Sulfur Starvation in *Lemna* Leads to Degradation of Ribulose-Bisphosphate Carboxylase without Plant Death*

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Ricardo M. B. Ferreira* and Artur R. N. Teixeira*

From the 1Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, 1399 Lisboa Codex, Portugal and 2Centro de Tecnologia Química e Biológica, Apartado 127, 2780 Oeiras, Portugal

Little is known about the degradation of the most abundant protein in nature, ribulose-bisphosphate carboxylase (RuBP carboxylase, EC 4.1.1.39), probably reflecting the fact that no stress situation has been identified capable of causing extensive RuBP carboxylase degradation without causing the death of the plant. We have subjected plants of *Lemna minor* L. to a variety of stress situations, nutritive deficiencies in particular, and have found a single condition—sulfur starvation—that caused almost complete degradation of RuBP carboxylase without causing plant death. Moreover, the enzyme was preferentially degraded under these conditions. However, when the plants were deprived of calcium, no RuBP carboxylase degradation was observed. Instead, the enzyme was oxidized and polymerized into high molecular mass aggregates. On the other hand, RuBP carboxylase shows an extreme stability when *Lemna* is deprived of some macronutrients (e.g. nitrogen, phosphorus, potassium, and magnesium) probably reflecting that this plant had to evolve in a way to cope with frequent shortages of such elements. The implications of these data for the role of RuBP carboxylase as a leaf storage protein are discussed.

Ribulose-bisphosphate carboxylase (RuBP carboxylase, EC 4.1.1.39) catalyzes the initial reactions of the photosynthetic and photorespiratory pathways (Pierce, 1988). In the leaves of higher plants, RuBP carboxylase often comprises 50–75% of the soluble protein, which makes it the most abundant protein in the biosphere (Kung, 1976; Tingey and Andersen, 1986). Several reasons have been advanced to try to explain the extraordinary abundance of this enzyme in green tissues. One reason appears to be that it is a sluggish catalyst. Under natural conditions, it is estimated that the carboxylase activity fixes CO$_2$ at a rate of 200 molecules/min/molecule of enzyme (Ellis, 1979). Nevertheless, the quantity of the protein present in leaf tissue may account for other noncatalytic functions in plant metabolism. Indeed, because RuBP carboxylase is present in the chloroplast stroma at greater concentrations than its substrates (Sicher and Jensen, 1979), it has been suggested that the enzyme may serve as a metabolite buffer in the chloroplasts (Ashton, 1982). On the other hand, RuBP carboxylase has also been considered as a storage protein. In fact, the stability of RuBP carboxylase prior to senescence, and its rapid degradation during the senescence of wheat and barley leaves led to its classification as a leaf storage protein (Kleinkopf et al., 1970; Huffaker and Miller, 1978). However, this hypothesis was based on studies using senescing leaves and has been tested only for a limited number of species. As a major protein component in leaf tissue, it becomes an important component for animal nutrition, since its amino acid composition is well balanced (Kawashima and Wildman, 1970).

Plants are often subjected to stress situations (Levitt, 1980). These conditions may alter the RuBP carboxylase content of the leaves in different but economically important ways. Thus, whereas some stress situations may not affect RuBP carboxylase concentration, modifying or not its structure, others may dramatically reduce the level of the enzyme (Wittenbach, 1979; Friedrich and Huffaker, 1980; Ferreira and Davies, 1987a, 1987b). In other words, the effect of plant stress on RuBP carboxylase may play an important role in the quality of most vegetable products. However, little is known about the degradation of this enzyme, paralleling the situation of proteins in general, in which so much more is known about their synthesis than about their degradation.

In the present work, we have studied the effect of stress on the degradation pattern of RuBP carboxylase from *Lemna minor* and also tested the possibility that this enzyme plays a role as a leaf storage protein.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

The Effect of Sulfur Starvation on *Lemna* RuBP Carboxylase—When the soluble proteins from unstressed *L. minor* fronds were separated by FPLC on the Mono Q anion exchange column, the pattern presented in Fig. 1A was obtained. The basic proteins are not retained by the column, and the more acidic proteins are eluted as the concentration of NaCl is increased. A two-step NaCl gradient was employed to obtain a good resolution between RuBP carboxylase (*peak 1*) and nucleic acids (*peak 2*) and to focus the different nucleic acid molecular species into a single peak (results not shown).

