Isolation and Some Properties of Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase from Red Kidney Bean Primary Leaves

GARY C. HARRIS and ARTHUR I. STERN
Department of Botany, University of Massachusetts, Amherst, Massachusetts 01003

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ABSTRACT

Purification of ribulose-1,5-bisphosphate carboxylase from primary leaves of Phaseolus vulgaris var. Red Kidney with ammonium sulfate precipitation, ion exchange chromatography, and gel filtration resulted in the complete loss of detectable oxygenase activity and the retention of a low velocity and a high K_m form of both the carboxylase and oxygenase. The polyethylene glycol-6000-purified ribulose-1,5-bisphosphate oxygenase displayed a broad pH optimum (7.9-9.4) and a high K_m for O_2 and ribulose 1,5-bisphosphate (0.90 mM and 0.25 mM, respectively). Initiation of the oxygenase reaction with protein rather than ribulose 1,5-bisphosphate resulted in reduced activity. The enzymes prepared by the two purification procedures were electrophoretically different.

Etiolated primary leaf tissue exhibited low rates of both carboxylase and oxygenase. Similar developmental kinetic activity was observed for both reactions during greening. Photosynthetic 14CO_2 fixation was inhibited 95% by 100% N_2 gas during the first 24 hours of greening, but the inhibition was rapidly overcome by 48 to 72 hours of light exposure.

The enzyme RuBP carboxylase can also act as an oxygenase, catalyzing the oxidative cleavage of RuBP producing phosphoglyceric acid and phosphoglyceric acid (2, 15, 20). In higher plants the properties of the RuBP oxygenase reaction have been characterized in both crude and purified extracts of tobacco, soybean, and spinach (10). The purified soybean and spinach oxygenases have been shown to have alkaline pH optima and K_m values for RuBP and O_2. These values can be contrasted with the broad pH optimum and the low K_m values for RuBP (45 µM [5]) and O_2 (0.22 mM [1]) exhibited by the enzyme in crude spinach extracts. The differences between the crude and the purified enzyme have recently been ascribed to the use of improper assay conditions with the purified enzyme (10). However, it has not been demonstrated with the purified enzyme that changes in the assay conditions will yield a high pH optimum and low K_m values for RuBP and O_2. The purified tobacco enzyme has been shown to exhibit oxygenase properties similar to those observed in crude spinach extracts (K_m [RuBP] of 22 µM and a pH optimum of 8.4-8.6 at 4-8 mM MgCl_2) when assayed in what can be referred to as the nonactivated form (19). Marsho and Kung (19) have postulated that the mild conditions employed in the purification of the tobacco enzyme may be the basis for these properties. We have purified RuBP carboxylase from red kidney bean using two different purification techniques to determine if the isolation procedures can affect the kinetics and properties of the RuBP carboxylase-oxygenase reaction.

Etiolated leaf tissue contains low levels of RuBP carboxylase, and the rapid de novo synthesis of additional enzyme protein occurs during greening (11). There is some evidence that indicates that the RuBP carboxylase and oxygenase activities are not tightly linked in vitro (2, 4, 13, 23) so that it is possible that the two activities may also exhibit different developmental kinetics during greening. Therefore, we have examined 14CO_2 fixation in intact leaf discs as well as RuBP carboxylase and oxygenase activities in primary leaf extracts during leaf development in red kidney bean.

MATERIALS AND METHODS

Plant Material. Seeds of Phaseolus vulgaris var. Red Kidney (Agway Inc., Syracuse, N.Y.) were surface-sterilized for 4 min with 5.25% sodium hypochlorite prior to planting in vermiculite in plastic flats (34 × 40 cm). The flats were watered with distilled H_2O and maintained in the dark at 26 C for 8 to 9 days. The etiolated plants were transferred to growth chambers maintained at 27 C and illuminated with continuous white light (300 ft-c) from cool white fluorescent lamps augmented with incandescent bulbs.

