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Communication

Protection of Tryptic-Sensitive Sites in the Large Subunit of Ribulosebisphosphate Carboxylase/Oxygenase by Catalysis[1]

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ABSTRACT

Limited tryptic proteolysis of spinach (*Spinacia oleracea*) ribulose bisphosphate carboxylase/oxygenase (ribulose-P_2 carboxylase) resulted in the ordered release of two adjacent N-terminal peptides from the large subunit, and an irreversible, partial inactivation of catalysis. The two peptides were identified as the N-terminal tryptic peptide (acetylated Pro-3 to Lys-8) and the penultimate tryptic peptide (Ala-9 to Lys-14). Kinetic comparison of hydrolysis at Lys-8 and Lys-14, enzyme inactivation, and changes in the molecular weight of the large subunit, indicated that proteolysis at Lys-14 correlated with inactivation, while proteolysis at Lys-8 occurred much more rapidly. Thus, enzyme inactivation is primarily the result of proteolysis at Lys-14. Proteolysis of ribulose-P_2 carboxylase under catalytic conditions (in the presence of CO_2, Mg^{2+}, and ribulose-P_2) also resulted in ordered release of these tryptic peptides; however, the rate of proteolysis at lysyl residues 8 and 14 was reduced to approximately one-third of the rate of proteolysis of these lysyl residues under noncatalytic conditions (in the presence of CO_2 and Mg^{2+} only). The protection of these lysyl residues from proteolysis under catalytic conditions could reflect conformational changes in the N-terminal domain of the large subunit which occur during the catalytic cycle.

Ribulose-P_2 carboxylase[2] (EC 4.1.1.39) occupies a key position in the biochemical pathway of photosynthetic CO_2 reduction. The bifunctionality of ribulose-P_2 carboxylase/oxygenase establishes the partitioning of carbon between the reductive and oxidative photosynthetic carbon cycles in plants (17, 20, 24). Identification of specific regions and sites in the primary structure of ribulose-P_2 carboxylase that are essential for enzyme function is necessary if potentially beneficial changes are to be engineered at the molecular level (2). The identity and location of amino acids participating in catalysis (7), subunit interactions (14, 15), activation (16), and influencing the CO_2/O_2 specificity (4) have been documented. Recent x-ray crystallographic studies have elucidated structural changes in the active site of ribulose-P_2 carboxylase in response to activation and reaction-intermediate analog binding (1, 18). Together, these studies provide a rational basis for attempts to improve ribulose-P_2 carboxylase activity through molecular engineering. However, characterization and location of catalytic-dependent conformational changes in the structure of ribulose-P_2 carboxylase, which may also influence catalysis, have received less attention.

Limited tryptic proteolysis has been used to probe the structure and function of ribulose-P_2 carboxylase (21). This study demonstrated that proteolysis of activated ribulose-P_2 carboxylase resulted in enzyme inactivation and the release of two N-terminal tryptic peptides and one C-terminal tryptic peptide from the LS. However, proteolysis of ribulose-P_2 carboxylase complexed with the reaction intermediate analog, carboxyarabinitol-P_2, resulted in the release of only the two N-terminal peptides. Thus, binding of carboxyarabinitol-P_2 protected the peptide bond at Lys-466 in the LS from tryptic proteolysis.

In this report, we demonstrate that partial inactivation of catalysis by ribulose-P_2 carboxylase during limited tryptic proteolysis is correlated with the loss of the penultimate N-terminal peptide, and that catalytic conditions retard the rate of proteolysis at two N-terminal tryptic sensitive sites.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea*) ribulose-P_2 carboxylase was purified according to McCurry *et al.* (19) except that 10 μM leupeptin was added to the extraction buffer and a food processor was used instead of a Waring Blendor. Ribulose-P_2 was synthesized as previously described (8) and purified by chromatography on a Dowex-1-Cl column with a linear gradient of 0 to 0.5 M LiCl in 5 mM HCl. Bicine, trypsin (type VIII, TPCK treated), trypsin inhibitor (type II-O), and carbonic anhydrase (bovine erythrocyte) were obtained from

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[2] Abbreviations: ribulose-P_2 carboxylase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; ribulose-P_2, D-ribulose-1,5-bisphosphate; carboxyarabinitol-P_2, 2-carboxyarabinitol 1,5-bisphosphate; LS, large subunit; SS, small subunit; 3-PGA, 3-phosphoglycerate.

