Rubisco Activase, a Possible New Member of the Molecular Chaperone Family†

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ABSTRACT: The present research addresses the question of whether Rubisco activase (R-A), the enzyme reported to activate Rubisco, is actually a molecular chaperone rather than a conventional enzyme. Several biochemical properties known to be characteristics of molecular chaperones were tested for R-A with positive results. The experiments were performed either *in vitro* with purified spinach Rubisco and Rubisco activase or *in vivo* in maize seedling leaves. Our results confirmed that activation of Rubisco by R-A is an ATP hydrolysis-dependent process and further demonstrated that (a) R-A binds preferably to non-native Rubisco protein, than to the native form, and dissociates from this complex after addition of ATP, (b) R-A increases during heat shock treatment in maize seedling leaves, and (c) a large recovery of Rubisco activity is achieved from heat-inactivated Rubisco by addition of R-A and an energy source. We conclude that R-A characteristics strongly suggest that this protein belongs to the molecular chaperone group. The possible role of R-A on maintaining Rubisco activity *in vivo* is discussed.

Advances on protein assembly research have led to establish the molecular chaperone concept (Ellis & van der Vies, 1991). This term is functional and has been applied to a class of unrelated families of proteins that assist on the correct noncovalent assembly of many other polypeptides but which are not components of these assembled structures when they are performing their normal biological function (Rothman & Kornberg 1989; Gatenby & Ellis, 1990; Ellis, 1993). The term assembly also includes changes in the degree of either folding or association that may occur when proteins carry out their functions, are transported across membranes, or are damaged after stresses such as heat shock (Ellis, 1993).

Rubisco[1] (ribulose-1,5-bisphosphate carboxylase/oxygenase) is the most abundant protein in green tissues of plants. This is a large oligomeric enzyme (550 kDa), responsible for combining ribulose bisphosphate (RuBP) and CO_2 to produce two molecules of 3-phosphoglycerate. It is conformed by large subunits (LS, 53 kDa) arranged as an octameric core and small subunits (SS, 14 kDa) in two layers of four, each on opposite sides of the molecule (Chapman et al., 1988). The LS peptide, synthesized in the chloroplast, is associated to a protein denominated large subunit binding protein (LSbp), which prevents its aggregation prior to assembly with SS. The discovery that LSbp is homologous to the prokaryotic GroEL heat shock protein (Hemmingsen et al., 1988) led to the definition of a distinct group of molecular chaperones, named chaperonins. These proteins are widely represented in bacteria, mitochondria, and plastids (Martel et al., 1990; Gatenby, 1992). The relevance of chaperonins in Rubisco assembly was demonstrated in engineered bacterial systems where the expression of prokaryotic Rubisco genes produced active enzyme only upon overexpression of GroEL and GroES chaperonins (Goloubinoff et al., 1989a,b). In addition, fully active plant Rubisco enzyme did not form efficiently in bacterial cells, even though the products of both genes were simultaneously expressed in the prokaryotic system (Gatenby et al., 1987). This might be due to the absence of the correspondent plant chaperonin in the prokaryotic cells.

A plant enzyme called Rubisco activase (R-A) has recently been described, which may serve in this role. This protein is often found as two closely related forms (41 and 43 kDa) produced by alternate splicing of the same primary transcript (Werneke et al., 1988). The reaction catalyzed by Rubisco requires that CO_2 adds to the enzyme prior to RuBP; otherwise, an inactive Rubisco−RuBP complex is formed that cannot be overcome by CO_2 but by R-A action. It has been postulated that R-A reactivates Rubisco by releasing it from the inactive complex, hence allowing the combination of free Rubisco with CO_2 (Robinson et al., 1988). Certain aspects of the mode of action of this protein, however, led us to consider the possibility that R-A might not be a conventional enzyme but a molecular chaperone. This postulate is supported by the important Rubisco conformational changes reported to occur in the Rubisco molecule through its activation−inactivation process (Bowien & Gottschalk, 1992). It is also based on the observation that Rubisco activation by R-A is an ATP hydrolysis-dependent process (Robinson & Portis, 1989). In addition, R-A is known to be in large concentrations in leaves of green plants (Salvucci et al, 1987), and we have found that it does not follow conventional enzyme kinetics and must reach non-

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[1] Abbreviations: BTP, Bis-Tris propane; DOC, deoxycholic acid sodium salt; DTNB, dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; LSbp, Rubisco large-subunit binding protein; PMSF, phenylmethanesulfonyl fluoride; R-A, Rubisco activase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate.