Purification of RuBP Carboxylase-Oxygenase. Spinach RuBP carboxylase-oxygenase was prepared by a modification of the procedures of Paulsen and Lane (21) and Andrews et al. (2). Locally purchased spinach was deveined and washed with cold tap water. A quantity of 200 to 250 g of washed spinach leaf fragments was then homogenized in a Waring Blender at full speed for 1 min in 2 volumes of an extraction medium containing 24 mM TES (pH 7.6), 1 mM EDTA, and 1 mM GSH. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth prior to the addition, with stirring, of solid ammonium sulfate (226 g of (NH_4)_2SO_4/l of extract). The dark green precipitate was removed by centrifugation (10,000g for 10 min), and additional solid ammonium sulfate was added, with stirring, to the supernatant (92.5 g of (NH_4)_2SO_4/l of original extract). The resultant precipitate was collected by centrifugation (10,000g for 10 min), and the pellets were resuspended in a minimum volume of 5 mM TES (pH 7.6), 1 mM EDTA, and 1 mM GSH. The resuspended pellets were pooled and centrifuged at 37,000g for 10 min prior to desalting on a G-25 (Pharmacia, Uppsala, Sweden) column (2.5 × 45 cm) previously equilibrated with 5 mM TES (pH 7.6), 1 mM EDTA, and 1 mM GSH. The dark, rapidly moving band was collected and immediately applied to a DEAE-cellulose column.
(4 x 15 cm) previously equilibrated with 5 mm TES (pH 7.6), 1 mm EDTA, and 1 mm GSH. The protein was then eluted with a 1-liter gradient of 0 to 0.5 m NaCl. The fractions containing the main protein peak were pooled and the protein precipitated by the addition, with stirring, of solid ammonium sulfate (318.5 g of (NH₄)₂SO₄/l of protein solution). The precipitate was collected by centrifugation (10,000g for 10 min) and resuspended in a minimum volume of 5 mm TES (pH 7.6), 1 mm EDTA, and 1 mm GSH prior to application to a Sepharose 4B (Pharmacia) column (2.5 x 44 cm) previously equilibrated with 5 mm TES (pH 7.6), 1 mm EDTA, and 1 mm GSH. The fractions containing the single protein peak were pooled and solid ammonium sulfate was added, with stirring, in a ratio of 318.5 g of (NH₄)₂SO₄/l of protein solution. The RuBP carboxylase-oxygenase was stored at this concentration of ammonium sulfate at 4 C. All of the above steps were carried out at 4 C within a time span of 48 to 72 hr.

Bean RuBP carboxylase-oxygenase was prepared by two alternative techniques designated the ammonium sulfate procedure and the PEG procedure. The ammonium sulfate procedure was similar to the one used to purify the spinach enzyme with the following modifications. For the initial homogenization, 50 to 100 g of bean primary leaf tissue were harvested from plants that had been greened in white light for 4 to 6 days. In contrast to the spinach technique, the initial crude bean homogenate was centrifuged at 37,000g for 30 min prior to the addition of ammonium sulfate. A sequential fractionation of the supernatant was not employed (28), and the fraction 1 protein was precipitated by the addition, with stirring, of solid ammonium sulfate (37 g/100 ml of extract) (18). The same ratio of ammonium sulfate to extract volume was used to precipitate active protein fractions recovered at all stages of purification. The bean protein bound to the DEAE-cellulose column (2.5 x 12 cm) was eluted with 0.6 m NaCl. Prior to enzyme assays the protein was dialyzed against 25 mm tris-HCl (pH 8.5), 1 mm MgCl₂, and 1 mm DTT.

