-Agricultural Research Service, Urbana, IL). The activase-coding sequences (*Nco*I to *Eco*RI fragments) were transferred to pET23d (Novagen, Madison, WI) for regulated high-level expression. *Escherichia coli* BLR(DE3)pLysS cells transformed with these plasmids were used for expression of recombinant activase (van de Loo and Salvucci, 1996). Recombinant Rubisco activase was purified from the cells, as described by Salvucci and Klein (1994), with the modifications noted by van de Loo and Salvucci (1996). Activase protein concentration was determined by the method of Bradford (1976).

Enzyme Assays

The temperature response of ATP hydrolysis was determined by measuring the rate of Pi formation from ATP. Reactions contained 50 mM Tricine, pH 8.0, 5 mM $MgCl_2$, 2

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Abbreviations: ATPγS, adenosine 5′-(γ-thio)triphosphate; T_{50}, temperature for 50% reduction in activity.

mM DTT, 2 mM ATP, and activase in a total volume of 0.35 mL. Reactions were initiated at the indicated temperatures by the addition of 12 μg of enzyme and terminated after 5 min by mixing a 0.15-mL aliquot with 0.15 mL of 12% (w/v) SDS (Chifflet et al., 1988). Pi in the aliquot was determined by the method of Chifflet et al. (1988).

ATP hydrolysis was also determined by monitoring the rate of ADP formation in a coupled spectrophotometric assay (van de Loo and Salvucci, 1996). For measurements of thermal stability, 1 mg mL^{-1} activase was preincubated at the desired temperature in 50 mM Tricine, pH 8.0, and 5 mM $MgCl_2$ in the presence and absence of nucleotide. After 15 min, ATPase activity was determined at 25°C by transferring a 10-μL aliquot to the cuvette. All assays of ATPase activity were performed at least in duplicate, and the results are presented as means ± SE. In most cases the SEs were smaller in magnitude than the data points on the figures. Activity is expressed as units mg^{-1} protein, where 1 unit is equivalent to 1 μmol ATP hydrolyzed min^{-1}.

Limited Proteolysis

Activase at 1 mg mL^{-1} was incubated at 25 or 42.5°C in 25 μL of a solution containing 50 mM Tricine, pH 8.0, 5 mM $MgCl_2$, and 2 mM ATPγS. After 15 min, 1 μL of freshly prepared trypsin or chymotrypsin (0.01, 0.001, or 0.0001 mg mL^{-1}) was added and the reactions were incubated for an additional 10 min in the presence of protease. Proteolysis was terminated by the addition of 15 μg of freshly prepared Bowman-Birk trypsin/chymotrypsin inhibitor (Sigma). Samples were immediately mixed with a solution containing 5% (w/v) SDS, 30% (w/v) Suc, 0.001% bromphenol blue, and 250 mM DTT, and electrophoresed in 12% SDS-PAGE gels (Chua, 1980).

Effect of Temperature on the Intrinsic Fluorescence of Activase

Intrinsic fluorescence was determined as described previously (Wang et al., 1993) in 100 mM Tricine-KOH, pH 8.0, 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. Fluorescence of the nucleotide-free enzyme was recorded for 1 min at 25°C, before adding ATPγS or ADP to 0.2 mM. After an additional 6 min, the temperature of the thermostatted cuvette was increased by increasing the temperature of the circulating water bath at a rate of 4°C min^{-1}. The temperature of the solution inside the cuvette was measured using a thermocouple.

Table I. *Kinetic properties of the ATPase activity of the 45- and 41-kD forms of recombinant spinach activase at 25°C*

Property	Activase Form	
	45 kD	41 kD
V_{max} (unit mg^{-1} protein)[a]	0.625	0.700
$S_{0.5}$ (μM ATP)[b]	88	128
Hill coefficient[c]	1.21	1.75

[a] V_{max} values were estimated from ATPase activity determined at a saturating ATP concentration of 2 mM. [b] $S_{0.5}$ values, the substrate concentration required for half-maximal velocity, were calculated from the Hill equation. [c] Hill coefficients were determined by regression analysis of the linear portion of the Hill plots.