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catalytic concentrations to activate Rubisco. In light of the above observations, we decided to test experimentally if R-A functions as a molecular chaperone.

MATERIALS AND METHODS

Biological Material. Maize seeds (*Zea mays* L. Cv Zacatecas 58, provided by Dr. J. Molina, Colegio de Posgraduados, Montecillo, Edo. Mexico) were grown in a greenhouse. The second fully expanded leaf appearing 10 days after sowing was used. Frozen leaves (approximately 1 g) were ground under liquid nitrogen in a chilled mortar, and the powder was homogenized with 2 mL of extraction buffer (50 mM Tricine−NaOH, pH 7.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, 1 mM ATP, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine, and 0.01 mM leupeptin). The extracts were clarified by centrifugation at 15000*g* for 5 min at 4 °C. The supernatant was saved and used for experimental measurements (leaf extract). For heat shock experiments the seedlings were treated at 45 °C for 4 h in a growth chamber (Labline Instruments, Inc.) at 90% relative humidity. For control of heat shock experiments we used *Escherichia coli* culture (strain MC 1061) grown in LB medium (GIBCO, BRL) at 27 °C and exposed to 42 °C for 15 min. Heated and nonheated cultures were collected by centrifugation, washed, and resuspended in a buffer containing 25 mM Tris-HCl (pH 8.0), 50 mM glucose, and 10 mM EDTA and lysed at 4 °C by addition of 4 mg/mL lysozyme. Five minutes later, the lysate was diluted with the same volume in ice-cooled buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% DOC, 1% NP40, 10 mM $MgCl_2$, 30 mM $NaHCO_3$, 1 mM DTT, and 1 mM PMSF) and centrifuged for 10 min at 10000*g* at 4 °C. The supernatant was used as the control for the heat shock experiments.

Rubisco and Rubisco Activase Purification. Approximately 100 g of spinach leaves was ground under liquid nitrogen in a chilled mortar, and the powder was homogenized with 200 mL of extraction buffer (100 mM BTP, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 1.4 mM ATP, 15 mM DTT, 1 mM PMSF, 2 mM benzamidine, and 0.01 mM leupeptin). From this material Rubisco and Rubisco activase were purified following the procedures reported by Salvucci et al. (1986) and Robinson et al. (1988), respectively. The purified proteins analyzed by gel electrophoresis showed homogeneity; that is, only the two expected bands for each protein—55 and 14 kDa for Rubisco and 43 and 41 kDa for R-A—were observed. These results were similar to the ones obtained by the authors of the original reports.

Rubisco Assay. Rubisco carboxylase activity was measured by the $^{14}CO_2$ fixation method (Robinson et al, 1988): 5μL aliquots of leaf extract were used as the source of enzyme, mixed with 60 μL of the assay mixture [100 mM Tricine−NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (New England Nuclear, 100 mCi/mmol, 1 Ci = 37 GBq), and 0.5 mM RuBP]. The assay was run for 0, 20, 40, and 60 s at 25 °C, stopped by addition of 200 μL of 3 N HCl in methanol, and dried at 65 °C (Loza-Tavera et al., 1990). Incorporation of $^{14}CO_2$ into acid-stable products was determined by liquid scintillation spectrometry (Packard Instruments). Total activity was calculated from the slope of the reaction values.

Rubisco Activase Assay. This assay was performed by reactivation of the inactive Rubisco−RuBP complex reaction (Robinson et al., 1988). Maize leaf extract was desalted by gel filtration in a Sephadex G-25 column (20 × 0.5 cm) equilibrated with 50 mM Tricine−NaOH (pH 8.0). For the formation of the Rubisco−RuBP complex, the filtered enzyme (1 mg/mL) was incubated in 1.5 mM RuBP final concentration for 30 min at 4 °C. The Rubisco reactivation assay mixture (100 μL) contained 50 mM Tricine−KOH (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 4 mM RuBP, 1 mM ATP, 3 mM creatine phosphate, 20 units/mL creatine kinase, 1 mg/mL BSA, 64.5 mg of the Rubisco−RuBP complex, and 15 mg of purified spinach R-A. R-A was added 1 min before initiation of the reaction at 25 °C; with the Rubisco−RuBP complex, Rubisco activity was measured 15 min later.