Since RuBP oxygenase activity was lost when purifying bean fraction 1 protein, an alternative technique designated the PEG procedure was developed using PEG fractionation and DEAE-cellulose ion exchange chromatography. The details of this procedure are as follows. The primary leaves of red kidney bean were harvested, quickly frozen in liquid N₂, and lyophilized for 2 to 3 days. The lyophilization step was included to facilitate clearing of the homogenate prior to the initial fractionation with PEG. (We had previously encountered difficulty in clearing crude homogenates of green particulate matter if fresh tissue was used and homogenized in a Waring Blender.) Approximately 12 g of lyophilized leaf material were added, with stirring, to a beaker containing 250 ml of an extraction medium containing 50 mm K-phosphate buffer (pH 7.4), 1 mm EDTA, 2 mm GSH, 1 mm diethyldithiocarbamate, 0.5% (w/v) PEG, and 6 g of insoluble PVP. When the leaf material was fully hydrated, it was poured into a Waring Blender and homogenized for 10 sec. The homogenate was then filtered through four layers of cheesecloth and one layer of Miracloth prior to centrifugation at 37,000g for 10 min. Solid PEG was then added, with stirring, to the pale yellow supernatant in a ratio of 12 g of PEG/100 ml of extract. When the PEG was in solution, the pH of the extract was adjusted to 6.7 with 10% (v/v) acetic acid. The resulting precipitate was removed by centrifugation (37,000g for 10 min) and the small green pellet was discarded. Additional solid PEG was added to the supernatant in a ratio of 6 g of PEG/100 ml of original extract. When the PEG was in solution, the pH of the extract was adjusted to 6.1 with 10% (v/v) acetic acid. The white precipitate was collected by centrifugation (37,000g for 15 min) and immediately resuspended in a minimum volume of 5 mm K-phosphate buffer (pH 7.6), 1 mm DTT. The resuspended pellets were then applied to a DEAE-cellulose column (2.5 x 15 cm) equilibrated with 5 mm K-phosphate buffer (pH 7.6), 1 mm DTT. The column was washed with 2 column volumes of 5 mm K-phosphate buffer (pH 7.6), 1 mm DTT, prior to elution of the protein with 5 mm K-phosphate buffer (pH 7.6), 1 mm DTT and 0.6 mm NaCl. The peak protein fractions were collected and aliquots were dialyzed against 25 mm tris-HCl buffer (pH 8.5), 1 mm DTT, and 1 mm MgCl₂, for 3 to 4 hr. These dialyzed fractions were then used directly for RuBP oxygenase assays or diluted with 25 mm tris-HCl (pH 8.5), 1 mm DTT, and 1 mm MgCl₂, for carboxylase assays. All of the above operations were carried out at 0 to 4 C within a time span of 5 to 6 hr.

Enzyme Assays and 14CO₂ Fixation by Leaf Discs. At the developmental times indicated, 11.8 g of primary leaf tissue were harvested and homogenized for 1 min in a Waring Blendor in 30 ml of extraction medium containing 50 mm sodium phosphate buffer (pH 7.8), 2 mm GSH, 1 mm EDTA, 1 mm diethyldithiocarbamate, 0.5% (w/v) PEG, and 2 g of insoluble PVP. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 20,000g for 20 min. An aliquot of the supernatant was removed and stored at 4 C prior to the enzyme assays.

RuBP oxygenase was assayed with a YSI Clark Oxygen Electrode connected to a Beckman 25-cm chart recorder. The temperature of the reaction was held constant by the continuous pumping of water from a large reservoir, which was maintained at 30 C, through a water jacket surrounding the sample chamber. The RuBP oxygenase reaction medium contained the following components: a buffer, either 0.1 mm glycine-NaOH, 0.1 mm tris-glycine, or 0.1 mm tris-HCl (the specific buffering system and pH are noted in the appropriate legends); 0.4 mm DTT; 0.1 mm EDTA; 10 mm MgCl₂. The RuBP concentrations for a given set of experiments are listed in the appropriate legends. Unless specifically stated otherwise, the RuBP oxygenase reaction was assayed in a medium saturated with 100% O₂ gas, and the reaction was initiated with RuBP following a 2-min preincubation of 500 to 1,000 μg of fraction I protein in an O₂-saturated reaction medium. The final total volume in the sample chamber was 2.1 ml. Generally, 2-min reaction times were employed. The O₂ electrode was calibrated with sodium dithionite, using a value of 0.23 μmol O₂/ml for air-saturated distilled H₂O at 30 C.