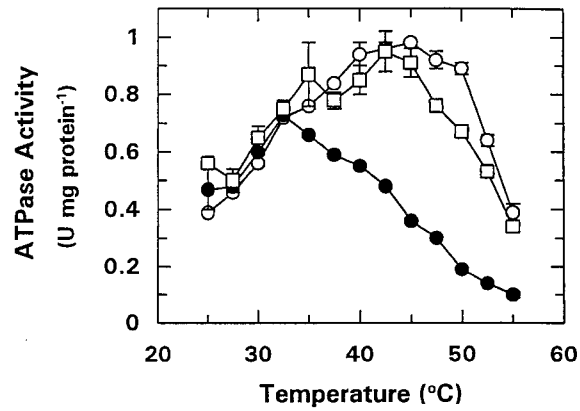

Figure 1. Effect of temperature on the ATPase activity of the 45-kD (○) and 41-kD (●) forms of recombinant spinach activase and a mixture of equal amounts (weight basis) of each form (□). The release of Pi from ATP was determined after incubation of the enzyme for 5 min at the various temperatures. U, Unit.

RESULTS

cDNAs encoding the 45- and 41-kD forms of Rubisco activase (Werneke et al., 1989) were subcloned into an expression system that produced copious amounts of active protein (van de Loo and Salvucci, 1996). Following purification the kinetics of ATPase hydrolysis were determined for each of the forms (Table I). At 25°C, the two forms of activase exhibited similar maximal rates of ATP hydrolysis, but the 45-kD form had a higher affinity for ATP and a lower Hill coefficient compared with the 41-kD form. Although we used the same clones, these results are almost exactly opposite of those reported by Shen et al. (1991) for the two forms of spinach activase.

The effect of assay temperature on ATP hydrolysis by the 45- and 41-kD forms is shown in Figure 1. The activity of the 41-kD form was greatest at 32.5°C and declined progressively with increasing temperature. In contrast, the activity of the 45-kD form increased with temperature to 45°C and remained high up to 50°C. When the two forms of activase were mixed prior to assay, the temperature profile was very similar to that observed for the 45-kD form alone (Fig. 1).

Time-course experiments showed that, when the 45-kD form was assayed continuously at 40°C, this form of activase maintained constant ATPase activity over a 27-min time course (Fig. 2). In contrast, the activity of the 41-kD form declined to near 0 under the same conditions. When the 45- and 41-kD forms were mixed, the activity remained high for about 15 min and then declined by about 10% over the next 12 min.

Both forms of activase were extremely sensitive to moderately elevated temperatures when incubated in the absence of adenine nucleotide prior to assay at 25°C (Fig. 3A). After 15 min at 30°C, ATPase activity decreased by about 50% (i.e. T_{50} = 30°C) compared with incubation at 25°C. Since it was not possible to test the effect of ATP because of

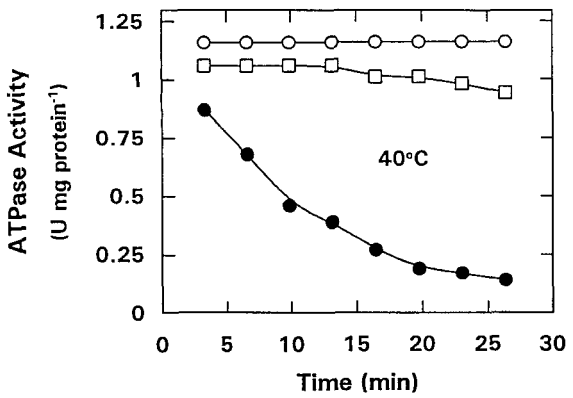

Figure 2. Time course of ATP hydrolysis at 40°C by the 45-kD (○) and 41-kD (●) forms of recombinant spinach activase and a mixture of equal amounts (weight basis) of each form (□). ATPase activity was determined spectrophotometrically by following the oxidation of NADH in an enzyme-linked assay system. U, Unit.

confounding effects of temperature on the ATP-regenerating system, we used the ATP analog ATPγS (Wang et al., 1993; van de Loo and Salvucci, 1996). Inclusion of 2 mM ATPγS during the 15-min incubation enhanced the thermal stability of the 45-kD form, increasing the T_{50} to 55°C (Fig. 3B). ADP was much less effective, increasing the T_{50} of the 45-kD form to about 36°C.

