Different location in dark-adapted leaves of *Phaseolus vulgaris* of ribulose-1,5-bisphosphate carboxylase/oxygenase and 2-carboxyarabinitol 1-phosphate

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Abstract In situ RuBisCO activity was analyzed in order to determine whether 2-carboxyarabinitol 1-phosphate (CAIP) interacts with the enzyme in dark-adapted leaves of *Phaseolus vulgaris*. Leaves ground to fine powder in liquid nitrogen were put directly into a reaction mixture containing a saturating concentration of ribulose 1,5-bisphosphate (RuBP) to preserve the activity of RuBisCO which was in the chloroplasts. Some 70% of the total catalytic sites of RuBisCO possessed carboxylase activity in this assay, however, RuBisCO was inhibited in the absence of RuBP. CAIP seemed to be concentrated in the veins. These results indicate that RuBisCO was not complexed with CAIP in leaves.

Key words: Ribulose-1,5-bisphosphate carboxylase/oxygenase activity; 2-Carboxyarabinitol 1-phosphate; Predawn leaves; Direct assay; Inorganic phosphate; *Phaseolus vulgaris*

1. Introduction

Several key enzymes in the photosynthetic carbon reduction (PCR) cycle are regulated for fine tuning of metabolic events depending on the rate of CO\(_2\) fixation. The light-dark regulation of some of these enzymes is also important to prevent artifactual interaction between RuBisCO and either leaf components or ingredients in the extraction buffer, leaves ground in liquid N\(_2\) should be put directly into the assay mixture as we suggested previously [19]. We used this direct assay method instead of extraction to measure directly the in situ RuBisCO activity in leaves of *Phaseolus vulgaris* collected before dawn. RuBisCO was not complexed with CA1P, but 70% of total potential activity was detected in this assay. RuBisCO and CA1P were in different parts of the leaves.

2. Materials and methods

2.1. Materials

*P. vulgaris* plants were grown on commercial fertilized soil in a controlled environment chamber at an irradiance of 550 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) for 16 h at 25°C and in the dark for 8 h at 20°C, with daily watering. Half-strength Hoagland’s solution was used instead of water once a week. 4-week-old plants were used for experiments. RuBP was purchased from Sigma (St. Louis, MO, USA) and NaH\(^{14}\)CO\(_3\) was purchased from Amersham (Tokyo, Japan). \(^{14}\)CABP was prepared from RuBP as reported elsewhere [20]. Onozuka-RS cellulase and pectinase were obtained from Yakult Biochemical Co. (Tokyo, Japan) and Fluka (Buchs, Switzerland), respectively. RuBisCO from fresh spinach leaves was purified as described before [21].

2.2. Measurement of RuBisCO activity

To avoid any artifactual event during extraction, we assayed the activity of RuBisCO after putting frozen leaf powder into the assay mixture using our direct assay method [19]. Fully expanded *Phaseolus* leaves were picked and immediately frozen in liquid N\(_2\) after which they were ground to fine powder in a mortar with a pestle. The nitrogen was evaporated off and 10–15 mg of powder was put into an assay mixture (0.5 ml) consisting of 0.1 M HEPES-KOH buffer (pH 8.0), 1 mM DTT, 1 mM EDTA, 20 mM MgCl\(_2\), 20 mM NaH\(^{14}\)CO\(_3\), and 1 mM RuBP to start the reaction. RuBisCO was assayed for 30 s at 25°C [22]. This activity is referred to as RuBisCO

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*Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; CA1P, 2-carboxyarabinitol 1-phosphate; RuBP, ribulose 1,5-bisphosphate; CABB, 2-carboxyarabinitol 1,5-bisphosphate; \(^{14}\)CABP, [2-\(^{14}\)C]carboxyarabinitol 1,5-bisphosphate; P, inorganic phosphate

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activity in the direct assay. The temperature of the mixture decreased by at most 1°C immediately after the frozen powder was added, but returned to the starting temperature within a few seconds. Another 10–15 mg of leaf powder was incubated for 10 min in the assay mixture without RuBP. RuBP was added to a final concentration of 1 mM and RuBisCO was assayed for 30 s. This activity is designated RuBisCO activity after incubation, which was the maximum of the RuBisCO activity remaining after RuBisCO inhibition by CA1P if present. The carboxylase reaction was stopped by the addition of 3% perchloric acid. A 200 μl portion of this acidified mixture was put into a scintillation vial and dried in an oven at 70°C. The fixed 14CO2 was counted as reported previously [22]. The remaining 500 μl portion was centrifuged at maximum speed for 5 min and the precipitate was extracted with 80% acetic acid and assayed for phytochrome [23].

