Reaction-intermediate analogue binding by ribulose bisphosphate carboxylase/oxygenase causes specific changes in proteolytic sensitivity: The amino-terminal residue of the large subunit is acetylated proline
(protein dynamics/amino-terminal derivatization/proteolysis)

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ABSTRACT Trypsin rapidly inactivated the catalytic activities of spinach ribulose bisphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] but the stoichiometry of binding of the reaction-intermediate analogue carboxyarabinitol bisphosphate was only slightly reduced after proteolysis. Electrophoretic analysis indicated that several forms of the large subunit were generated during proteolysis but that the small subunit was resistant. Three tryptic peptides were isolated and characterized after digestion of the activated enzyme; the tryptic-sensitive sites were identified at Lys-8, Lys-14, and Lys-466 of the large subunit. Tryptic digestion of the enzyme complexed with the reaction-intermediate analogue released only two peptides by hydrolysis at Lys-8 and at Lys-14. The loss of susceptibility of Lys-466 to trypsin may be the result of a conformational change that limits the accessibility of the carboxyl-terminal region after binding of the reaction-intermediate analogue. Analysis of the amino-terminal tryptic peptide by composition and fast atom bombardment mass spectrometry demonstrated that the actual amino-terminal residue is proline at position 3 of the DNA-deduced sequence and that this proline is blocked with an N-acetyl moiety. Thus, posttranslational processing of the chloroplast-encoded large subunit of the enzyme must occur to remove Met-1 and Ser-2 and to acetylate the amino terminus.

Ribulose bisphosphate carboxylase/oxygenase [RuBP2 carboxylase, 3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] catalyzes both the carboxylation and oxygenation of RuBP2, which represent the first committed reactions of both the reductive and oxidative photosynthetic carbon cycles (1). Although considerable detail is known about the mechanism of the carboxylation reaction (2, 3), little is known about the dynamics of the protein during activation, 2'-C-carboxyarabinitol 1,5-bisphosphate (CABP) binding, or catalysis. Activation of RuBP2 carboxylase by CO2 and Mg2+ results in conformational changes as measured by changes in sedimentation velocity (4), circular dichroism (5), and reactivity with active-site probes (6, 7). Binding of CABP also results in conformational changes in the protein as documented by changes in absorbance spectroscopy (8), electrophoretic mobility (9), and x-ray crystallography patterns (10). With the exception of active-site derivatization, these studies have not provided specific molecular information, such as regions or individual amino acid residues that change in conformation or reactivity.

In this report proteolysis has been utilized to probe the structure and function of RuBP2 carboxylase. Susceptibility of native proteins to proteases may increase or decrease due to ligand-induced conformational changes (11). A specific peptide bond at Lys-466 in the carboxyl-terminal region of RuBP2 carboxylase radically changed sensitivity to trypsin after CABP binding. In addition, characterization of the peptides released from spinach RuBP2 carboxylase showed that the amino-terminal amino acid Pro-3 is acetylated and that the amino acid sequence predicted from the DNA sequence is in error at residue 12. A preliminary report of these results has been published (12).

MATERIALS AND METHODS

Enzyme Preparation, Assay, and Treatment with Trypsin. RuBP2 carboxylase was purified to homogeneity from spinach leaves, activated, and assayed as described (13, 14). RuBP2 carboxylase was activated at 2 mg/ml and exposed to trypsin (10 μg/ml) at 30°C. The initial proteolysis was terminated by addition of 150 μg of trypsin inhibitor per μg of trypsin before assaying enzyme activity. The stoichiometry of CABP bound by RuBP2 carboxylase was determined as described (13).

Electrophoresis. Discontinuous NaDodSO4/polyacrylamide gels were prepared with a concave gradient resolving gel of 10–20% acrylamide and electrophoresed as described by Laemmli (15). Gels were fixed with 12% (wt/vol) trichloroacetic acid and 5% (vol/vol) methanol and stained with Coomassie brilliant blue R-250.