Under the conditions of study, it is reasonable to assume that, when *Lemna* fronds are subjected, for various periods of time, to a nutritive deficiency that causes extensive RuBP carboxylase degradation, the other protein bands would have a similar behavior.

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†To whom correspondence should be addressed. Tel.: 351-1-3698161 (ext. 363); Fax: 351-1-3650503; Telex 44700 ISATEL P.

‡The abbreviations used are: FPLC, fast protein liquid chromatography; RuBP, ribulose-bisphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
carboxylase degradation, it is possible to detect the proteolytic process by following the decrease in the size of RuBP carboxylase peak isolated by anion exchange chromatography. However, as far as we are aware, no stress situation has been identified capable of causing extensive RuBP carboxylase degradation without causing the death of the plant. This may explain why so little is known about the cellular mechanism(s) responsible for the degradation of this enzyme.

When *Lemna* fronds were incubated in growth medium lacking sulfur, they continued to divide, and remained viable for at least 26 days. However, as shown in Fig. 1, RuBP carboxylase is extensively degraded during the first 17 days of sulfur deprivation.

To assess further the degradation of RuBP carboxylase in *Lemna* subjected to sulfur starvation, a modification of the double-isotope technique of Arias et al. (1969) was used to study protein degradation under stress conditions (Ferreira and Davies, 1987b). This widely used technique has the advantage of ease and simplicity in operation, but gives relative rather than absolute values of degradation. The values for protein degradation rates are presented as the direct $^{14}$C/$^3$H ratios, rather than as $K_0$ or $t_{1/2}$, since the assumption of a linear relationship between $^{14}$C/$^3$H and $K_0$ is not valid if amino acid recycling occurs (Zak et al., 1977), and Davies and Humphrey (1978) have shown that, under normal growth conditions, 50% of the leucine released in *L. minor* cells during protein degradation undergoes recycling. Furthermore, growth that occurs during the chase period further distorts the relationship.

Two batches of *Lemna* fronds were grown in complete medium (750 ml) containing either 2.78 MBq of L-[3,4,5-$^3$H] leucine or 0.62 MBq of L-[1-$^4$C]-leucine for 25.5 h. During that time, 76% of the L-[3,4,5-$^3$H]leucine and 95% of the L-[1-$^4$C]-leucine were taken up by the fronds, as judged by measuring the loss of radioactivity from the solutions. After the labeling period, the *^3$H* labeled fronds were washed in unlabeled medium and transferred, as approximately equal samples, to five flasks containing growth medium lacking sulfur. The number of fronds in each flask was counted, and the fronds were incubated for 0, 2, 4, 8, and 14 days before being harvested and combined with the corresponding samples of *$^3$H*-labeled fronds, as described under "Experimental Procedures." Protein was extracted and fractionated by FPLC using the Mono Q ion exchange column. The fractions (1 ml) were counted for $^{14}$C and $^3$H. The design of this experiment is such that a comparison of the $^{14}$C/$^3$H ratios of the isolated protein fractions provides a measure of the relative rates of in vivo degradation; a high $^{14}$C/$^3$H ratio is indicative of a relatively rapid rate of protein breakdown. The data shown in Fig. 2A constitutes a control, giving the noise level in the experiment. The results presented in Fig. 2 show that with increasing periods of time, there is a general increase in the ratio $^{14}$C/$^3$H, corresponding to an intense protein degradation under conditions of sulfur starvation. However, the fractions containing RuBP carboxylase had the highest $^{14}$C/$^3$H ratios, suggesting that this enzyme is preferentially degraded under these conditions. A number of fractions eluting before the RuBP carboxylase peak showed low $^{14}$C/$^3$H ratios throughout the experiment, indicating the presence of proteins with very low rates of degradation under conditions of sulfur deprivation.

Samples of the double-labeled total soluble proteins for each of the five times specified in Fig. 2 were counted for $^{14}$C and $^3$H. The results are presented in Fig. 3A together with data on RuBP carboxylase degradation obtained by anion exchange chromatography (Fig. 2). This figure further supports the hypothesis of RuBP carboxylase being preferentially degraded under conditions of sulfur starvation.