Sigma. Trifluoroacetic acid (sequanal grade) was obtained from Pierce Chemical Co., and HPLC grade acetonitrile was obtained from Fisher. All aqueous solutions were prepared with Milli-Q deionized water (18 $M\Omega \cdot cm^{-1}$). Sodium [^{14}C] bicarbonate (54 Ci/mol) was obtained from Amersham Corp.

Proteolysis and Inactivation of Catalysis

Ribulose-P_2 carboxylase was activated at 2 to 4 mg/mL in activation buffer (100 mM Bicine-NaOH [pH 8.2], 20 mM $MgCl_2$, 10 mM $NaHCO_3$) at 30°C for 30 min (22). Experiments which measured CO_2 fixation included $H^{14}CO_3^-$ at 0.2 to 0.4 Ci/mol. Proteolysis at 30°C was initiated by the addition of activated enzyme (final concentration 320 μg/mL) to 2 mL of activation buffer with 60 μg/mL trypsin in the presence or absence of 1.5 mM ribulose-P_2. The rate of inactivation of ribulose-P_2 carboxylase activity under noncatalytic conditions (ribulose-P_2 absent) was determined by termination of proteolysis with a 100-fold excess of trypsin inhibitor (weight/weight) after various time periods and assay of carboxylase activity as previously described (22). The rate of proteolytic inactivation under catalytic conditions (ribulose-P_2 present) was determined by following a time course of ^{14}C incorporation from $H^{14}CO_3^-$ into acid stable radioactivity. Sufficient ribulose-P_2 was present in these experiments to maintain saturating substrate concentration (>10-fold K_m) and maximal velocity for at least 2 min. In both experiments controls were conducted in the absence of trypsin. In addition, 50 μL aliquots were removed at 10-s intervals for gel electrophoresis.

Peptide Analysis

Proteolysis conditions for subsequent peptide analysis were as described above except that the reaction volume was 30 to 50 mL and the $NaHCO_3$ was not ^{14}C-labeled. At various intervals after initiation of proteolysis at 30°C, a 10-mL aliquot (containing a total of 3.2 mg of ribulose-P_2 carboxylase) was withdrawn from the reaction mix and rapidly mixed with a 1 mL solution of water containing 60 mg of trypsin inhibitor. The two peptides described in this study have been previously characterized (9, 21) and were isolated by a combination of gel-permeation chromatography and HPLC as described previously (21). Column effluent during HPLC was monitored at A_{214} and peaks integrated with a Hewlett Packard model 3390A Integrator. An extinction coefficient for the tryptic peptides was determined by digestion of 3, 6, and 9 mg of purified ribulose-P_2 carboxylase. The tryptic digestions were allowed to proceed until exhaustive release of N-terminal peptides, as judged by gel electrophoresis. A molar extinction coefficient (ϵ_{214}) was determined based on integrated peak area units from HPLC and nanomoles of LS digested. This method of peptide quantitation was within 10 to 20% of yields obtained from amino acid compositional analyses. Peptide recovery after gel-permeation chromatography and HPLC was approximated at 85% by chromatography of known peptide quantities.

Gel Electrophoresis

Ribulose-P_2 carboxylase (1–2 μg) was electrophoresed on discontinuous SDS polyacrylamide slab gels (0.75 mm), which were constructed with a concave gradient resolving gel of 10 to 20% acrylamide and electrophoresed according to Laemmli (13). Gels were stained with Coomassie brilliant blue R-250.

