Hydrolysis of Ribulose-1,5-bisphosphate Carboxylase by Endoproteinases from Senescing Barley Leaves

Received for publication March 16, 1981 and in revised form August 10, 1981

Bruce L. Miller and Ray C. Huffaker
Plant Growth Laboratory and the Department of Agronomy & Range Science, University of California at Davis, Davis, California 95616

ABSTRACT

The hydrolysis of ¹⁴C-labeled ribulose-1,5-bisphosphate carboxylase (RuBPCase) by two partially purified endoproteinases from senescing barley (Hordeum vulgare v. Numae) leaves is described. The major thiol proteinase, EP₁, exhibits biphasic kinetics which appear to be caused by a region of the large subunit of RuBPCase that is highly sensitive to attack by EP₁. This proteinase further hydrolyzes both the large and small subunit to smaller peptides. A second proteinase, EP₂, appears to convert the small subunit of RuBPCase rapidly to a 13.7-kilodalton fragment during initial stages of hydrolysis and then to degrade both this fragment and the large subunit. The presence of a third endoproteinase, EP₃, was discovered when [¹⁴C]RuBPCase, which appeared to be homogeneous by sodium dodecyl sulfate polyacrylamide electrophoresis, seemed to undergo very low but significant rates of "autolysis." The large molecular weight fragments produced by EP₂ were different from those of EP₁ and EP₃.

RuBPCase is the chloroplastic protein responsible for CO₂ fixation in photosynthetic organisms. RuBPCase is synthesized predominantly during leaf expansion (10) or during the greening of etiolated leaf tissue (17), after which the cellular concentration of RuBPCase (which can constitute 50–70% of the total soluble leaf protein) remains nearly constant for several days with little or no apparent turnover (23). Total protein is rapidly degraded during senescence, and RuBPCase is the predominant protein lost during the initial stages (10, 24). Thus, in many higher plants, RuBPCase also appears to serve as a leaf storage protein that can be hydrolyzed during leaf senescence (16), thereby providing reduced nitrogen that can be transported to developing leaves or fruits (6). Much information is available concerning the synthesis of RuBPCase (2, 3, 14), but knowledge about the control of RuBPCase turnover in the mature or senescent leaf is lacking. Although exo- and endoproteinases in green and senescing leaf tissue have been described (6, 9, 16, 20, 26, 28), little is known about their function in cellular protein turnover or in senescence. No RuBPCase-specific proteinases have been reported.

Endoproteinases have been purified or partially purified from leaf tissue (7, 11, 25), and some of these enzymes are reported to hydrolyze RuBPCase in addition to other protein substrates (16, 22, 28). However, the manner in which RuBPCase is hydrolyzed and the major degradative products formed have not been described. This report describes the hydrolysis of RuBPCase by three endoproteinases from intact senescing barley leaves.

EXPERIMENTAL PROCEDURES

Purification of EPs. Two major EPs were purified from senescing 12-day-old primary barley leaves by a combination of ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. The leaves were homogenized in a Waring Blender with 0.1 g insoluble PVP and 3 ml 0.1 M K-phosphate (pH 6.0) containing 2 mM DTT and 1 mM EDTA for each g of tissue. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 20 min at 27,000g. Solid ammonium sulfate was added to the resulting supernatant, and the 35 to 70% pellet was recovered. This pellet was resuspended in 50 mM K-phosphate (pH 6.0), containing 1 mM DTT and dialyzed for 20 h against the same buffer. The proteolytic activity was resolved into two separate activity peaks by gel filtration on a 3-× 50-cm column of Sephadex G-100, which had been equilibrated with 50 mM K-phosphate (pH 6.0) containing 1 mM DTT. The activities eluted at the trailing edge of the major nonactive protein peak (21). Any cross-contamination of the two activities was removed by chromatography on a 2-× 20-cm column of DEAE-cellulose equilibrated with 50 mM K-phosphate (pH 6.0) containing 1 mM DTT. One of the two enzymes, EP₁, did not bind to the column. whereas the other enzyme, EP₂, was eluted from the column with a 0.0- to 0.2-M NaCl gradient. This DEAE-cellulose preparation of EP₂ (28,300 ± 2,000) was then dialyzed against 50 mM K-phosphate (pH 6.0) containing 1 mM DTT, and a major nonactive protein (65,000 ± 3,000) was removed by gel filtration on a Sephadex G-75 superfine equilibrated with the same buffer (21).