Peptide Characterization. Tryptic peptides were purified by molecular-sieve chromatography and HPLC. Activated carboxylase (20 mg/ml, 1.8 ml) was treated with 100 μg of trypsin for 30 min at 30°C. In these experiments for peptide isolation the digestion was terminated by addition of 200 μg of trypsin inhibitor. The peptide fractions were separated from the undigested protein by column chromatography with Sephadex G-75, or Sephadex G-50 followed by Sephadex G-10, and eluted with 2 mM Tris-Cl (pH 8.2). Those fractions that contained peptides were pooled, lyophilized, and dissolved in a minimal volume of 0.07% trifluoroacetic acid. The peptides were separated by HPLC with an octadecyl silica reverse phase column (10-μm particle size, 4.6 x 220 mm Aquapore RP-300 column, Brownlee Lab, Santa Clara, CA). Peptides were eluted with a linear gradient of aqueous 0.07% trifluoroacetic acid to 60% (vol/vol) acetonitrile with 0.07% trifluoroacetic acid. Fractions were collected and

Abbreviations: CABP, 2'-C-carboxyarabinitol 1,5-bisphosphate; RuBP2 carboxylase, ribulose bisphosphate carboxylase/oxygenase; RuBP3, ribulose 1,5-bisphosphate; LS, large subunit of RuBP2 carboxylase; SS, small subunit of RuBP2 carboxylase.

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lyophilized or evaporated at 50°C under nitrogen, and the HPLC separation was repeated with each peptide fraction to ensure purity. Amino acid composition of the purified peptides was determined after hydrolysis with the Pico-Tag amino acid analysis system (Waters Chromatography Division). Amino acid sequence analysis was performed with a gas-phase sequencer [Applied Biosystems (Foster City, CA) 470A protein sequencer] and HPLC of the phenylthiodyantoin derivative of the amino acids. Fast atom bombardment (FAB) mass spectrometry was performed with a JEOL JMS-HX110HF, a double-focusing Matsuda geometry (16) mass spectrometer, operated at a 10-kV accelerating voltage. Ionization by FAB used the hot-filament JEOL FAB (17) gun, 6-kV Xe atoms, 5-mA filament emission current. Data were acquired and processed with a JEOL DA-5000 data system. Details of the fragmentation pattern and fragment ions produced by collisional activation dissociation, as well as identification of amino-terminal modifications in Rbup2 carboxylase from other plant species, will be published elsewhere.

Materials. Trypsin was obtained from Worthington or Sigma and had been treated with N-p-toluenesulfonyl-l-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Trypsin was dissolved at 0.5–5 mg/ml in 1 mM H2SO4. Trypsin inhibitor was type II-0 from Sigma.

Rbup2 was synthesized from ribose 5-phosphate and ATP and purified by chromatography on Dowex-1-chloride with a linear gradient of 0–0.5 M LiCl prepared in 5 mM HCl (18). CABP and [2'2-14C]CABP were prepared as described (19).

RESULTS

Proteolysis of activated Rbup2 carboxylase with trypsin resulted in rapid loss of carboxylase activity (Fig. 1). Oxy-
genase activity was equally sensitive to proteolysis, and neither activity was selectively inhibited (data not shown). In contrast to the sensitivity of the catalytic activities during initial proteolysis, the ability of the activated protein to bind CABP, a reaction-intermediate analogue, was relatively insensitive to proteolysis by trypsin (Fig. 1), as reported for wheat Rbup2 carboxylase (20). Earlier results demonstrated that the stoichiometry of CABP bound by Rbup2 carboxylase was proportional to carboxylase activity when the protein was partially inactivated by heat denaturation (21). Thus, the binding of CABP correlated with the catalytic competence of the protein after partial inactivation with heat. CABP binds to native activated Rbup2 carboxylase with a Kd of ≤10 pM (3, 19) and a half-life for dissociation of the activated-enzyme inhibitor complex of ≥16 days. Therefore, CABP is probably a transition-state analogue of the six-carbon intermediate formed during carboxylation of Rbup2. Dissociation of CABP from Rbup2 carboxylase after tryptic digestion occurred with a first-order rate constant of $3 \times 10^{-3}$ hr$^{-1}$ or a half-life of the CABP–enzyme complex of 10 days (data not shown). Thus, the proteolytically inactivated enzyme retained the ability to tightly bind CABP, although the very slow rate of release of this ligand may be somewhat greater than from the native activated protein. Kinetic analysis of native and proteolysed Rbup2 carboxylase (prepared as in Fig. 1) indicated that the proteolyzed form behaved with a $V_{max}$ of ~20% of the native form and that the apparent $K_m$ for Rbup2 increased from 30 μM to 70 μM after proteolysis. The change in $K_m$ is an indication that the proteolysed form of the protein has distinct kinetic parameters and that the catalytic activity is not solely due to residual undigested Rbup2 carboxylase.

Denaturing gel electrophoresis of activated Rbup2 carboxylase after initiating proteolysis indicated that a lower molecular weight form of the large subunit (LS) was generated by the tryptic digestion (Fig. 2, lane 3). No change was perceptible in the electrophoretic mobility of the SS. Proteolysis of unactivated Rbup2 carboxylase, activated Rbup2 carboxylase, or activated Rbup2 carboxylase with 10 mM gluconate 6-phosphate (lanes 2, 3, and 5, respectively) gave a similar product of proteolysis. However, tryptic treatment of the activated Rbup2 carboxylase–CABP complex resulted in a different product (lane 4), apparently from the decreased reactivity of a tryptic-sensitive site.