Kinetic Analyses

Activated ribulose-P_2 carboxylase (2 mg/mL) was diluted to 320 μg/mL in activation buffer, and proteolyzed with 60 μg/mL trypsin at 30°C for 100 s. The reaction was terminated by addition of trypsin inhibitor (6 mg/mL). Aliquots (15 μL) of proteolyzed or nonproteolyzed (control) enzyme were removed and used for determination of kinetic constants. Assays were initiated with enzyme in a 0.5 mL final volume of activation buffer and terminated 15 s later with 200 μL of 2 N HCl. Substrate consumption was less than 15% at all substrate concentrations. Determination of K_m(ribulose-P_2) was performed with assays which contained 102 μM CO_2 and 0.005 to 1.28 mM ribulose-P_2 for control enzyme and 0.05 to 6.4 mM ribulose-P_2 for proteolyzed enzyme. Different ranges of ribulose-P_2 concentration were required in these experiments because the K_m(ribulose-P_2) is fivefold greater after proteolysis.

Determination of $K_m(CO_2)$ was performed with assays which contained 0.822 mM ribulose-P_2 for control enzyme and 1.85 mM ribulose-P_2 for proteolyzed enzyme. Concentrations of CO_2 ranged from 5 to 300 μM. All buffers and reagents except $NaH^{14}CO_3$ (1.35 Ci/mol) were prepared from "CO_2-free" solutions and assays were performed under anaerobic conditions in serum-stoppered vials (22). In addition, assays for determining the $K_m(CO_2)$ contained 600 Wilbur-Anderson units of carbonic anhydrase. Kinetic constants were obtained from equations derived from fitting the observed data to the Michaelis-Menten equation using maximum likelihood estimation.

RESULTS

Proteolytic Inactivation of Ribulose-P_2 Carboxylase

Tryptic proteolysis of CO_2- and Mg^{2+}-activated ribulose-P_2 carboxylase resulted in the formation of two forms of the LS with increased electrophoretic mobility and no change in the mobility of the SS (Fig. 1, lanes 2, 4, 6, 8). Maintenance of the enzyme under catalytic conditions by the addition of ribulose-P_2 during tryptic proteolysis resulted in identical forms of the LS, although the rate of formation was retarded (Fig. 1, lanes 3, 5, 7, 9).

The rate of proteolytic inactivation was followed under activated (−ribulose-P_2) or catalytic (+ribulose-P_2) conditions (Fig. 2). The experimental protocol included a control for the determination of ribulose-P_2 carboxylase specific activity in which trypsin was omitted during the activity measurements under noncatalytic or catalytic conditions. Each control gave identical rates for 30 s after the initiation of catalysis; however, after 30 s enzyme under catalytic conditions, a decline in specific activity occurred (see refs. 2 and 5 for a discussion of this inhibition of enzyme activity). Therefore, we limited our analysis of the relationship between peptide release and enzyme inactivation to the first 30 s of proteolysis. Proteolytic removal of the two N-terminal peptides from the large subunit of ribulose-P_2 carboxylase resulted in approximately 66% loss