Purification of [¹⁴C]-Labeled RuBPCase. Barley seeds (Hordeum vulgare v. Numae) were planted in 15.24-cm pots containing vermiculite. Nutrient solution was supplied continuously by cotton wicks that linked the pots to reservoir jars below. Seedlings were grown in continuous darkness for 6 days and then placed in a self-contained chamber under light (~550 μE/m² s) and allowed to green in the presence of CO₂. After 48 h, the green leaves were harvested and homogenized in a Waring Blender using 5 ml buffer and 0.1 g of insoluble PVP per g of tissue. Homogenization buffer was 0.2 M Tris-SO₄ (pH 8) containing 10 mM MgCl₂, 10 mM NaHCO₃, 2 mM DTT, and 1 mM EDTA. The crude homogenate was filtered through eight layers of cheesecloth and centrifuged at 27,000g for 20 min. Solid ammonium sulfate was added to the resulting supernatant, and the 35 to 65% pellet was recovered. This pellet was resuspended in 50 mM Tris-SO₄ (pH 8.0), 2 mM DTT, and 1 mM EDTA, and 15 ml was chromatographed on a 3-× 50-cm Sephadex G-100 column equilibrated with the same buffer. Only the first one-half of the RuBPCase peak was kept to prevent possible contamination by the major leaf proteinases (B. L. Miller, unpublished). The pooled fractions (~45 ml) were applied to a 2-× 20-cm DEAE-cellulose column equilibrated with

1 Supported in part by National Science Foundation Grant PFR 77-07301.

2 Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; EP, endoproteinase; PMSF, phenylmethyl sulfonfluoride.
are cpm/min-mg EP1, DEAE-cellulose (O-O), Hydrolysis rate was after 30 min, one set was added 2 μg EP1 (DEAE-cellulose preparation) was added to one set of reactions. (●●), Hydrolysis by 1 μg EP1; (○○○), hydrolysis after an additional 2 μg EP1.

Fig. 1. Hydrolysis of [14C]RuBPCase by EP1. The final reaction mixture was 4 mg/ml [14C]RuBPCase and 1 μg EP1, DEAE-cellulose preparation. Hydrolysis was run, and TCA-soluble counts were determined. After 30 min, an additional 2 μg EP1 (DEAE-cellulose preparation) was added to one set of reactions. (●●), Hydrolysis by 1 μg EP1; (○○○), hydrolysis after an additional 2 μg EP1.

50 mm Tris-SO₄ (pH 8.0), 2 mm DTT, and 1 mm EDTA. After the column was washed with the same buffer, the RuBPCase was eluted with 0.0- to 0.2-m ammonium sulfate gradient. The RuBPCase had a specific radioactivity of 4.0911 × 10⁶ cpm/mg protein and appeared to be homogeneous by SDS-polyacrylamide gel electrophoresis at three different acrylamide concentrations.

Hydrolysis of Azocasein. The reaction mixture contained 4 mg/ml azocasein, 40 mm K-phosphate (pH 5.7), 1 mm DTT, and 1 μg of EP1 (DEAE-cellulose preparation). After 1 to 3 h at 40°C, the reaction was stopped by adding 1.0 ml 14% HClO₄, and the protein was pelleted by centrifugation. Hydrolysis was measured as the increase in A₅₄₀ of the supernatant at 340 nm (5).

Hydrolysis of RubPCase. The final reaction mixture contained 90 mm K-phosphate (pH 5.7), 1 mm DTT, and 2 to 4 mg/ml [14C]RuBPCase. One μg of EP1 (DEAE-cellulose preparation) or 5 to 15 μg of EP2 (DEAE-cellulose preparation) were added to 250 μl of reaction mixture. Assay temperature was 40°C. An aliquot was removed at designated times and added to an equal volume of 10% TCA. The aliquot was chilled on ice for 0.5 h; the precipitated protein was pelleted, and the supernatant was used to determine TCA-soluble counts. A second aliquot of reaction mixture was added to an equal volume of SDS denaturation buffer containing 4% SDS, 20 μm leupeptin, and 2 mm PMSF. The presence of leupeptin and PMSF was required to inhibit EP1 and EP2, respectively. These enzymes had residual activity in SDS, even after the samples had been boiled for 2 min.

SDS Electrophoresis and Fluorography. SDS electrophoresis of
the \[^{14}C\]RuBPCase hydrolysis products was performed in 10% polyacrylamide slabs (1 mm) using the buffer system of Laemmli (18). Current was 15 mamp/slab. Each sample well included a small amount of SDS standard proteins (BioRad) as internal mol wt markers. The gels were stained according to Fairbanks et al. (8). After destaining, the gels were treated with ENHANCE (New England Nuclear) for 30 min, washed with distilled H\(_2\)O for several h, and placed upon Whatman 3 MM paper and dried. The dried gels were exposed to Kodak NS-2T No-Screen x-ray film for 7 to 14 days at -80°C. The film was developed and scanned with a Cary 219 recording spectrophotometer.