RuBP carboxylase was assayed at 30 C in a reaction medium which contained the following components: 0.1 mm tris-HCl buffer (pH 8.5); 0.1 mm EDTA; 0.4 mm DTT; 10 mm MgCl₂; 25 mm 14C-bicarbonate (specific activity of 0.66 μCi/μmol; New England Nuclear) unless otherwise indicated in the appropriate legends; ± 0.5 mm RuBP; 10 to 20 μg of protein; in a final total volume of 0.5 ml. The protein was preincubated in the reaction medium for 2 min prior to the initiation of the reaction with RuBP. The reaction time was for 3 min and was terminated with 200 μl of 23.5 N formic acid containing a small amount of Triton X-100. From each reaction vessel a 200-μl aliquot was removed and spread on planchets and dried overnight. The planchets were counted in a Nuclear-Chicago model 1042 gas flow detector. CO₂ concentration was calculated from total bicarbonate according to Andrews et al. (1). A pH of 6.29 was employed.

Leaf discs, 7 mm in diameter, were floated, adaxial side up, on 1 ml of distilled H₂O in Amino double sidearm Warburg flasks (five discs/flask). The flasks were flushed for 8 min with either 100% N₂, 100% O₂ or compressed air, which were initially passed through water and soda lime filters. The flasks were sealed and 1.87 μCi of NaH¹⁴CO₃ (7 μCi/μmol) were injected into the sidearm. The ¹⁴CO₂ gas (0.03%) was liberated by the injection of 0.1 ml of 10% (v/v) phosphoric acid. In those flasks requiring 1% O₂ (v/v) by volume, a sufficient volume of 100% O₂ was injected into flasks previously flushed.
with 100% N₂. The flasks were shaken in the bath maintained at 29 °C for 4 min in the dark prior to either a 4- or 8-min period of exposure to light. The flasks were illuminated from below by two rows of Westinghouse reflector spot lamps providing 2,500 ft-c at the level of the flasks. The flasks were then opened to the air and the discs rapidly plunged into hot 80% (v/v) ethanol. After a period of extraction, the leaf discs were ground with a homogenizer and 500-μl aliquots were mixed with 250 μl of 24 N formic acid on planchets. The planchets were dried with a heat lamp and counted in a Nuclear-Chicago model 1042 gas flow detector.

Electrophoresis. Electrophoresis was performed by a technique modified from that of Laemmli (12). A stock solution of 19% (w/v) acrylamide and 1% (w/v) bis-acrylamide was used to make the 5% (w/v) and 2.5% (w/v) acrylamide solutions in an Anqebogue slab gel apparatus. The 5% (w/v) running gel acrylamide solution contained 0.089 m tris-HCl, 0.0025 m EDTA, 0.089 m boric acid (pH 8.3), and was polymerized with 0.016% (w/v) ammonium persulfate and 0.06% (v/v) TEMED. The 2.5% (w/v) stacking gel acrylamide solution contained 8.9 m tris-HCl, 0.25 mm EDTA, 8.9 mm boric acid (pH 6.8), and was polymerized with 0.016% (w/v) ammonium persulfate and 0.1% TEMED. The electrophoresis buffer was 0.089 m boric acid (pH 8.3). The protein (15 μg) was run through the stacking gel at 30 V, and the current was then increased to 100 V for 3 hr. After electrophoresis the gel was fixed in 10% (w/v) trichloroacetic acid, 20% (w/v) methanol at 37 °C overnight, stained with 0.25% (w/v) Coomassie blue in 7% (v/v) acetic acid, 10% (w/v) methanol for 2 hr, and then destained in 7% (v/v) acetic acid at 37 °C.

Protein Determinations. Protein content of all extracts was determined by the Lowry method (16). Aliquots of extracts were dialyzed against 25 mm buffer for 24 hr prior to protein determinations. The protein content of purified enzyme solutions was determined according to the method of Paulsen and Lane (21).

RESULTS AND DISCUSSION

Purification of RuBP Carboxylase-Oxygenase. Preliminary purification studies indicated that the addition of solid ammonium sulfate (37 g/100 ml) to crude bean homogenates resulted in the complete loss of detectable oxygenase activity after 24 hr at 4 °C (data not shown). Subsequent work demonstrated that if all of the purification steps of the ammonium sulfate procedure were completed rapidly (24-30 hr), RuBP oxygenase activity was detectable at the G-25 and DEAE-cellulose steps; however, the Sepharose 4B fractions invariably lacked RuBP oxygenase activity (Table I). Therefore, with bean, the ammonium sulfate procedure resulted in a rapid loss of detectable RuBP oxygenase activity and the retention of a low velocity form of RuBP carboxylase (Table I).