RuBP carboxylase isolated by ion exchange chromatography on the Mono Q column for each time period (Fig. 2) was resolved into its subunits by SDS-PAGE using a 12.5% (w/v) polyacrylamide gel. The double-labeled protein bands corresponding to the large and small subunits of RuBP carboxylase were sliced, digested, and counted for $^{14}$C and $^3$H, as described under "Experimental Procedures." The $^{14}$C/$^3$H ratios for each of the RuBP carboxylase subunits increase with time (Fig. 3B), paralleling the increase observed for the native enzyme (Fig. 3A). This result suggests that RuBP carboxylase subunits degrade at the same rate when *L. minor* is incubated in the absence of sulfur.

The Effect of Calcium Starvation on *Lemna RuBP Carboxylase*—Ferreira and Davies (1989) and Ferreira and Shaw (1989) have shown that although many proteins from *L. minor* undergo enhanced degradation during osmotic stress, RuBP carboxylase is not degraded. Instead, RuBP carboxylase is converted in a series of steps to a very high molecular mass form. The first step involves the action of an oxidase system that after 24 h of stress converts RuBP carboxylase to an acidic and catalytically inactive form. These changes in the oxidation state of RuBP carboxylase were detected by FPLC by following the changes in the binding properties of the enzyme to the Mono Q anion exchange column. Subsequently, the oxidized RuBP carboxylase protein is gradually polymerized to a number of very large aggregates.

*Lemna* fronds were incubated in growth medium lacking calcium for 0 (control), 6, 10, and 14 days. After each time period, the fronds were harvested and the protein was extracted and fractionated by anion exchange chromatography on the FPLC Mono Q column. The isolated RuBP carboxylase peak was further purified by gel filtration on the FPLC Superose 6 column. The results presented in Fig. 4 clearly show that when *Lemna* fronds are deprived of calcium for a number of days, RuBP carboxylase is first oxidized to an acidic form (Fig. 4C, peak 3) and then polymerized into high molecular mass aggregates (Fig. 4, B, D, F, and H). These aggregates were eluted from the gel filtration column at the void volume (Fig. 4, peak a) and at peaks corresponding in molecular mass to 670 kDa (Fig. 4, peak b) and 532 kDa (native *Lemna RuBP carboxylase* (Fig. 4, peak c)). The size of these high molecular mass forms, which are stable during boiling in the presence of SDS and dithiothreitol, is also illustrated by the fact that they do not enter the stacking gel (5% w/v acrylamide, 0.13% w/v bisacrylamide) when subjected to SDS-PAGE (results not shown). This process parallels the situation observed during osmotic shock. Identical results were obtained when the fronds were incubated in the simultaneous absence of calcium and sulfur.

The Effect of Other Nutritive Deficiencies on the Degradation Pattern of *Lemna RuBP Carboxylase*—When *Lemna* fronds were incubated, for extended periods of time, in growth medium lacking nitrogen, phosphorus, potassium, magnesium, or iron, no RuBP carboxylase degradation was detected. As expected, a specific sharp decrease in the size of the nucleic acids peak was observed when the plants were deprived of phosphorus. However, the five nutritive deficiencies induced in *Lemna* by the lack of nitrogen, phosphorus, potassium, magnesium, or iron in the growth medium produced different responses in the plants. Thus, whereas iron starvation seemed to be a heavy stress for the plants, affecting the size and color of the fronds, the lack of nitrogen, phosphorus, or potassium in the growth medium for periods up to 15 days did not produce any visible harm in the fronds apart from a lower
rate of division than that observed in the absence of stress.

Ferreira and Davies (1987b) have shown that when Lemna fronds are subjected to total nutrient starvation both in the light and in the dark, the degradation of RuBP carboxylase is induced. However, this process leads irreversibly to the death of the fronds. To test whether the presence of some specific nutrients would prevent the degradation of RuBP carboxylase, Lemna fronds were incubated under selected conditions for various periods of time. After each period of time, the fronds were harvested and their RuBP carboxylase relative content was determined by anion exchange chromatography. The results obtained show that there is extensive RuBP carboxylase degradation when Lemna fronds are incubated in water containing either calcium, calcium + sulfur, sulfur, sucrose, or sulfur + sucrose. However, the proteolytic processes accompany irreversibly the death of the plants. In fact, the conditions in which the fronds died more quickly were precisely the ones that showed the fastest degradation of RuBP carboxylase.