Reactivation of Heat-Inactivated Rubisco by R-A. Purified spinach Rubisco was desalted through a G-50 column (20 × 0.5 cm) equilibrated with 50 mM Tricine−NaOH (pH 8.0), and the solution (2 mg/mL) was heated for 1 h at 55 °C. After this treatment Rubisco lost about 65% of the original activity. To restore Rubisco activity, 20 mg of this Rubisco sample was mixed with varying amounts of R-A in a reactivation mixture containing 50 mM Tricine−NaOH (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM ATP, 3 mM creatine phosphate, 20 units/mL creatine kinase, and 1 mg/mL BSA. R-A was added 1 min before initiation of the reaction at 25 °C, measuring Rubisco activity 15 min later.

Interaction between Rubisco and R-A and Immunoprecipitation. Purified spinach Rubisco samples (either heat-inactivated or native) were mixed with R-A and incubated in the reaction mixture described above for R-A reactivation of heat-inactivated Rubisco. For some samples ATP was omitted from the mixture. The reaction was stopped with 200 μL of buffer A (10 mM Tricine, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% NP40, 1 mM PMSF, and 15 mM leupeptin) and addition of 30 μL of polyclonal anti-Rubisco antibodies. The mixture was allowed to stand overnight at 4 °C. Then the samples were supplemented with 20 μL of protein A−Sepharose and incubated for an additional 2 h at 25 °C, and the immunoprecipitate was collected by centrifugation, dissolved in 20 μL of sample-loading buffer, and analyzed by SDS−PAGE (Laemmli, 1970). The gel was stained with Coomassie blue. Other gels were immunoblotted onto nitrocellulose sheets for Western blot analysis and revealed by R-A antibodies, as described below.

Modification of Sulfhydryl Groups with DTNB. Native and heat-denaturated Rubisco samples were used to measure sulfhydryl group modification in Rubisco with DTNB reagent according to Chollet and Anderson (1977). Increase in absorbance at 412 nm of 2-nitro-5-thiobenzoate liberated (E = 13600 M^{-1} cm^{-1}) was followed spectrophotometrically. The assay tubes contained 50 mM Tris (pH 7.4), 67 mM NaCl, 1.1 mg of purified Rubisco, and 100 mM DTNB in a final volume of 1.0 mL. Following 5-min preincubation at 25 °C, the reaction was initiated with DTNB addition, and ΔA_{412nm} was monitored over a 30-min period. Controls without DTNB were also performed.

Immunoblotting. Protein samples were transferred onto nitrocellulose sheets at 50 mA for 2.5 h using a semidry blotting apparatus (LKB, Bromma, Sweden). After protein transfer, the blots were processed according to Ferreira and Shaw (1989). The membranes were probed with anti-spinach R-A mouse antibody (kindly donated by Dr. W. L. Ogren, University of Illinois) and rabbit anti-GroEL (kindly donated by Dr. David Gobezensky, Weizmann Institute of Science,

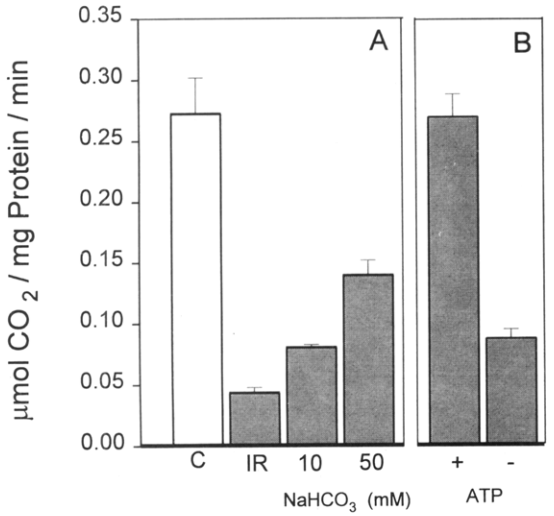

FIGURE 1: Effect of R-A on the Rubisco−RuBP Inactive Complex. Rubisco activity from maize leaf extracts was inhibited by RuBP treatment (see Materials and Methods). Enzyme reactivation was measured either as a function of $NaHCO_3$ (A) or of R-A treatment (B). Panel A: (C) Rubisco activity in leaf extracts (control); (IR) remaining Rubisco activity after RuBP inhibition; (10 and 50) Rubisco reactivation after incubation with 10 or 50 mM $NaHCO_3$, respectively. Panel B: Rubisco activity of the Rubisco−RuBP inactive complex after addition of purified spinach R-A and 10 mM $NaHCO_3$, in the presence (+) or absence (−) of the ATP-generating system. Each value represents the mean of three independent experiments. Vertical lines represent standard deviations.