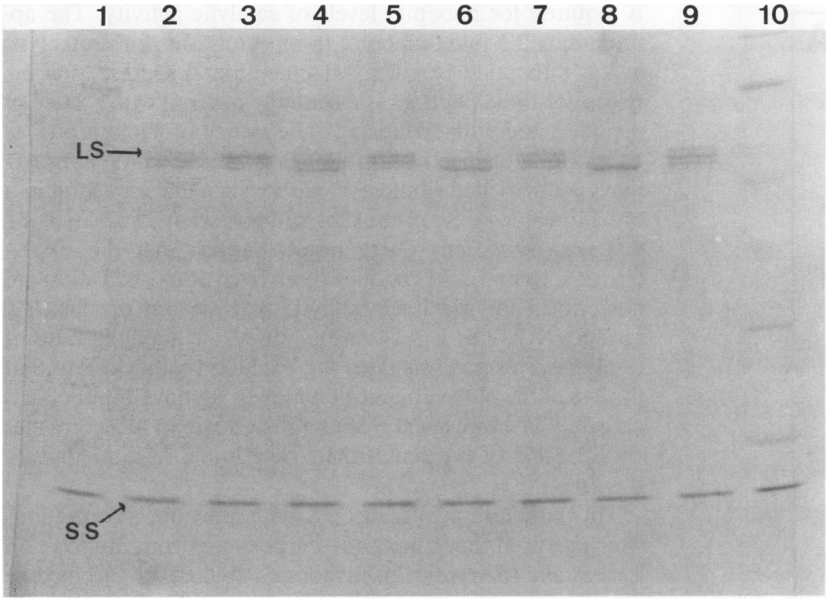

Figure 1. SDS-PAGE of ribulose-P_2 carboxylase after tryptic proteolysis. Activated ribulose-P_2 carboxylase (320 μg/mL) was proteolyzed with 60 μg/mL trypsin and terminated after 10, 20, 40, and 80 s (lanes 2, 4, 6, and 8). Activated ribulose-P_2 carboxylase (320 μg/mL) was incubated with 1.5 mM ribulose-P_2 and 60 μg/mL trypsin and terminated after 10, 20, 40, and 80 s (lanes 3, 5, 7, and 9). Lanes 1 and 10 are molecular mass standards: 92.0, 66.2, 45.0, 31.0, 21.5, and 14.4 kD. All lanes contain ~1.5 μg of ribulose-P_2 carboxylase. LS and SS identify the positions of the native large and small subunits of ribulose-P_2 carboxylase, respectively.

in catalytic activity. The residual activity (33%) did not represent residual unproteolyzed enzyme (discussed below). The activity of proteolyzed ribulose-P_2 carboxylase, and the rate of catalytic inactivation during limited tryptic proteolysis was corrected for the residual activity before log transformation and derivation of rate constants.

The pseudo first-order rate constant for inactivation of carboxylase activity under activated (−ribulose-P_2) conditions was 0.034 s^{-1} (Fig. 2A). However, under catalytic (+ribulose-P_2) conditions ribulose-P_2 carboxylase was not appreciably inactivated by trypsin in the 30 s assay (Fig. 2B). Thus, the activated form of the enzyme was more susceptible to tryptic inactivation.

Kinetics of Peptide Release from Ribulose-P_2 Carboxylase

Fractionation by HPLC of the peptides released from ribulose-P_2 carboxylase during 2 min of tryptic proteolysis demonstrated that only two peptides were released (data not shown). Previous characterization of these peptides by compositional analyses, Edman degradative sequence analyses and fast-atom bombardment mass spectroscopy identified these peptides as the acetylated N-terminal tryptic peptide (9, 21):

$$\text{Peptide 1:}\quad \overset{3}{\text{Acetyl-Pro-Gln-Thr-Glu-Thr-Lys}}\overset{8}{};$$

and the penultimate N-terminal tryptic peptide:

$$\text{Peptide 2:}\quad \overset{9}{\text{Ala-Ser-Val-Gly-Phe-Lys}}\overset{14}{}.$$

In addition to these peptides, a third tryptic peptide was found to be released from the C-terminus of the LS in a previous study (21), but the kinetics for hydrolysis at this peptide bond are much slower, so that the release of this peptide is not observed in the much shorter time frame of the present studies.

The kinetics of proteolysis at Lys-8 and Lys-14 were examined by termination of proteolysis with trypsin inhibitor at various intervals and quantitation of the peptides by HPLC as described in experimental procedures. The rate of release of peptide 2 was pseudo first-order with a rate constant of 0.036 s^{-1} (Fig. 2A). The tryptic reactivity of Lys-8 was much greater than Lys-14, as evidenced by the rapid release of peptide 1 (approximately 80% complete in 15 s after proteolysis). Therefore, the rate constant can only be estimated at 0.087 s^{-1} for the release of peptide 1.

The release of both peptides exhibited a lag during the first 10 s of proteolysis; however, data for proteolytic inactivation of ribulose-P_2 carboxylase activity derived from similar experiments, but with much smaller volumes, did not show any such lag. This lag may represent the time required for mixing the larger volumes required for these peptide determinations.

The rapid release of peptide 1 ($k \cong 0.087\ s^{-1}$) and the slower rate of catalytic inactivation of catalysis ($k = 0.034\ s^{-1}$) suggests that proteolysis at Lys-8 did not have a major effect on catalytic activity. The similar kinetics of hydrolysis at Lys-14 ($k = 0.036\ s^{-1}$) and rate of inactivation ($k = 0.034\ s^{-1}$) suggests that proteolysis at Lys-14 and loss of the penultimate N-terminal tryptic peptide was largely responsible for the partial inactivation of carboxylase activity.