Exposure of activated Rbup2 carboxylase to trypsin for 30 min resulted in the release of three peptides (Fig. 3A). Peptide 1 could not be sequenced; no phenylthiodyantoin derivatives were obtained by Edman degradation, indicating that the amino terminus was blocked. Amino acid composition of this peptide indicated that it was an amino-terminal tryptic peptide composed of six amino acids from Pro-3 to Lys-8 (Thr$_3$Glx$_2$Pro$_1$Glx$_2$Ser$_2$Gly$_2$) (Fig. 4). The sequence and composition of the amino terminus was analyzed by FAB mass spectrometry. The parent ion of this peptide (m/z 745) and amino-terminal fragments derived from collisional-activated dissociation were shifted in mass by 42, indicating the amino-terminal residue was acetylated (Fig. 5). The acetylated dipeptide, acetyl-Pro-Gln, was represented as the ion peak at 268. Although further fragment-

![Fig. 1. The effect of trypsin on carboxylase activity and CABP binding by Rbup2 carboxylase. Activated spinach Rbup2 carboxylase (2 mg/ml) was treated with trypsin (10 μg/ml) at 30°C. Aliquots were removed at various intervals and assayed for carboxylase activity (■) and CABP binding (○). Zero-time values were determined before the addition of trypsin.](image-url)
tation of this acetylated dipeptide was not observed, the amino-terminal blockage with the presence of the acetylated dipeptide indicates that the amino-terminal residue of the spinach LS is acetylated Pro-3.

Sequence analyses of peptides 2 and 3 are summarized in Fig. 4. Peptide 2 is the penultimate amino-terminal tryptic peptide composed of amino acid residues from Ala-9 to Lys-14. The original reported DNA sequence predicted that amino acid 12 was a glutamyl residue (22); however, subsequent sequence analysis predicted a glycine at residue 12 (23), which is confirmed by these results. Peptide 3 is the carboxyl-terminal tryptic peptide composed of amino acid residues from Phe-467 to Val-475. Thus, the tryptic-sensitive sites of the spinach LS are Lys-8, Lys-14, and Lys-466.

The tryptic peptides released from activated RbuP$_2$ carboxylase and the activated RbuP$_2$ carboxylase–CABP complex are compared in Fig. 3. Only peptides 1 and 2 were released from the activated RbuP$_2$ carboxylase–CABP complex, although less rapidly than from the activated enzyme. Peptide 3 was not generated in appreciable quantities by tryptic digestion of the activated RbuP$_2$ carboxylase–CABP complex. Consequently the electrophoretic mobility of the remaining LS was less than the LS from trypsin-treated activated RbuP$_2$ carboxylase (Fig. 2). CABP binding to activated RbuP$_2$ carboxylase caused a change in the protein that radically altered the sensitivity of the peptide bond at Lys-466 to trypsin.

**DISCUSSION**

Exposure of activated RbuP$_2$ carboxylase to trypsin resulted in rapid loss of carboxylase and oxygenase activities, but the ability of the protein to tightly bind CABP was only slightly reduced. Thus, the proteolyzed protein was unable to carry out some step(s) in catalysis (e.g., enolization, carboxylation, or hydrolysis), although the structure of the catalytic site was relatively intact as judged by the ability of the protein to tightly bind the reaction-intermediate analogue.

Similar conclusions were reported in a study of proteolytic inactivation of wheat RbuP$_2$ carboxylase (20). Related observations have been made on a mutant RbuP$_2$ carboxylase from *Chlamydomonas reinhardtii* (24) that lacked catalytic activity but still bound CABP, although these investigators did not assess the tightness of binding. In addition, the isolated LS of RbuP$_2$ carboxylase from *Synechococcus* binds CABP but lacks catalytic activity (25). Thus, although CABP binding correlates with catalytic activity remaining during heat denaturation (21), this correlation does not apply under conditions that are less disruptive of polypeptide folding, such as limited proteolysis or loss of SS. Consequently the use of CABP to quantify RbuP$_2$ carboxylase catalytic sites must be used with caution due to these complications.