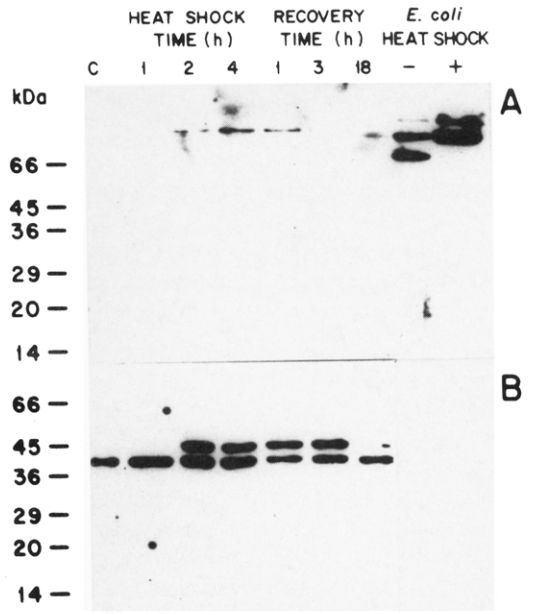

FIGURE 2: Heat shock effect on GroEL-like proteins and Rubisco activase. Soluble protein from maize leaf and *E. coli* extracts, before and after heat shock treatment, was resolved by SDS−PAGE and blotted as indicated in Materials and Methods. Antibodies raised against either GroEL (A) or spinach Rubisco activase (B) were used in the Western blot. Periods of leaf heat shock, 1, 2, and 4 h at 45 °C, and recovery, 1, 3, and 18 h, are indicated for both blots. C stands for leaf control. *E. coli* (control), before and after heat shock, is also included in (A). This panel is representative of three independent repetitions.

Israel). Antibodies against IgG, conjugated to peroxidase, were diluted 1:400 for R-A and 1:1500 for GroEL and applied as a second antibody. The blots were developed with the ECL system (Amersham).

Other Procedures. Molecular weight was estimated in an Ultrogel ACA-24 column (90 × 0.5 cm) equilibrated with 50 mM Tricine (pH 8.0). Protein determinations were made by the method of Bradford (1976) using BSA as the standard. SDS−PAGE was performed in 10% acrylamide gels according to Laemmli (1970) and stained with Coomassie blue.

RESULTS

Binding of RuBP to Rubisco protein produces an inactive Rubisco−RuBP complex and prevents CO_2-mediated Rubisco activation. Rubisco activity can, however, be restored when Rubisco is released from this complex by R-A and an ATP-generating system (Robinson et al., 1988). Figure 1 shows that addition of RuBP to maize leaf extract causes 90% inhibition of Rubisco activity. Addition of large amounts (up to 50 mM) of $NaHCO_3$ to the system can only partially alleviate this inhibition (Figure 1A). In contrast, when the same inhibited-leaf extract was treated with R-A and the ATP-regenerating system in the presence of 10 mM $NaHCO_3$, the original Rubisco activity was fully restored. These data suggest that R-A efficiently restores the original Rubisco CO_2-activable form by interacting with the Rubisco−RuBP inactive complex and that this activity depends on ATP hydrolysis (Figure 1B). This particular mode of R-A action resembles the behavior of chaperones rather than the behavior of conventional enzymes. Following this rationale, several experiments were designed to test whether R-A functions as a molecular chaperone. Since many molecular chaperones are known to increase their concentration after cells have been heat-shocked, maize seedlings were exposed to 45 °C during 4 h followed by an 18-h recovery period at room temperature. As a control, an *E. coli* culture received the heat shock treatment for 15 min. *E. coli* and leaf extracts, from different times within the experimental period, were analyzed by gel electrophoresis and Western blot. Recognition of heat shock proteins was performed in the bacterial and leaf extracts using anti-GroEL antibody (Figue 2A). Increasing concentrations of cross-reacting heat shock proteins were found 2−4 h after heat shock application, followed by restoration to the original levels during the subsequent recovery period (Figure 2A). Similarly, R-A cross-reacting bands were revealed by spinach R-A antibodies in the same nitrocellulose sheets (Figure 2B). Seedling leaf extracts obtained before the heat shock treatment showed one 41 kDa R-A cross-reacting band. As heat shock progressed, this band increased and a second heavier R-A band (43 kDa) appeared. During the recovery period, the heavier R-A band slowly disappeared whereas the intensity of the 41 kDa R-A band decreased to basal levels at 18 h of recovery (Figure 2B). In parallel, Rubisco activity and Rubisco protein were measured on the same leaf samples tested for heat shock and R-A proteins. Rubisco activity was found to increase during the heat shock period in a fashion similar to that of the R-A protein and also returned later to a basal level at the end of the recovery period (Figure 3A). There was no change on Rubisco protein content during the experimental period, as judged by the Western blot Rubisco analysis (Figure 3B).