Proteolysis of activated ribulose-P_2 carboxylase for 100 s with trypsin, which resulted in nearly complete loss of peptide 1 and peptide 2, altered the kinetic constants for substrate CO_2 and ribulose-P_2 (Table I). The proteolyzed form of ribulose-P_2 carboxylase exhibited a 66% reduction in K_{cat}, a 4.6-fold increase in the K_m(ribulose-P_2), and a 1.7-fold increase in the $K_m(CO_2)$. The changes in K_m for the carboxylation substrates indicate that the residual 33% catalytic activity is the result of a modified form of ribulose-P_2 carboxylase, and not from unproteolyzed protein which would exhibit native kinetic constants.

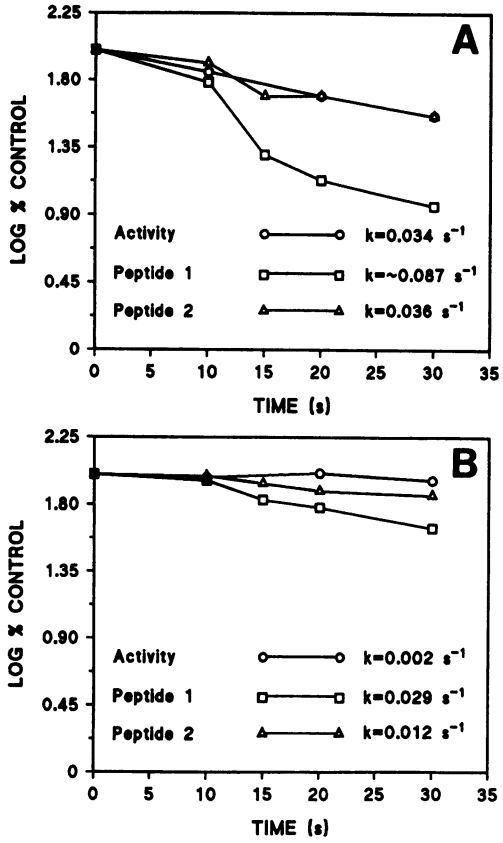

Figure 2. Peptide release and proteolytic inactivation of ribulose-P_2 carboxylase under activated or catalytic conditions. A, Specific activity of ribulose-P_2 carboxylase was followed at 10, 15, 20, and 30 s after addition of trypsin. Trypsin was omitted from the control reactions. B, Specific activity of ribulose-P_2 carboxylase was followed at 10, 15, 20, and 30 s after the addition of 1.5 mM ribulose-P_2 and trypsin. In both panels, samples were removed from duplicate experiments and analyzed for peptides 1 and 2 (see text) as described in "Materials and Methods." Rate constants were derived from linear regression analysis. Results shown are the mean from two experiments each with duplicate determinations.

Catalytic Protection of Tryptic Proteolysis Sites

Under catalytic conditions, proteolytic inactivation of ribulose-P_2 carboxylase activity was not detectable, and the release of peptides 1 and 2 was reduced to one-third of the rates observed under activating conditions (Fig. 2, A *versus* B). The protective effect of ribulose-P_2 against tryptic proteolysis was not due to the inhibition of trypsin by the substrates or products of the ribulose-P_2 carboxylase reaction; trypsin was assayed spectrophotometrically with *p*-toluene-sulfonyl-L-arginine methyl ester, and 2 mM ribulose-P_2 or 5 mM 3-PGA did not affect activity (data not shown). The rate constant for inactivation in the presence of ribulose-P_2 was negligible; however, hydrolysis at Lys-8 (0.029 s^{-1}) and Lys-14 (0.012 s^{-1}) did occur under such conditions, but were greatly reduced relative to noncatalytic conditions.