Numerous efforts were made to restore RuBP oxygenase activity to the purified protein. These included: (a) the addition of various metal ions to the assay medium (Fe²⁺, Cu²⁺, Mn²⁺, Co²⁺); (b) heating the protein for 10 min at 50 °C in the presence of DTT and Mg²⁺ (22); (c) initiation of the reaction with protein rather than RuBP (25); (d) varying the pH of the assay medium (8.5-9.5); (e) varying the protein concentration in the assay; and (f) combining various fractions from different steps in the purification procedure. All attempts to restore detectable RuBP oxygenase failed.

Andrews et al. (2) reported that the RuBP carboxylase and oxygenase activities in purified spinach enzyme followed dissimilar decay kinetics. It was found that both activities decayed to a considerable degree with time, with the RuBP oxygenase activity exhibiting a greater degree of stability than the carboxylase. They also found that the pH optimum of the oxygenase was quite alkaline (9.4) whereas previous work had demonstrated that the pH optimum for carboxylation was pH 7.8 (25). Subsequent work has shown that the oxygenase to carboxylase ratio can also vary as a function of temperature (4, 13). These reports and the present results with bean indicate that the ratio of carboxylase to oxygenase may vary and can be altered in vitro.

The complete loss of detectable oxygenase activity following ammonium sulfate precipitation necessitated the development of an alternative purification technique designated the PEG procedure. It was found that quick freezing the leaf tissue with liquid N₂ and subsequent lyophilization and PEG fractionation resulted in a stable preparation. High yields of high velocity bean RuBP carboxylase and RuBP oxygenase could be obtained with this technique (Table II). Also, it was found that the oxygenase activity of the PEG-purified enzyme remained high during the same time period in which complete loss of detectable oxygenase activity was observed with the ammonium sulfate purification procedure (data not shown).

Characteristics of Bean RuBP Carboxylase. A double reciprocal plot (Fig. 1) of RuBP carboxylase velocity versus bicarbonate concentration indicated that the bean enzyme can exist in at least two distinct forms. The enzyme prepared by the PEG procedure has a much higher velocity than the protein prepared with ammonium sulfate at all bicarbonate concentrations tested. The RuBP carboxylase velocities at a bicarbonate concentration of 12.5 mmol 14C-bicarbonate (0.66 μCi/μmol). RuBP oxygenase was assayed in a two min reaction in a medium containing 0.1 M Tris-HCl, pH 8.5; 0.4 mm DTT; 0.1 mm EDTA; 10 mm MgCl₂; 1 mM RuBP; and saturated with 100% O₂ gas. Protein determinations for the crude and DEAE Cellulose purification steps were according to Lowry (16). Protein determinations for the Sepharose 4B fractions were according to Paulsen and Lane (21).

<table>
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<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
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</thead>
<tbody>
<tr>
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<td>1301</td>
<td>46.8 104.0</td>
<td>0.036 0.080</td>
<td>100</td>
</tr>
<tr>
<td>DEAE Cellulose</td>
<td>125</td>
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<td>0.033 0.117</td>
<td>9</td>
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<tr>
<td>Sepharose 4B</td>
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<td>(not detectable)</td>
<td>--- 0.143</td>
<td>0 5</td>
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</table>

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<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>744</td>
<td>53.6 212.0</td>
<td>0.072 0.285</td>
<td>100</td>
</tr>
<tr>
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<td>0.202 1.102</td>
<td>42 50</td>
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</table>

Table I. Ammonium Sulfate Procedure for the Purification of Fraction I Protein from Red Kidney Bean.

Table II. PEG Procedure for the Purification of Fraction I Protein from Red Kidney Bean.