Proteins in unfolded or partially folded states can form stable binary complexes with chaperones. In order to verify the strength and specificity of the R-A−Rubisco interaction, experiments were performed where either native or non-native (desalted and heat-inactivated) spinach Rubisco and

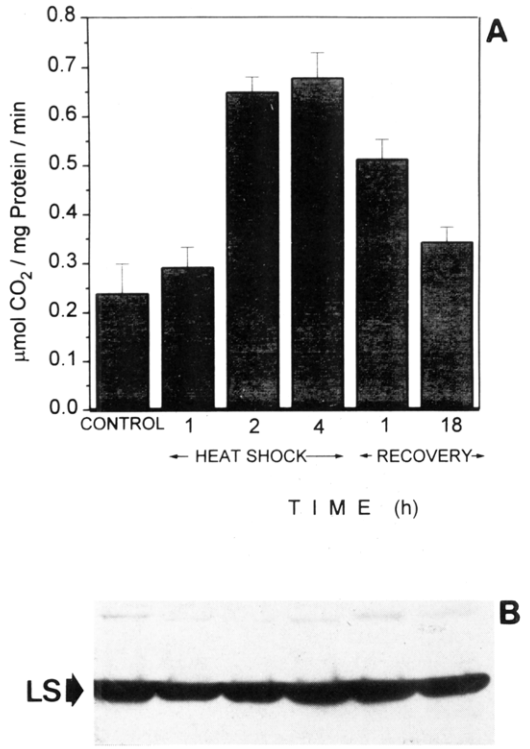

FIGURE 3: Maize leaf Rubisco activity during heat shock and recovery periods. Rubisco activity measurements were performed in leaf extracts before heat shock (control), during heat shock treatment for 1, 2, and 4 h, and during the recovery period for 1 and 18 h, by the $^{14}CO_2$ fixation procedure as indicated in Materials and Methods (A). Rubisco protein concentration was tested in the same leaf samples by Western blot analysis with antibodies raised against spinach Rubisco LS (B). Each value represents the mean of three independent experiments. Vertical lines represent standard deviations.