ATPγS also increased the thermal stability of the 41-kD form, but the increase was much less than for the 45-kD form; the T_{50} was 41°C in the presence of ATPγS (Fig. 3B). The effect of ADP on the thermal stability of the 41-kD form was similar to the effect with ATPγS. When the 45- and 41-kD forms of activase were mixed and then incubated in the presence of ATPγS, the temperature response of the hybrid enzyme resembled the 45-kD form at temperatures up to 50°C (data not shown).

The concentrations of ATPγS or ADP required for stabilizing activase against thermal inactivation were different for the two forms (Fig. 4). At 35°C, protection of the 45-kD form against thermal inactivation required about 0.1 mM ATPγS or about 2 mM ADP. Under the same conditions, the 41-kD form required a much higher ATPγS concentration for complete protection (i.e. 0.4 mM ATPγS) or a much lower concentration of ADP (i.e. 0.25 mM), compared with the 45-kD form.

In the presence of 2 mM ATPγS, incubation of activase for 15 min at 42.5°C caused complete inactivation of the ATPase activity of the 41-kD form but did not affect ATP hydrolysis by the 45-kD form (Figs. 3 and 4). After digestion with trypsin (Fig. 5A) or chymotrypsin (data not shown) at 42.5°C, SDS-PAGE showed that the relative susceptibility of the two forms to proteolysis, based on degradation of the intact polypeptides, was quite similar. Complete degradation of the intact 45- or 41-kD forms occurred at the highest trypsin level, and minimal degradation was observed at the lowest trypsin level. When this experiment was conducted at 25°C, there was slightly less proteolysis, but the fragmentation patterns and the susceptibilities of the intact polypeptides to proteolysis were relatively the same as for the 42.5°C experiment (Fig. 5B). Thus, the intact 45- and 41-kD forms appeared to be equally susceptible to proteolytic digestion when the ATPase activity of the 41-kD form was completely heat-inactivated and the activity of the 45-kD form was unaffected.

To analyze for differences in subunit interactions, the effect of temperature on the intrinsic fluorescence of the two forms of activase was determined (Fig. 6). In the absence of nucleotide, the fluorescence signal of both the 45- and the 41-kD forms declined at about the same rate with time, and the rate was relatively unaffected by temperature. A similar effect was observed in the presence of 0.5 mM ADP (data not shown). For both the 45- and the 41-kD forms, addition of 0.2 mM ATPγS at 25°C caused a rapid increase in fluorescence, indicative of subunit aggregation (Wang et al., 1993; van de Loo and Salvucci, 1996). Increasing the temperature from 25 to 62°C in the presence of