The results of this direct assay and the extraction assay as carried out in other laboratories were compared. Frozen leaf powder was extracted as described by Seemann et al. [3] and Holbrook et al. [13] with 0.1 M HEPES-KOH buffer (pH 8.0) containing 5 mM DTT, 5 mM EDTA, 5 mM MgCl2, 1 mM PMSF, 10 μM leupeptin, and 2% PVP-40 at 0°C. The RuBisCO activity in the supernatant was measured within 5 min after the extraction began with and without incubation as described above.

Another portion of leaf powder was extracted with 0.1 M HEPES-KOH buffer (pH 8.0) containing 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 10 μM leupeptin, and 2% PVP-40 after centrifugation for 5 min at 10 000 × g, the supernatant was obtained and ammonium sulfate was added to 50% saturation to eliminate possible interactions between CA1P and RuBisCO [4]. The precipitate was dissolved in a small volume of 25 mM HEPES-KOH buffer (pH 8.0) containing 1 mM DTT and put on a column (1 × 30 cm) of Sephadex G-75 equilibrated with the same buffer. The protein fractions were measured for the total potential activity of RuBisCO free from possible inhibition by CA1P. The activity was measured as described above for the maximum activity.

The active catalytic sites of RuBisCO were determined with [14C]CABP. RuBisCO (0.2 mg) was incubated for 10 min at 25°C in 0.5 ml of buffer mixture (pH 8.0) containing 0.1 M HEPES-KOH, 1 mM DTT, 1 mM EDTA, 20 mM MgCl2, and 20 mM NaHCO3. Then, [14C]CABP was added to a final concentration of 10 μM (4273 dpm/nmol) to the activated RuBisCO solution and incubation was continued for 1 h. The quaternary RuBisCO complexes were fractionated on a Sephadex G-75 column (1 × 30 cm) with the buffer used in incubation. The number of the active RuBisCO sites was determined by centrifugation of the first extract were measured as reported before [23].

2.3. Isolation of chloroplasts and protoplasts
Mid ribs and branched large ribs of fully expanded leaves were removed and photosynthetically active chloroplasts were isolated according to the method of Asada and Badger [24] with some modifications. All steps for isolation of chloroplasts were performed under illumination at room-light intensity of about 150 μmol quanta m−2 s−1 for midday leaves and under dim light for predawn leaves. Pellet of intact chloroplasts were suspended in HEPES buffer (pH 8.0 and 7.0 for the midday and predawn leaves, respectively) and layered on a discontinuous Percoll gradient.

Photosynthetically active protoplasts were isolated from 4-week-old Phaseolus leaves as described by Kobza et al. [25] with some modifications. All procedures were conducted in darkness and the pH of the medium was 7.0.

2.4. Perchloric acid extraction
Fine powder of midday and predawn leaves was extracted with 3% perchloric acid in the presence of the medium used for isolation of protoplasts. The acidified mixture was kept on ice for 30 min and centrifuged at 10 000 × g for 10 min. The supernatant was neutralized with KOH and the debris was extracted with 80% acetic to measure phytochrome as above. Potassium perchlorate was removed by centrifugation at 10 000 × g for 10 min. The supernatant was lyophilized and dissolved in a small volume of distilled water.

3. Results
First, we confirmed that the Phaseolus leaves contained CA1P. The total potential activity of RuBisCO in both midday leaves and predawn leaves was close to 450 μmol mg−1 Chl h−1 (Table 1). The activity in the extract from predawn leaves without incubation was 39% of the potential activity. Incubation without RuBP for 10 min decreased the activity to 34% of the potential activity (Table 1). Since the concentration of the catalytic site of RuBisCO in the assay mixture was routinely about 30 nM and the dissociation constant in the binding of CA1P to activated RuBisCO is reportedly 32 nM [7], the observed decrease in activity to 34% was plausible if the concentration of CA1P in the predawn leaves of P. vulgaris reported by others [3,9].

In Table 1 the activity in the direct assay is compared with the maximum activity after incubation without RuBP and the total potential activity in midday and predawn leaves. The potential activity corresponded to the activity of all catalytic sites in the extract, and was 3.5–3.8 mol CO2 fixed s−1 mol−1 of catalytic sites measured with [14C]CABP. The result of the direct assay of predawn leaves was 70% of the potential activity. The RuBisCO activity was inhibited by 87% after incubation. The conventional extraction method [3,13] caused the activity to be inhibited by 39 and 34% without and with incubation, respectively. This stronger inhibition after incubation in the direct assay than in the extraction assay was due to the concentrations of RuBisCO and metabolites being higher during incubation in the direct assay. The inhibition was time-dependent during incubation and the half-time for inhibition to be completed was 70 s at 25°C (Fig. 1). These results led us to the idea that most of RuBisCO in the predawn leaves was not complexed with CA1P, but highly activated. CA1P may have been able to bind to RuBisCO only during extraction of leaves and incubation in the assay mixture without RuBP. With midday leaves, activity was not inhibited after incubation in the direct assay, further evidence from the previous observation [9,11,13] that CA1P is not synthesized in the light.