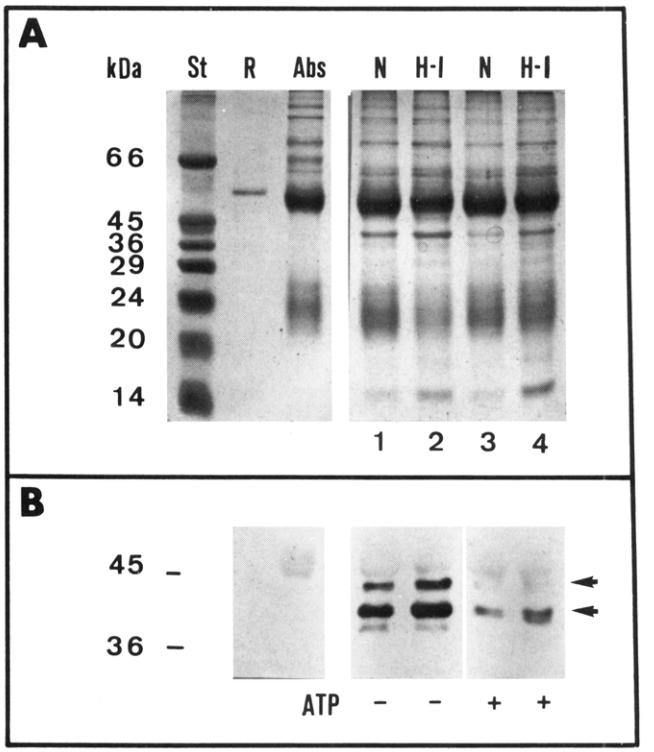

FIGURE 4: Immunoprecipitation of R-A associated to Rubisco. Either native (N) or heat-inactivated (H-I) spinach Rubisco was mixed with R-A (20 μg each) in the absence (lanes 1 and 2) or in the presence of ATP and the ATP-generating system (lanes 3 and 4) and immunoprecipitated with antibodies against Rubisco (Abs). The immunoprecipitates were analyzed by gel electrophoresis and stained by Coomassie blue (A) or transferred to nitrocellulose membranes for Western blot analysis and revealed with antibodies raised against R-A (B). Arrows point to 41 and 43 kDa R-A peptides (B). R, spinach Rubisco; St, molecular mass markers. This plate is representative of three independent experiments.

R-A were mixed. Antibodies against Rubisco were then added to the Rubisco−R-A mixture in the presence or absence of the ATP-generating system and allowed to react for 15 min at 25 °C. The immunoprecipitated proteins were separated by centrifugation with protein A−Sepharose and analyzed by Western blot, using R-A antibodies to reveal the bands. The results showed that the two R-A bands (41−43 kDa) coprecipitated with both native and non-native Rubisco samples when ATP was not present in the mixture, although in less amount with the native sample (lanes 4 and 5, Figure 4B). On the other hand, the samples that received the ATP treatment showed only slight or no R-A coprecipitation, especially for the non-native Rubisco samples (lanes 6 and 7, Figure 4B). These data confirm the formation of a tight Rubisco−R-A complex when these two proteins are mixed. This complex, however, can be easily separated if ATP is generated in the system (Figure 4B). A similar behavior has been documented for many molecular chaperones.

Finally, an experiment was performed to test the R-A ability to restore Rubisco activity lost by alteration of the native protein conformation. For this means, purified spinach Rubisco was filtered through a G-50 column (to devoid Rubisco of small molecules and ions, CO_2, Mg^{2+}, and RuBP, that stabilize the enzyme) and heat-inactivated at 55 °C for 1 h. Measurements of Rubisco activity in these samples indicated that, after this treatment, only approximately 35% of the original activity remained in the Rubisco samples.

To provide evidence of the conformational changes caused by the desalting and heat treatment, the amount of titrable −SH residues in the protein was determined following the time course reaction of Rubisco with DTNB reagent. A clear diminution in −SH reactivity was observed in the treated sample as compared to the native Rubisco sample (Figure 5). This difference is in agreement with a conformational change in the Rubisco structure caused by the heat treatment of the desalted enzyme.

Another indication of structural alteration of the treated Rubisco protein was obtained by filtration of Rubisco samples through the Ultrogel ACA-24 column. In this experiment, both native Rubisco and heat-inactivated Rubisco showed different elution profiles (Figure 6). The apparent molecular mass of the non-native sample was displaced from 550 up to approximately 700 kDa, suggesting a large change in conformation.

The role of molecular chaperone proteins on restoring native protein structure—and therefore function—constitutes the main characteristic of these proteins. To test if R-A can serve this function, experiments on restoration of Rubisco activity from heat-inactivated Rubisco were performed. Rubisco activity was determined in both native (control) and heat-inactivated Rubisco, and the effect of increasing $NaHCO_3$ concentrations on Rubisco activity recovery was tested. Rubisco activity remained low, and no signs of recovery were observed even at concentrations as high as 50 mM $NaHCO_3$ (Figure 7B). On the other hand, samples of the same