RESULTS AND DISCUSSION

General Properties of EPs of Barley Primary Leaves. Sephadex G-100 elution profiles obtained during enzyme purification indicated that the major EP (EP\(_1\)) contributed approximately 85% of the total proteolytic activity in crude extracts if azocasein or \[^{14}C\]RuBPCase were the substrates (21). This enzyme appeared to be a sulfhydryl proteinase which required reduced sulfhydryl reagents for maximum activity and was inhibited 100% by 10 \(\mu\)M leupeptin in the azocasein or \[^{14}C\]RuBPCase assays. EP\(_1\) had been purified 5,800-fold (21), but, because of low yields, the enzyme from the DEAE-cellulose stage of purification was used for the information reported here. This preparation contained an inactive protein contaminant, but it appeared to be homogeneous with regard to proteolytic activity. Hydrolysis time courses performed using purified enzyme gave the same results as did those shown for the DEAE-purification in Figures 1 and 2.

A second proteinase, EP\(_2\), contributed the remaining 15% of the total activity in a test-tube assay. It was inhibited 50% by 1 mm PMSF in the azocasein or \[^{14}C\]RuBPCase assays. The DEAE-cellulose stage of purification of EP\(_2\) also yielded a preparation that appeared homogeneous with respect to proteolytic activity but contained inactive contaminating proteins. EP\(_2\) was purified about 50-fold. A detailed description of the purification and characterization of EP\(_1\) and EP\(_2\) is the subject of a separate publication (21).

Hydrolysis of \[^{14}C\]RuBPCase by EP\(_1\). EP\(_1\), hydrolyzed \[^{14}C\]RuBPCase at an initially rapid rate, followed by a constant rate beginning at about 30 min (Fig. 1). Preincubation of the substrate for various times before the addition of EP\(_1\) gave the same kinetics. However, if the reaction was allowed to proceed for 30 min and then more EP\(_1\) was added, the rate from the time of addition was linear (Fig. 1). These results indicate the presence of a region of RuBPCase that may be particularly susceptible to hydrolysis by EP\(_1\). This leads to unusual kinetics for the hydrolysis of RuBPCase (Fig. 2, C and D). In contrast, the rate of hydrolysis by EP\(_2\) was constant, and Michaelis-Menten kinetics were normal when azocasein was used as the substrate (Fig. 2, A and B). Therefore, the role of an endoproteinase in the \(in\) \textit{vivo} hydrolysis of specific proteins (such as RuBPCase) may not be clarified by a comparison of the \(in\) \textit{vitro} rates of hydrolysis for different protein substrates. A protein cannot be considered to be a uniform substrate, and the interpretation of \(in\) \textit{vitro} assays may be complicated by the existence of hypersensitive regions in a protein substrate and changing kinetics as that protein is fragmented into smaller polypeptides.

The degradative products formed by the hydrolysis of \[^{14}C\]RuBPCase by EP\(_1\) were separated by SDS polyacrylamide gel electrophoresis. Results showed that the large subunit (57.5 kD) was converted very rapidly to a major fragment of 54.5 kD (Fig. 3, A and B). Other major fragments that appeared during hydrolysis had mol wt of 49.0, 37.7, 34.5, 32.6, 27.4, 19.1, 17.7, and 13.7 kD (Fig. 3A) (the small subunit had a mol wt of 14.7 kD). Figure 4, A and D, show the large-to-small subunit ratio, which reflects the initially rapid rate of hydrolysis. Apparently, the large and small subunits were degraded at nearly equal rates after about 70% of the large subunit had been converted to the 54.5-kD fragment. The pH optimum for RuBPCase hydrolysis was 5.5 to 5.7, and the specific activity of purified EP\(_1\) was 12,800 nmol \(\alpha\)-amino-N solubilized/h · mg, as determined by measurement of 5% TCA soluble products with ninhydrin (21).

Hydrolysis of \[^{14}C\]RuBPCase by EP\(_2\). There was a very rapid rise in the ratio of the large subunit to small subunit when EP\(_2\) hydrolyzed \[^{14}C\]RuBPCase (Fig. 4E). Initially, there was a rapid loss of the small subunit (14.7 kD) and a corresponding rise in a protein fragment of 13.7 kD (Figs. 5A and 6). The amount of large subunit changed little or not at all during the initial 15 min (Fig. 4B). Apparently, EP\(_2\) first cleaved a sensitive region of the small subunit but then hydrolyzed the large subunit at a slightly faster rate after about 70% of the small subunit had been processed to the 13.7-kD fragment. Other fragments found later had mol wt of 40.2, 34.6, 18.5, and 16.8 kD. The pH optimum for RuBPCase hydrolysis was also 5.5 to 5.7, and the specific activity of partially purified EP\(_2\) was about 195 nmol \(\alpha\)-amino-N solubilized/h · mg, as determined by measurement of 5% TCA soluble products with ninhydrin (21).