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equivalent to 10.9 \mu M CO_2 (physiological concentration [27]) have been expressed on a Chl basis (Table III). At this CO_2 concentration the enzyme prepared by the PEG procedure had approximately a 10-fold greater velocity than the enzyme prepared by the ammonium sulfate procedure, and the calculated velocity of 126 \mu mol CO_2 fixed/mg of Chl-hr for the PEG-prepared enzyme compares favorably with reported rates of intact bean leaves (29).

The \( K_m \) values (bicarbonate or CO_2) of the enzymes prepared by the two alternative procedures were very similar and quite high (22 mM bicarbonate or 135 \mu M CO_2 [pH 8.5, 30 C]). These \( K_m \) values (bicarbonate) were similar to the \( K_m \) value (bicarbonate) reported by Paulsen and Lane (21) for highly purified spinach RuBP carboxylase. Bahrs and Jensen (6), using freshly lysed spinach chloroplasts, detected a low \( K_m \) (bicarbonate or CO_2) form of RuBP carboxylase. The \( K_m \) values reported ranged from 0.5 to 0.8 mM bicarbonate (11-18 \mu M CO_2 [pH 7.8, 25 C]) and were accompanied by a velocity of 24 \mu mol/mg of Chl-hr at 9 \mu M CO_2 (pH 7.8, 25 C). In spite of its low velocity, this kinetic form was referred to as a physiological form of the enzyme, because its range of \( K_m \) values for CO_2 was similar to that reported for intact leaves and isolated chloroplasts.

Lilley and Walker (14) and Andrews et al. (1), using crude enzyme preparations, have obtained a high velocity form of RuBP carboxylase at physiological concentrations of CO_2. Lilley and Walker (14), using an improved assay technique, have reported a \( K_m \) of 46 \mu M CO_2 (pH 7.6, 20 C) and velocities in the range of 100 \mu mol/mg of Chl at 10 \mu M CO_2. Andrews et al. (1), using the low \( K_m \) (bicarbonate or CO_2) form of the enzyme, have reported data from which we have calculated velocities of approximately 132 and 171 \mu mol/mg of Chl-hr at 8.3 \mu M CO_2 (pH 8.4, 25 C) and 9 \mu M CO_2 (pH 8.2, 25 C), respectively.

It is evident that preparations of RuBP carboxylase can have similar \( K_m \) values yet widely divergent maximal velocities. This may reflect isolation-induced modifications or variations in the assay techniques employed (10). Our results indicate that the isolation technique can modify the protein such that the maximal velocity of the nonactivated form of the carboxylase can vary significantly while the \( K_m \) remains constant.

### Characteristics of Bean RuBP Oxygenase

A double reciprocal plot of the RuBP oxygenase velocity as a function of O_2 concentration indicated that the bean enzyme prepared by the PEG procedure had a high \( K_m \) (O_2) of 0.90 mM. Low (0.22 mM O_2) \( K_m \) forms of RuBP oxygenase have been reported for crude spinach extracts and the purified soybean and spinach enzymes.

Bean fraction I protein prepared by the PEG procedure exhibited a relatively high oxygenase velocity (Table II) when compared to values reported by others for the purified enzyme (2,19). The velocity of the bean enzyme was, however, too low at 21% O_2 (pH 8.5, and 30 C) to account for rates of glycolic acid synthesis and photorespiration (29). When expressed on a Chl basis (Table III), the oxygenase velocity was significantly less than that reported by Andrews et al. (1) for the low \( K_m \) (O_2) form of the oxygenase detected in crude and partially purified spinach leaf homogenates (0.21 \mu mol of O_2/min · mg of protein or 75.6 \mu mol of O_2/mg of Chl-hr using a ratio of 6 mg of protein/mg of Chl [17]). However, Tobert and Ryan (26) have noted that the velocity of the low \( K_m \) form of the spinach oxygenase was 0.05 \mu mol/min · mg of protein in air, which was similar to our bean preparation (0.056 \mu mol/min · mg of protein).