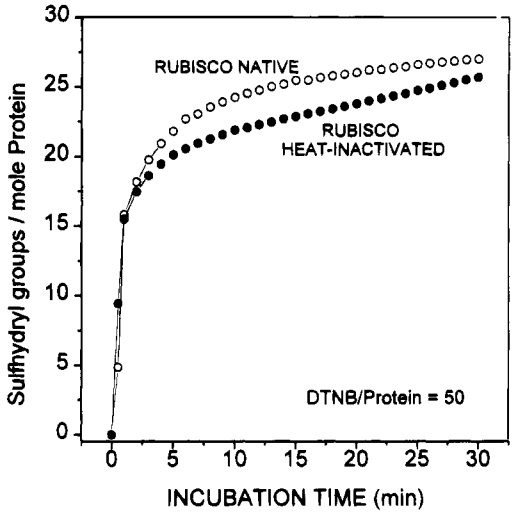

FIGURE 5: Sulfhydryl group reactivity with DTNB. Reaction of either native (○) or heat-inactivated (●) purified spinach Rubisco with DTNB at 25 °C. The cuvettes contained 50 mM Tris (pH 7.4), 67 mM NaCl, 1.1 mg of Rubisco, and 100 mM DTNB in a final volume of 1.0 mL. The reaction was initiated with DTNB and the ΔA_{412nm} determined over a 30-min period.

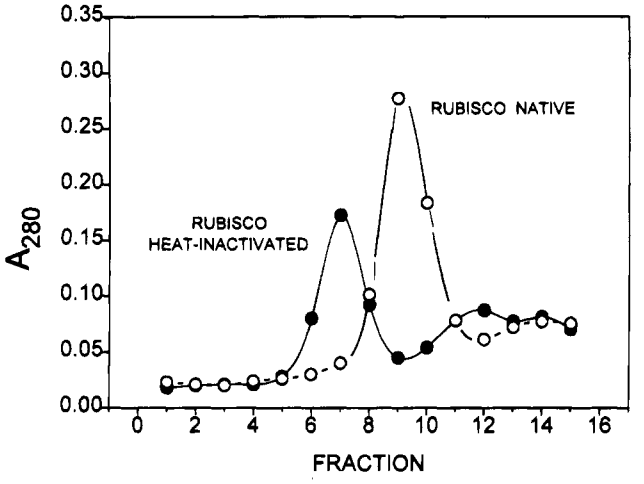

FIGURE 6: Filtration profile of native and heat-inactivated Rubisco. Five milligrams of native (○) or heat-inactivated (●) spinach Rubisco was filtered through an Ultrogel ACA-24 filtration column (see Materials and Methods). Rubisco elution was performed with 50 mM Tricine–NaOH (pH 8.0) at an effluent rate of 0.25 mL/min. Apparent molecular masses calculated from the protein mobility were 700 kDa for heat-inactivated Rubisco and 550 kDa for the native sample. This profile illustrates a representative result from three independent repetitions.

Rubisco preparation in the presence of the ATP-generating system and increasing R-A concentrations showed a clear recovery of Rubisco activity, which was not observed if the ATP generating system was omitted in the assay (Figure 7C). It is evident in this last figure that the amount of Rubisco activity recovered is a function of R-A concentration. With the highest concentration of R-A used, final Rubisco activity was very similar in the heat-treated and native preparations.

DISCUSSION

The data presented here provide evidence that R-A shares functional characteristics with many reported molecular chaperones. As previously described, molecular chaperones comprise groups of unrelated protein families that assist other proteins in their correct assembly (Ellis, 1993). The complex