Hydrolysis of \[^{14}C\]RuBPCase by EP\(_3\). The third endoproteinase (EP\(_3\)) was first detected when purified \[^{14}C\]RuBPCase was incubated at 5.7 and 40°C in the absence of any added proteinase. Although the RuBPCase appeared to be homogeneous on the basis of native and SDS polyacrylamide gel electrophoresis, there
that of was not detected during subunit preparation. (However, EP3 may observed as a contaminating protein in the purified products had mol wt 50%o, when EP1 was when EP1 was a contaminating protease in the chloroplasts (14) and etioplasts (12) has been reported, it is unknown whether EP3 is located in the chloroplast and copurifies with RuBPCase, because it has a high affinity for RuBPCase.

EP1 and EP2 made up the bulk of the proteolytic activity in in vitro assays of crude leaf extracts, but the role they may play in general protein turnover or in the rapid loss of protein during senescence remains uncertain. They may hydrolyze RuBPCase independently or act together in a manner similar to the hydrolysis of pumpkin-seed globulin. Two proteolytic activities have been therefore, is indistinguishable from these.) When compared with EP1 and EP2, EP3 had a broader pH optimum, which averaged pH 5.2. There were also differences in the major degradation products formed, as shown in Figure 5B versus Figures 3 and 5A. EP3 was not inhibited by 10 μM leupeptin, 1 mm PMSF, 10 μM pepstatin, or 1 or 10 mm EDTA. It is unknown, at this point, to which class of proteinases EP3 belongs. Casein, hemoglobin, and myoglobin competitively inhibited the hydrolysis of [14C]RuBPCase when they were included in the reaction mixture. However, BSA did not inhibit the hydrolysis. EP2 could not be removed from the purified RuBPCase by means of affinity chromatography on hemoglobin-Sepharose 4B affinity columns. The inability to remove EP3 from the [14C]RuBPCase did not appear to complicate the analysis of the products formed by EP1 or EP2, because hydrolysis was much slower by EP2 than it was by EP1 or EP2 (Figs. 3-5). Although the presence of specific plastid proteinases in chloroplasts (14) and etioplasts (12) has been reported, it is unknown whether EP3 is located in the chloroplast and copurifies with RuBPCase, because it has a high affinity for RuBPCase.

was a minor number of TCA-soluble counts released (~4% of the rate when EP2 was added) at a linear rate during 8 h of incubation at 40°C. This slow increase in TCA-soluble counts was observed even though RuBPCase was carefully purified from isolated chloroplasts (27). When the degradation products were separated by SDS electrophoresis, results showed that a significant amount of hydrolysis had occurred (Fig. 4C). The large subunit had declined by 50%, and the small subunit by 30%, after 2 h. However, these products had mol wt of 49.9, 39.7, 36.5, 35.4, 33.0, 18.5, 16.8, and 13.8 kD and were, therefore, not soluble in 5% TCA (Fig. 5B). EP3 degraded both subunits of RuBPCase but hydrolyzed the large subunit more readily (Fig. 4F).

EP2 was apparently present in only minor amounts, because it was not detected during purification of EP1 and EP2 and was not observed as a contaminating protein in the purified [14C]RuBPCase preparation. (However, EP2 may have a mol wt similar to that of either the large or the small subunit of RuBPCase and...
detected in pumpkin seeds that apparently work in a concerted manner to hydrolyze the pumpkin-seed globulin (13). Activity I gave only limited products, hydrolyzing the α and β subunits of globulin to a product the authors have called Fαβ. This product was 13 to 20 kD smaller than were the original subunits. Activity II then hydrolyzed the Fαβ fragment to small peptides and amino acids.

It is also possible that a particular protein, such as RuBPCase, is not available for proteolysis by EP2; or by EP2 in vivo because of compartmentation. The cellular locations of EP1 and EP2 are unknown. Some authors have suggested that leaf proteinases may be in the cytoplasm (23), while other reports (19) indicate that they are located in the vacuole. In addition, not all the proteinases present in the cell may have the same role. Some proteinases of *Escherichia coli* appear to be involved in breakdown of protein during starvation, some in protein turnover, and others in the degradation of aberrant proteins (1, 4). Furthermore, an endopeptidase may not be involved in the *in vivo* degradation of RuBPCase. There are some proteins that appear to be degraded entirely by exopeptidases (15).

The very significant hydrolysis of RuBPCase by EP2 further suggests that this enzyme or some other minor proteinase, not detected by standard proteolytic assays, may be important in the turnover and rapid hydrolysis of RuBPCase during senescence.

**LITERATURE CITED**