**DISCUSSION**

Most cellular proteins are in a continuous state of turnover. Moreover, they usually undergo enhanced degradation when the organism is subjected to a stress situation (Goldberg and St. John, 1976). However, when L. minor fronds are incubated under normal growth conditions, RuBP carboxylase appears to undergo little or no degradation (Ferreira and Davies, 1987a). This situation is similar to that reported for the enzyme from wheat and barley (Peterson et al., 1973; Huffaker and Miller, 1978). The stability of RuBP carboxylase prior to senescence, and its rapid degradation during the senescence of wheat and barley leaves led to its classification as a leaf storage protein (Kleinkopf et al., 1970; Huffaker and Miller, 1978). According to this hypothesis, RuBP carboxylase would be degraded when the plant needs nutrients because of changing environmental conditions, leaf senescence, or newly developing sink demands (e.g. seed formation).

Plant RuBP carboxylase contains no detectable carbohydrate residues, phosphorylated side chains, prosthetic groups, or any cations other than magnesium ions (Ellis, 1979). Thus, the only eligible nutrients able to account for RuBP carboxylase degradation is a storage protein. Previous studies (Ferreira and Davies, 1987a, 1987b) have shown that RuBP carboxylase is not degraded when L. minor is incubated in complete growth medium in the dark or subjected to nitrogen starvation, suggesting that the enzyme does not function as a storage of nitrogen or carbon skeletons. Furthermore, the incubation of Lemna fronds in the presence of sucrose did not prevent the degradation of the enzyme observed when the plants are incubated in water (Ferreira and Davies, 1987b). On the other hand, no degradation of RuBP carboxylase was detected when Lemna fronds were deprived of magnesium for up to 14 days.

The number of SH groups in RuBP carboxylase isolated from L. minor has been estimated as 89 per enzyme molecule (Ferreira and Davies, 1989), compared with the 95 or so residues reported for several other species (Kung, 1976). Assuming an RuBP carboxylase concentration of 0.5 mM in the chloroplast stroma (Ellis, 1979), it is easy to calculate that this enzyme alone is responsible for a 50 mM concentration of sulfur inside the chloroplasts. Sulfur deprivation was the only single nutrient deficiency able to induce RuBP carboxylase degradation in a process that did not lead to the death of the fronds. This observation suggests that, in L. minor, RuBP carboxylase may function as a storage of sulfur. The double-labeling experiments illustrated in Fig. 2 show that there is a large increase in general protein degradation when Lemna is incubated in the absence of sulfur. However, the fractions containing RuBP carboxylase had the highest 14C/ 3H ratios, indicating that this enzyme is preferentially degraded under such conditions. A comparison between the rates of degradation of the total soluble protein and of RuBP carboxylase under conditions of sulfur starvation (Fig. 3) further supports the possibility of the enzyme functioning as a storage of sulfur in the leaf. In this respect, it is interesting to note that those conditions that provoke degradation of Lemna RuBP carboxylase in a process leading to the death of the fronds do not cause preferential degradation of the enzyme (Ferreira and Davies, 1987b).

When Lemna fronds are incubated in the absence of calcium, a classic membrane stabilizer, RuBP carboxylase is not degraded (Fig. 4). Instead, the enzyme undergoes a sequence of structural changes involving the oxidation of the enzyme to an acidified and catalytically inactive form (Fig. 4), followed by the polymerization of its subunits into high molecular mass aggregates (Fig. 4). This process is identical with that observed in Lemna during osmotic shock (Ferreira and Davies, 1989; Ferreira and Shaw, 1989). Experiments recently performed in our lab suggest that a similar process occurs when Lemna is incubated under conditions affecting membrane integrity. The physiological significance of the polymerization process is unknown.

We have therefore grouped all stress situations investigated into four categories according to their effect on Lemna RuBP carboxylase: (i) stress situations that do not cause RuBP carboxylase degradation (normal growth conditions, in the light or in the dark, and nitrogen, phosphorus, potassium, magnesium, or iron starvation); (ii) stress situations that cause oxidation and polymerization of RuBP carboxylase (osmotic shock, calcium or calcium + sulfur starvation, and apparently, all conditions that affect membrane integrity); (iii) stress situations that cause RuBP carboxylase degradation in a process leading to plant death (total nutrient starvation, in the light or in the dark); (iv) stress situations that cause RuBP carboxylase degradation in a process that does not lead to plant death, i.e. sulfur deprivation. Apparently, RuBP carboxylase shows an extreme stability when Lemna fronds are deprived of some macronutrients (e.g. nitrogen, phosphorus, potassium, magnesium), probably reflecting that Lemna had to evolve in a way to cope with frequent shortages of such elements.