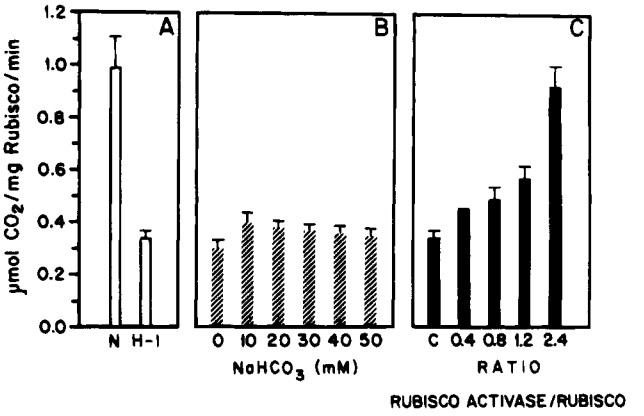

FIGURE 7: Reactivation of heat-inactivated Rubisco. (A) Native spinach Rubisco (N) was filtered through a Sephadex G-50 column (20 × 0.5 cm) equilibrated with 50 mM Tricine–NaOH (pH 8.0) and then heat-inactivated at 55 °C for 1 h (H-I). (B) Heat-inactivated Rubisco (20 μg) was incubated for 15 min at 4 °C with increasing $NaHCO_3$ concentrations. (C) Aliquots of 20 μg of heat-inactivated Rubisco were mixed with varying R-A concentrations and 1 mM ATP plus the ATP-generating system (see Materials and Methods) in a final volume of 100 μL. In each case, Rubisco activity was measured by the $^{14}CO_2$ fixation procedure. Control determinations were performed in the absence of ATP and the ATP-generating system at all R-A/Rubisco ratios. Rubisco activity at 2.4 R-A/Rubisco ratio is illustrated (C). Each value represents the mean of three independent experiments. Vertical lines represent standard deviations.

regulatory process of Rubisco activity by its substrates involves important Rubisco conformational changes (Bowien & Gottschalk, 1992), and it has been documented that Rubisco acquires the active carbamylated form by allosteric interaction with CO_2 whereas the other substrate downregulates the enzyme through the formation of an inactive Rubisco–RuBP complex (Portis, 1986). Restoring Rubisco activity from this complex, and thus the active Rubisco conformation, requires R-A action in an ATP hydrolysis-dependent process (Figure 1), similar to that reported for molecular chaperones (Goloubinoff et al., 1989b; Skowyra et al., 1990). It shall be noticed that this process does not seem to produce phosphorylated Rubisco and that the catalysis of the ATP hydrolysis is an intrinsic characteristic of R-A independent of the presence of Rubisco (Robinson et al., 1988; Martínez-Barajas et al., unpublished data). R-A, as well as other molecular chaperones, is widely distributed in large amounts in living organisms (Salvucci et al., 1987; Ellis & van der Vies, 1991). Some are constitutively expressed (Parsell & Lindquist, 1993), but many of them, including R-A, respond to heat shock induction (Jaenicke & Creighton, 1993) (Figure 2). In the latter case, it is interesting to note that Rubisco activity was maintained and even increased in heat–shocked plants concomitant to R-A increase, without significant alteration of Rubisco protein concentration (Figure 3). Recognition between molecular chaperones and their target proteins does not require precise amino acid sequences; rather, it seems to be based on protein–protein interactions (Rothman & Kornberg, 1989) that allow them to tightly associate with their target proteins for chaperone-assisted refolding (Ellis & van der Vies, 1991). Addition of an ATP-generating system to those protein complexes allows release of the chaperone (Gatenby, 1992), as was demonstrated to occur in the R-A–Rubisco complex (Figure 4). Finally, the strongest evidence to define R-A as a molecular chaperone is provided by the restoration of

Rubisco activity from the heat-inactivated enzyme. Purified Rubisco protein, altered in its conformational structure as well as in its activity by the desalting and heat treatments (Figures 5−7A), recovered its original activity when incubated with R-A and the ATP-generating system (Figure 7C), similar to that reported for other heat- or high salt-inactivated enzymes (Goloubinoff, 1989b; Skowyra et al., 1990). However, large concentrations of R-A were required to obtain full recovery of Rubisco activity from heat-inactivated enzyme (Figure 7C). The correspondent R-A/Rubisco ratio, calculated on molecular mass bases, indicates that there are approximately 30 R-As (41−43 kDa) per molecule of Rubisco (550 kDa). This ratio suggests that R-A might surround the Rubisco molecule to induce the conformational changes needed to restore Rubisco activity. Actually, this type of mechanism has been proposed before for other chaperones (Saibil et al., 1993).

It has recently been recognized that different types of chaperones seem to intervene at different steps of the refolding protein process (Langer et al., 1992). Within this context, chaperone function is now considered not only related to folding nascent peptides and protein assembly but also related to stabilizing, protecting, and repairing protein structures (Chitnis & Nelson, 1991; Ellis, 1993). In this regard, it is interesting to mention that during the grain-filling period of maize, a period of strong photosynthate demand, a significant increase of Rubisco activity was observed in the leaf above the ear. This was not supported by a change of Rubisco protein but rather by an increase of R-A leaf content (Martínez-Barajas et al., unpublished results). During this period of very low Rubisco turnover, lasting about 60 days, it is possible that a R-A-mediated path exists to restore Rubisco structure, disrupted by unfavorable environmental conditions and/or accumulation of endogenous RuBP in the leaves. Work is being performed in our laboratory to further test this hypothesis.

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