It has been reported (26) that to obtain the low \( K_m \) (O_2) form of the purified spinach RuBP oxygenase, the oxygenase assay must be initiated with large amounts of activated (preincubated with CO_2 and Mg^{2+}) enzyme protein. Associated with the low \( K_m \) (O_2) form was a broad pH optimum for the oxygenase. With the bean enzyme, the high \( K_m \) (O_2) form exhibited a broad pH optimum (Fig. 2), indicating the lack of a correlation between the \( K_m \) (O_2) and the pH optimum. In contrast to previously reported results with spinach, we have found that initiating the oxygenase reaction with bean protein led to very low velocities (Fig. 3). Similar results were obtained with crystalline fraction I protein from tobacco when the same preincubation and reaction conditions were employed (19). However, with the tobacco enzyme, preincubation with CO_2 and Mg^{2+} eliminated the inhibition observed when initiating the reaction with protein (19). There may be species-specific kinetic differences associated with the enzyme, or the absence of ammonium sulfate in the preparations of the tobacco and bean enzymes may have led to similar kinetic forms. There are, however, major differences between the oxygenase properties of the tobacco (19) and bean enzymes. The bean enzyme had a \( K_m \) (RuBP) of 0.25 \mu M which was similar to the purified soybean (13) and spinach enzymes (2), while the tobacco enzyme had a \( K_m \) (RuBP) of 22 \mu M. Also, the tobacco enzyme

### Table III. RuBP Carboxylase-Oxygenase Activity of Fraction I Protein from Red Kidney bean

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>RuBP Carboxylase Activity</th>
<th>RuBP Oxygenase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulfate</td>
<td>13.9 (not detectable)</td>
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</tr>
<tr>
<td>PEG</td>
<td>126.0</td>
<td>20.5</td>
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</table>

RuBP carboxylase was assayed in a 3 min reaction. Bicarbonate concentration was attained by adding unlabeled bicarbonate to reaction tubes containing 2.09 \mu Ci of 14C-bicarbonate (7 \mu Ci/\mu mol). The CO_2 concentration (10,9 \mu Ci CO_2) was calculated from total bicarbonate according to Andrews et al. (1). RuBP oxygenase was assayed in a 2 min reaction in a medium containing 0.1 n tris-HCl, pH 8.5, 0.4 \mu M DTT, 0.1 \mu M EDTA; 10 \mu M MgCl_2; 1 \mu M RuBP; and saturated with compressed air (21% O_2). Activity on a chlorophyll basis was computed by using a factor of 6 mg of carboxylase protein / mg Chl (17).
Fig. 2. RuBP oxygenase activities as a function of pH. Bean RuBP oxygenase was prepared by the PEG procedure. Reaction time was 2 min in a medium containing 0.1 M tris-glycine (various pH values), 0.4 mM DTT, 0.1 mM EDTA, 10 mM MgCl₂, 1 mM RuBP, and saturated with 100% O₂ gas.

![Graph showing RuBP oxygenase activities as a function of pH](image)

Fig. 3. Effect of initiating the RuBP oxygenase reaction by the enzyme or the substrate. Bean RuBP oxygenase was prepared by the PEG procedure, and the reaction medium contained 0.1 M tris-HCl (pH 8.5), 0.4 mM DTT, 0.1 mM EDTA, 10 mM MgCl₂, and saturated with 100% O₂ gas. R: Reaction initiated with 1 mM RuBP following a 2-min preincubation of the enzyme protein in the reaction medium. P: Reaction initiated by injection of enzyme protein into reaction medium containing 1 mM RuBP.

![Graph showing effect of initiating the RuBP oxygenase reaction](image)

Fig. 4. Slab gel electrophoresis of fraction I protein from spinach and bean. Spinach fraction I protein prepared by the ammonium sulfate procedure (A) and the PEG procedure (B). A 5% (w/v) acrylamide slab gel was employed with a 2.5% (w/v) stacking gel. Electrophoresis buffer was tris-HCl, boric acid (pH 8.3). The gel was stained with Coomassie blue and destained with acetic acid.

![Slab gel electrophoresis image](image)

had a pH optimum for the oxygenase between 8.4 and 8.6, and the bean enzyme had a broad pH optimum.