Little is known in relation to the degradation of RuBP carboxylase, probably because such studies have been undertaken using senescing tissues in which the degradation of the enzyme occurs as part of a process leading to cell death. Indeed, a study on the proteolysis of RuBP carboxylase during leaf senescence is difficult to undertake, since chloroplasts do not senesce synchronously and the isolation of senescing chloroplasts is probably very difficult due to their lability (Miyadai et al., 1990). Besides, the cellular mechanism(s) responsible for RuBP carboxylase degradation during leaf senescence is probably different from the one(s) induced by some environmental conditions that cause RuBP carboxylase degradation without causing cell death. Here, we present evidence for a stress situation that induces extensive RuBP carboxylase degradation without causing cell death. Work is in progress to study the cellular mechanism(s) responsible for the preferential degradation of RuBP carboxylase observed when L. minor is deprived of sulfur, as well as to characterize...
the process of the enzyme degradation in plants of economic interest.

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Supplemental Material:

SULFUR STARVATION IN LEMNA LEADS TO DEGRADATION OF RuBP CARBOXYLASE WITHOUT PLANT DEATH

Ryuichi M. S. Fonseca and Anton R. N. Tanaka

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions - Lemma minor L., grown autotrophically under sterile conditions at 25°C under continuous light, was used as the source of plant material in all the experiments. Table 1 lists the composition of the growth media used.

Table 1: Composition of the growth media

| Complex | /  | / |  |  |  |  |
|---------|---|---|---|---|---|---|
| T | / | / | / | / | / | / |
| K | / | / | / | / | / | / |
| Mg | / | / | / | / | / | / |
| Ca | / | / | / | / | / | / |
| Cu | / | / | / | / | / | / |
| Fe | / | / | / | / | / | / |
| Mn | / | / | / | / | / | / |
| Zn | / | / | / | / | / | / |
| Mo | / | / | / | / | / | / |
| B | / | / | / | / | / | / |

Sulfur starvation was performed in the presence of sodium dodecyl sulfate (SDS) in 12.5% (w/v) acrylamide gel casts as previously described (Pierce and Darvin, 1987). The proteins were separated on a combibeads brilliant blue R. The radioactive protein bands were visualized using the chemiluminescent detection method (Long, 1980). Sulfuric acid was added to SDS-PAGE and scanned with a gel documentation system (Bio-Rad, Herlev, Denmark).
The Degradation of RuBP Carboxylase

Figure 1 - The effect of buffer treatment on Lema RuBP carboxylase. Lema minor fronds were incubated in complete growth medium lacking sulfur for 0 days (A), 4 days (B), 7 days (C), 13 days (D), and 17 days (E). After each period of treatment, the plants were harvested, and soluble protein extracts and RuBP carboxylase were analyzed by anion exchange chromatography as described under "Experimental Procedures." Arrows: 1. RuBP carboxylase; 2. nucleic acids.

Figure 2 - The effect of buffer treatment on the relative, in vivo degradation rate of Lema RuBP carboxylase. Double-labelled soluble protein from Lema were isolated and loaded into the TFEU Mono Q columns equilibrated with 10 mM Tris-HCl buffer (pH 7.5) as described under "Experimental Procedures." The bound protein was eluted with a gradient of NaCl; eluted fractions were collected and counted for "C and "H. A high "C/H ratio indicates a high rate of degradation. A, B, C, D, and E: degradation of soluble protein after 0, 2, 4, 6, and 14 days in complete medium lacking sulfur, respectively. F, G, and H: control of the fractions, corresponding to degradation after 14 days (I) and sample (A) of the NaCl gradient used. 1. "C/H ratio; 2. "C/H. Arrows: 1. RuBP carboxylase; 2. nucleic acids.

Figure 3 - The effect of buffer treatment on Lema RuBP carboxylase. Lema minor fronds were incubated in complete growth medium lacking sulfur for 0 days (A), 4 days (B), 7 days (C), 13 days (D), and 17 days (E). After each period of treatment, the plants were harvested, and soluble protein extracts and RuBP carboxylase were analyzed by anion exchange chromatography as described under "Experimental Procedures." A high "C/H ratio indicates a high rate of degradation.