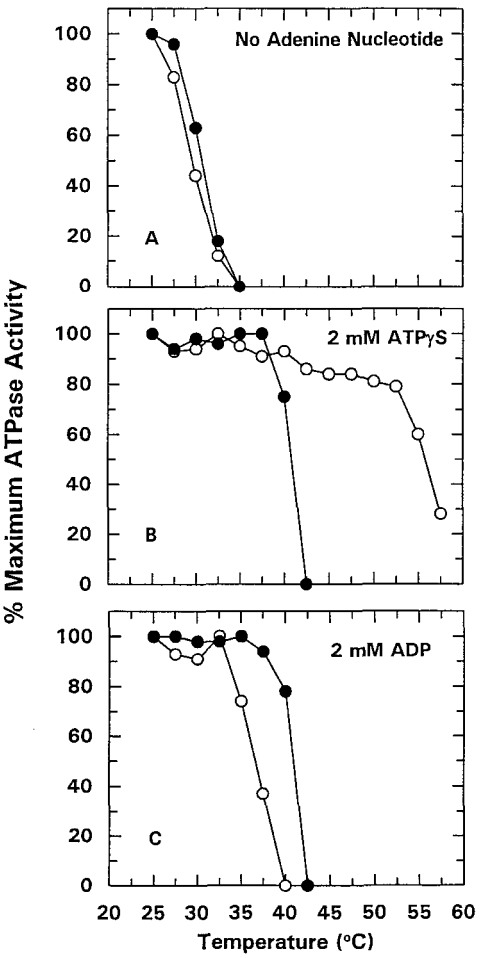

Figure 3. The effect of preincubation temperature on the ATPase activity of the 45-kD (○) and 41-kD (●) forms of recombinant spinach activase. Enzyme was preincubated for 15 min at the indicated temperature either in the absence of adenine nucleotide (A) or in the presence of 2 mM ATPγS (B) or ADP (C) and then assayed at 25°C. ATPase activity was determined spectrophotometrically by following the oxidation of NADH in an enzyme-linked assay system. Maximum activities of the 45- and 41-kD forms were 0.46 and 0.54 unit mg^{-1} protein, respectively.

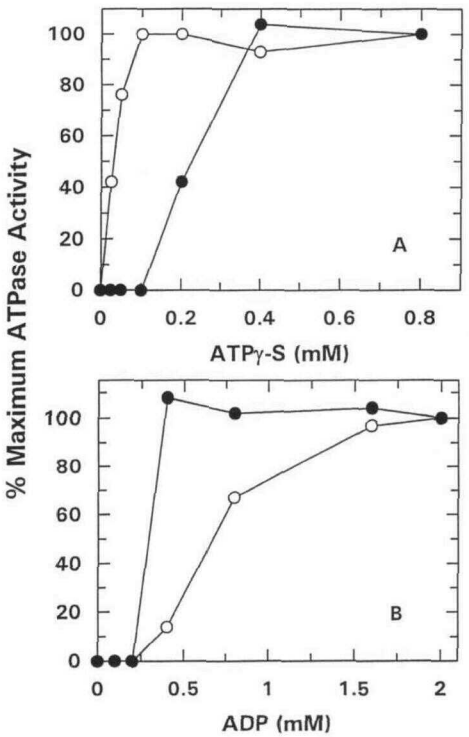

Figure 4. Effect of nucleotide concentration on thermal inactivation of the 45- (○) and 41-kD (●) forms of recombinant spinach activase. Enzyme was preincubated at 35°C for 15 min in the presence of the indicated concentrations of ATPγS (A) or ADP (B) and then assayed at 25°C. ATPase activity was determined spectrophotometrically by following the oxidation of NADH in an enzyme-linked assay system. Maximum activities of the 45- and 41-kD forms were 0.38 and 0.52 unit mg^{-1} protein, respectively.

ATPγS caused a rapid decay in the fluorescence signal for both the 45- and 41-kD forms, but the decay occurred at different temperatures (Fig. 6A). The difference plot, corrected for the time-dependent decay of fluorescence in the absence of nucleotide, showed that a sharp decrease in the ATPγS-induced component of fluorescence occurred at about 40°C for the 41-kD form and at about 55°C for the 45-kD form (Fig. 6B). The fluorescence signal of a mixture of the 41- and 45-kD forms followed a decay pattern more similar to the 45-kD form, but the sharp decrease occurred at about 50°C.

DISCUSSION

Produced by alternative splicing of pre-mRNA species, the 45- and 41-kD forms of spinach activase are identical, except for an extra 37 amino acids on the C terminus of the 45-kD form. Until now, the only known functional difference between the 45- and 41-kD forms was a difference in the kinetics of ATP binding (Shen et al., 1991). To our knowledge, our investigation revealed the first potential physiological difference between the two forms, a difference in thermal stability. We also observed differences in the kinetics of ATP binding. However, in our study the affinity for ATP was greater for the 45- than for the 41-kD form, which is in opposition to the result of Shen et al. (1991).