DISCUSSION

These results demonstrate that the penultimate N-terminal region on the large subunit of spinach ribulose-P_2 carboxylase is required for maximal levels of catalytic activity. The approximate 2.5-fold difference in rate constants for proteolysis at Lys-8 (peptide 1) and Lys-14 (peptide 2) suggests that the release of these peptides is essentially ordered with release of peptide 1 preceding peptide 2. The rate of proteolysis at Lys-14 and rate of inactivation (0.036 s^{-1} and 0.034 s^{-1}, respectively) of activated ribulose-P_2 carboxylase are very similar; a much greater rate constant for proteolysis at Lys-8 (~0.087 s^{-1}) was estimated. These results suggest that the six N-terminal amino acid residues (acetylated Pro-3 to Lys-8) are not critical for maximal catalytic activity, but the next six residues (Ala-9 to Lys-14) are required for maximal rates of catalysis. This analysis does not exclude the possibility that the loss of the N-terminal region may have some minor effects on catalysis; however, the dramatic decrease in K_{cat} correlates with the loss of the penultimate N-terminal region (Ala-9 to Lys-14).

This conclusion is in accord with previous observations which showed that ribulose-P_2 carboxylase from muskmelon is resistant to tryptic inactivation. Ribulose-P_2 carboxylase from muskmelon is posttranslationally modified by ^{1}N-methylation at Lys-14; the trimethyllysyl residue is not hydrolyzed by trypsin and Lys-18 is hydrolyzed very slowly (9). Studies with wheat ribulose-P_2 carboxylase (6, 11) also suggest that the N-terminal region of the large subunit from Ala-9 to Lys-14 plays a role in catalysis, although these studies did not determine kinetics or order of N-terminal peptide release during tryptic proteolysis, or the effects of catalysis on proteolytic inactivation.

The rate of tryptic proteolysis at Lys-8 and Lys-14 was reduced under catalytic conditions as judged by SDS-PAGE and quantitation of the two peptides released. It seems likely that the reduction in proteolytic sensitivity of lysyl residues 8 and 14 during catalysis is a consequence of conformational changes that may occur at or near these sites; however, structural changes in ribulose-P_2 carboxylase at sites remote from the N-terminus could also result in differences in proteolytic sensitivity through tertiary or quaternary changes in structure. Cooperativity between active sites in the large subunits of ribulose-P_2 carboxylase in response to ligand binding has been demonstrated (10), and could relate to these changes in proteolytic sensitivity. The activation status of ribulose-P_2 carboxylase, and the ability of the enzyme to bind the reaction-intermediate analog carboxyarabinitol-P_2, are not influenced by limited tryptic proteolysis (6, 21). In addition, sub-

Table I. *Kinetic Constants for Proteolyzed and Native Ribulose-P_2 Carboxylase*

Ribulose-P_2 carboxylase (320 μg/mL) was proteolyzed with 60 μg/mL trypsin for 100 s at 30°C and 15-μL aliquots removed for determination of kinetic constants after addition of 100× (weight/weight) trypsin inhibitor. Kinetic constants were derived as described under "Materials and Methods."

Enzyme	K_m (CO_2)	K_m (ribulose-P_2)	K_{cat}
		μM	s^{-1}
Native	16.6	32.7	2.1
Proteolyzed	28.0	150.5	0.7

unit stoichiometry is not perturbed as judged by native gel electrophoresis (21; RL Houtz, unpublished observation).

Although none of the amino acid residues that form the catalytic site are located in the N-terminal region (residues acetyl-Pro-3 to Lys-14) (7), structural investigations of ribulose-P_2 carboxylase have demonstrated that the active site is composed of amino acid residues that are contributed by both the C- and N-domains of pairs of adjacent LS (3, 14, 15, 23). Thus, the active site is formed at the interface of the adjacent LS pairs and interaction occurs between these domains. Amino acid residues from the N-terminal region (Phe-13, Ala-15, Gly-16, and Val-17) are located at the interface of LS dimers (12). The side chain of Phe-13 interacts extensively with a conserved 3_{10} helix (residues 70–74) in the N-domain of the same LS. Thr-71, one residue of this helix, hydrogen bonds with the carbonyl oxygen of Lys-175, an active site residue that forms multiple interactions with carboxyarabinitol-P_2 and other active site amino acid residues. Thus, proteolytic removal of the penultimate tryptic peptide (Ala-9 to Lys-14) would eliminate the interaction of Phe-13 with Thr-71, and potentially result in additional conformational changes, that may consequently perturb the structure of the active site.

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