The three peptides released by tryptic digestion of spinach RbuP$_2$ carboxylase represent the amino-terminal, penultimate amino-terminal, and carboxyl-terminal tryptic peptides of the LS. The composition of these peptides showed that the "mature" form of the LS was composed of amino acid residues from Pro-3 to Val-475. We will show elsewhere that this composition is similar for RbuP$_2$ carboxylase from other plant species. Early reports indicate that the LS of RbuP$_2$ carboxylase was synthesized as a precursor and posttranslationally processed to a form 1–2 kDa smaller (26). Other amino-terminal determinations have indicated that Ala-15 in barley (27) or both Ala-15 and Thr-5 in tobacco (28) were the amino-terminal residues. The amino-terminal tryptic peptide

<table>
<thead>
<tr>
<th>composition of peptide 1</th>
<th>sequence of peptide 2</th>
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<tr>
<td>(thr:glx:pro:lys=2:2:1:1) ala ser val gly phe lys</td>
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<td></td>
<td>5 10 15</td>
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<tr>
<td>sequence of peptide 3</td>
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<tr>
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<tr>
<td>... ala cys glu val trp lys glu ile lys phe glu phe pro ala met asp thr val</td>
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**Fig. 4.** Comparison of tryptic peptides with the predicted amino acid sequence for spinach RbuP$_2$ carboxylase LS. The lower lines are amino acid sequence predicted from the spinach LS DNA sequence (22). Underlined sequences correspond to the tryptic peptides identified. The upper lines of italic letters are results of the compositional and sequence analyses. Residue 12 is confirmed to be glycine (23).
of the wheat LS has been reported to contain Ser-2, but apparently lacked Met-1 (20). Amino-terminal analyses have undoubtedly been confounded by amino-terminal derivatization and the action of proteases that leaves unreactive polypeptides intact while proteolytically degraded forms bear unblocked termini. We propose that, although the spinach LS is not processed to a form 1–2 kDa smaller than the "precursor," the LS is processed by removal of Met-1 and cis Ser-2 and subsequent amino-terminal acetylation. Thus, the true molecular weight of the spinach LS is 52,569, assuming no other derivatizations and accurate DNA sequencing. The functional significance and mechanism of this processing is unknown, but it could be involved in the assembly of the holoenzyme or in some way related to the function of the protein. An additional possibility is that the amino-terminal processing is related to the stability of RbuP2 carboxylase in the chloroplast since the nature of the amino terminus is related to protein turnover in some systems (29). Removal of the initiator methionine residue is common in many amino-terminally processed proteins such as actin (30). Posttranslational proteolytic processing and acetylation of the membrane associated chloroplast 32-kDa herbicide-binding protein has been reported (31). Proteolytic processing of one mitochondrial-encoded polypeptide, cytochrome oxidase subunit II, has been reported in yeast mitochondria (32), although amino-terminal formylated methionine residues have been reported for other polypeptides synthesized in the mitochondria (33). RbuP2 carboxylase is only the second protein to be reported to have a amino-terminal acetylated proline. A survey of 250 amino-terminally acetylated proteins cited only rabbit myosin A1/A2 catalytic light chain as having an acetylated proline at the amino terminus (34).

Three tryptic sensitive peptide bonds in the LS of spinach RbuP2 carboxylase have been identified at Lys-8, Lys-14, and Lys-466. Since these peptide bonds are accessible to trypsin, they are apparently present at the surface of the protein and are unhindered by subunit interactions. The tryptic sensitivity of the activated RbuP2 carboxylase–CABP complex at Lys-466 differed markedly from activated RbuP2 carboxylase. Thus, a conformational change in the protein occurs upon CABP binding that affects the accessibility or reactivity of Lys-466 with trypsin. Since CABP is a transition-state analogue of the carboxylation reaction (19), CABP may stabilize the protein in a conformation that reflects a transient state that exists during the catalytic cycle. Thus, conformational changes in the region of Lys-466 may play a role in the dynamics that occur in the LS during catalysis. The conformational change could involve changes in the LS tertiary structure or changes in the subunit interactions. A nearby cysteine (Cys-459) has been derivatized by an active-site probe (6) and could contribute structurally to the formation of the active site. A conformational change in the spinach protein after CABP binding could be readily transmitted to Lys-466 through Cys-459, although other less-direct interactions are certainly possible. Three-dimensional structural studies of the distantly related, but functionally homologous, Rhodospirillum rubrum RbuP2 carboxylase indicated that the homologous region to Cys-459 may not be near the active site of this protein (35). However, since this protein with two LSs is dissimilar in subunit composition to the higher plant form (8 LS and 8 SS) and crystallography studies were conducted with unactivated enzyme, it is difficult to evaluate the significance of this region of the LS in the structure and function of spinach RbuP2 carboxylase.

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