Previous studies have shown that purified leaf and recombinant activase are extremely heat-labile if incubated in the absence of ATP (Robinson and Portis, 1989; Holbrook et al., 1991; Eckardt and Portis, 1997). Our results corroborated these findings, because we observed inactivation of both forms upon exposure to relatively moderate temperatures (i.e. >25°C) in the absence of nucleotide. Inclusion of ATPγS during the temperature treatment (Fig. 3A) or catalysis in the presence of saturating ATP (Figs. 1 and 2) increased the thermal stability of both forms but to different extents. It is interesting that when both forms were mixed the thermal stability of the hybrid was very similar to the 45-kD form. These results indicate that, in the presence of ATP or an ATP analog, the 45-kD form exhibits greater thermal stability than the 41-kD form and it can confer thermal stability to the 41-kD form when the two forms are mixed.

Compared with ATP or ATPγS, ADP was comparatively ineffective in protecting against thermal inactivation of the 45-kD form of activase. Wang et al. (1993) have shown that ATP and ATPγS promote subunit association, whereas ADP does not. Subunit association of activase appears to be a prerequisite for activity (Wang et al., 1993; Salvucci and Ogren, 1996), reminiscent of the GroE chaperonins. In fact, like the GroEL toroids, the activase subunits display con-

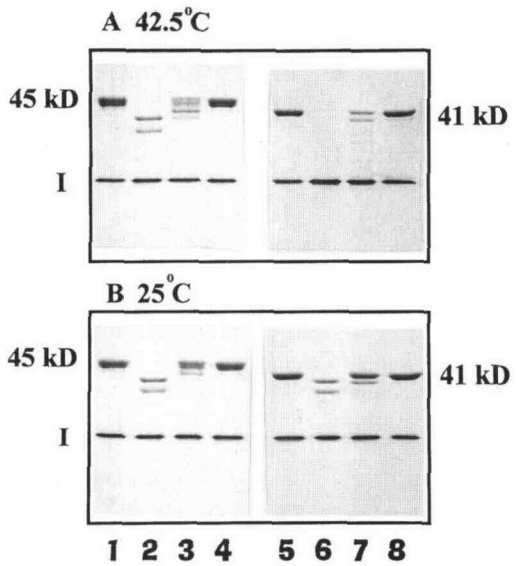

Figure 5. Tryptic digestion of the 45- and 41-kD forms of Rubisco activase. Activase was incubated for 15 min at 42.5°C (A) or 25°C (B) in the presence of 2 mM ATPγS followed by incubation for an additional 10 min in the presence of trypsin. Reactions were terminated with trypsin/chymotrypsin inhibitor. Lanes 1 to 4, The 45-kD form incubated with trypsin/chymotrypsin inhibitor and 10 ng of trypsin (lane 1), 10 ng of trypsin (lane 2), 1 ng of trypsin (lane 3), or 0.1 ng of trypsin (lane 4). Lanes 5 to 8, The 41-kD form incubated with trypsin/chymotrypsin inhibitor and 10 ng of trypsin (lane 5), 10 ng of trypsin (lane 6), 1 ng of trypsin (lane 7), or 0.1 ng of trypsin (lane 8). The position of the trypsin/chymotrypsin inhibitor on the gel is indicated by I.