**Electrophoresis.** Fraction I protein from bean and spinach purified by ammonium sulfate fractionation, ion exchange chromatography, and gel filtration was subjected to electrophoresis, and the results (Fig. 4) indicated that the bean enzyme was electrophoretically different from the spinach enzyme. Wilson and McCalla (28) also purified fraction I protein from spinach, pea, bean, and Chlorella using ammonium sulfate fractionation and gel filtration. Electrophoresis of the purified enzymes indicated that the bean and Chlorella enzymes were electrophoretically different from each other, and from the pea and spinach enzymes. When fraction I protein of bean was prepared by the PEG procedure, it was found that the product was electrophoretically similar to the spinach enzyme purified with ammonium sulfate (Fig. 4). As already noted, the bean enzymes prepared by these two alternative procedures had markedly different properties, but it is not known at this time if a cause and effect relationship exists between these differences and the observed electrophoretic patterns. Ammonium sulfate-induced structural modifications have also been noted in *Euglena* fraction I protein preparations (J. Schiff, personal communication).

**Developmental Studies.** Etiolated leaf tissue has low levels of RuBP carboxylase, and the stimulus of light results in the rapid *de novo* synthesis of additional enzyme protein (11). Since it was noted that under certain conditions the carboxylase and oxygenase activities of the enzyme were not tightly linked, it was of interest to study the developmental kinetics of RuBP carboxylase and RuBP oxygenase during greening in bean. Low but detectable levels of both RuBP carboxylase and RuBP oxygenase activity were observed in etiolated primary leaf tissue. During greening there was an apparent lag of approximately 24 hr prior to a period of rapid increase in the specific activity of carboxylase and oxygenase during a subsequent 48-hr period. Previous studies on the development of RuBP carboxylase activity in *Phaseolus vulgaris* have described induction lags of different durations (3, 7, 8). However, we found that the RuBP carboxylase and oxygenase activities followed very similar developmental kinetics, indicating that the activities were linked at all developmental stages.

We also studied the photosynthetic *14CO₂* fixation in bean primary leaf discs to determine if there was a correlation between the RuBP carboxylase and oxygenase activities at a given developmental stage and the ability of leaf discs to photosynthesize under different gas regimes (Fig. 5). There was an initial rapid increase in the leaves' ability to fix *14CO₂* under atmosphere of 1% and 21% O₂. The rate of increase slowed between 24 and 48 hr and then increased to its maximum at 72 hr. There were indications of differences in the ability of leaf discs to photosynthesize under 1% and 21% O₂ after 72 and 96 hr of greening; because of the variability encountered, we do not believe these differences to be significant at this time.

It was found that 100% N₂ (Fig. 5) strongly inhibited photosynthesis initially, but by 72 hr this inhibition had been largely...
overcome. Leaf discs, greened for 24 and 96 hr, were assayed for differences in stomatal aperture after a period of photosynthesis under a 100% N₂ atmosphere (30). No apparent differences in stomatal aperture were observed. The initial N₂ inhibition may reflect the importance of either pseudoplastic photo-phosphorylation (24) and/or the participation of mitochondrial respiration in light-driven CO₂ fixation at early stages of development. The ability of leaf discs to photosynthesize under atmospheres of 100% O₂ (Fig. 5) increased slowly at first and reached a maximum at 72 to 96 hr. At all developmental stages investigated, photosynthetic CO₂ fixation was severely inhibited by 100% O₂. It appears that photosynthesis during early stages of development was more sensitive to high partial pressures of O₂ (Fig. 5).

The results from the developmental studies indicated that the 48- to 72-hr period is an extremely important interval in the greening process. It was during this period that carboxylase and oxygenase showed the greatest percentage increase in specific activity. It was also the period when N₂ inhibition of photosynthesis was overcome, and the leaf discs under atmospheres of 1%, 21%, and 100% O₂ attained their maximum rates of photosynthesis. Howes and Stern (9) found that in chloroplasts isolated from primary red kidney bean leaves, grown and greened under similar conditions, photosynthetic phosphate reduction appeared early in development and reached maximum rates in vivo before the maximum rates of ¹⁴CO₂ fixation were observed in vitro as determined in this study. It would appear that factors other than functional light reactions may be limiting the capacity of the chloroplasts to fix CO₂ at maximal rates during the first few days of development.

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