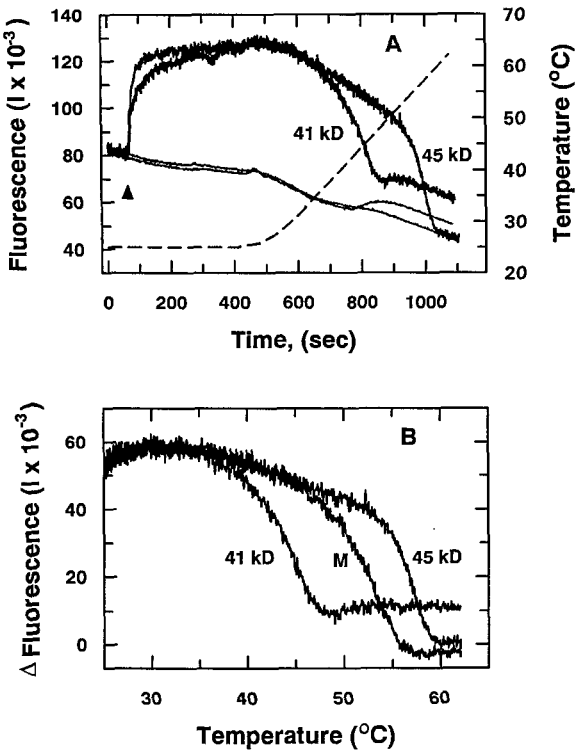

Figure 6. The effect of temperature on the intrinsic fluorescence of the 45- and 41-kD forms of recombinant spinach activase and a mixture of equal amounts (weight basis) of each form (M). A, Time course of intrinsic fluorescence during a gradual increase in temperature (– – – – –). Fluorescence was determined in the absence (bottom traces) or presence (top traces) of 0.2 mM ATPγS. The arrowhead denotes the time when ATPγS was added. B, Response of ATPγS-induced fluorescence to temperature replotted from the data in A. The traces were corrected for the changes in fluorescence that occurred in the absence of nucleotide.

siderable cooperativity in ATP hydrolysis (van de Loo and Salvucci, 1996). Unfortunately, the propensity of activase to self-associate (Salvucci, 1992) has made it difficult to determine the precise subunit composition of the functional enzyme. Using gel-filtration chromatography, Wang et al. (1993) showed that the molecular mass of the heat-inactivated enzyme was 140 kD, whereas the fully aggregated enzyme (i.e. in the presence of Mg^{2+} and ATPγS) has a molecular mass of greater than 600 kD. Thus, like the GroEL chaperonin (Hayer-Hartl et al., 1995), subunit association of activase appears to involve aggregation of assemblies of subunits into higher-order oligomers.

For activase, the conformation changes that occur upon ATP- or ATPγS-induced subunit aggregation shift one or more of the Trps to a more hydrophilic environment, causing an increase in the intrinsic fluorescence of the protein (Wang et al., 1993). In the present study the individual forms of activase and a mixture of the two forms all exhibited an abrupt decline in the ATPγS-induced component of intrinsic fluorescence. This decline occurred at approximately the same respective temperatures that inactivated ATP hydrolysis. We also found that there were no major differences in the susceptibility to proteolysis after exposure to temperatures that caused complete inactivation of the 41-kD form but not the 45-kD form. Taken together these results suggest that thermal inactivation of activase is caused by subunit dissociation and not subunit denaturation. It is unclear whether the tighter binding of ATP by the 45-kD form is the primary cause of the enhanced subunit interactions. Nevertheless, the differences in thermal stability between the 45- and 41-kD forms indicate that the C-terminal extension of the 45-kD form enhances subunit association.

Physiological Implications

It has been known for some time that light activation of Rubisco is one of the first processes adversely affected by elevated temperature (Weis, 1981; Kobza and Edwards, 1987). Activase is thought to be the critical component in the process, since the isolated enzyme is extremely susceptible to thermal inactivation in the absence of nucleotide. The results presented here show that the two forms of activase differ in their thermal stabilities and that, by enhancing subunit association, the 45-kD form confers protection to the 41-kD form against thermal inactivation. These observations may have physiological significance, since Jiménez et al. (1995) have shown that the 45-kD form of activase, which is not normally present in maize at ambient temperatures, accumulates and then declines in maize leaves following exposure to and recovery from 45°C. Differential expression of two biochemically different forms of activase could provide a mechanism for optimizing Rubisco activation to the prevailing environmental conditions.

It is interesting to speculate that alternative splicing in plants may be regulated by environmental cues. In animals alternative splicing is regulated according to development stages or cell types, but there are relatively few described cases of alternative splicing in plants and little evidence for this type of regulation (Nussaume et al., 1995). In contrast, it has been shown that cold stress can induce a form of alternative splicing (exon skipping) in the invertase transcript of potato but not whether the change is adaptive (Bournay et al., 1996). Different levels of the alternatively spliced RcaA1 and RcaA2 Rubisco activase transcripts accumulate in barley during the diurnal cycle and to a lesser extent during leaf development. However, it has not yet been demonstrated that the differences in these mRNA pool sizes are regulated at the level of splicing rather than degradation (Rundle and Zielinski, 1991b). It will be interesting to determine whether the pattern of Rubisco activase splicing changes in response to temperature.

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