To assay RuBisCO activity more directly as the enzyme was in chloroplasts, we put chloroplasts and protoplasts isolated from midday and predawn leaves into assay mixture containing 0.1% Triton X-100. RuBisCO activity of chloroplasts and protoplasts of midday leaves was more than 200 μmol mg−1 Chl h−1 (Table 2). As expected, inhibition was not observed in the assay mixture containing 0.1% Triton X-100.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of extraction method on the activity of RuBisCO from predawn and midday leaves of Phaseolus vulgaris</th>
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<tr>
<td>% RuBisCO activity*</td>
<td>Without incubation</td>
</tr>
<tr>
<td>Predawn leaves</td>
<td></td>
</tr>
<tr>
<td>Direct assay</td>
<td>70.1 ± 2.0</td>
</tr>
<tr>
<td>Extraction assay</td>
<td>38.7 ± 2.3</td>
</tr>
<tr>
<td>Midday leaves</td>
<td></td>
</tr>
<tr>
<td>Direct assay</td>
<td>100.1 ± 17.4</td>
</tr>
</tbody>
</table>

*Values are means ± S.D. of the ratios of the activity found in six experiments to the total potential activity found in that experiment, multiplied by 100. The total potential activity of RuBisCO from midday and predawn leaves was 450 ± 17 and 448 ± 14 μmol mg−1 Chl h−1, respectively.
with the idea that the absence of inhibition in the direct assay procedure, since the kcat value after digestion was 3.5-3.8 s⁻¹ site⁻¹. These results are consistent with the existing form of RuBisCO in the night: Why the direct assay method at the reported Km for RuBP (20 μM). From these considerations, it cannot be true that CA1P artifactually binds to the enzyme before and during the assay in our direct assay method. This method may also be useful for measurements of the activation states of the enzymes involved in the PCR cycle.

The direct assay method showed that up to 70% of RuBisCO was in the activated state without any interaction with CA1P in Phaseolus leaves adapted to the dark. The activity was, however, decreased to 13% of the total potential activity during incubation in the presence of CO₂ and Mg²⁺ but in the absence of RuBP in a time-dependent manner. The stromal pH decreases to 7.0–7.2 and concomitantly the stromal Mg²⁺ concentration is lowered to 1–3 mM in the dark [17,18]. Nevertheless, RuBisCO was highly active under these conditions. RuBisCO may not be in the complex with CA1P, as has been discussed [14], but was in the activated form by some factor involved in the PCR cycle.

The possibility that RuBisCO and CA1P were in different parts of the leaf was examined in a different way in Fig. 2. The predawn leaves were cut into segments of 1 mm width and then, the RuBisCO activities in leaf powder were measured directly (0 time) in our direct assay system [19] and for different intervals of incubation (as indicated) of leaf powder in the assay buffer as performed in Table 1. Specific activity of ¹⁴CO₂ was 123 dpm/nmol.

[Fig. 1. Time course of inhibition of the in situ RuBisCO activity in predawn leaves of Phaseolus vulgaris during incubation in assay buffer in the absence of RuBP. The predawn leaves were made powder in liquid N₂o as explained in Section 2. Then, RuBisCO activities in leaf powder were measured directly (0 time) in our direct assay system [19] and for different intervals of incubation (as indicated) of leaf powder in the assay buffer as performed in Table 1. Specific activity of ¹⁴CO₂ was 123 dpm/nmol.]

During incubation without RuBP. The enzyme activity of chloroplasts and protoplasts of predawn leaves was low, as has been reported [26]. Besides, it has been suggested that a requirement for illumination at 200 μmol quanta m⁻² s⁻¹ is essential for a high yield of intact protoplasts and chloroplasts from Phaseolus leaves [25]. It may be plausible to assume that RuBisCO and CA1P are sequestered in chloroplasts both as a complex between them and as their free forms. Damage of protoplasts or chloroplasts, by any means during isolation, will lead to a release of both complexes and free forms of the enzyme and CA1P at the same rates into the medium and the remaining bound and free forms of the enzyme and CA1P in protoplasts or chloroplasts would exhibit responses similar to those in the in vitro states. On the basis of this assumption, inhibition of the enzyme in dark-adapted chloroplasts and protoplasts was not observed. The lower activity was due not to a consequence of inhibition of the enzyme by CA1P but to the release of the enzyme from chloroplasts and protoplasts during their preparation, since the kcat value after incubation was 3.5–3.8 s⁻¹ site⁻¹. These results are consistent with the idea that the absence of inhibition in the direct assay in Table 1 may be ascribed to different locations of RuBisCO and CA1P in Phaseolus leaves.

The possibility that RuBisCO and CA1P were in different parts of the leaf was examined in a different way in Fig. 2. The predawn leaves cut into segments of 1 mm width were digested, as in the isolation of protoplasts, with cellulase and pectinase 3 times each for 30 min. After each digestion, the released protoplasts were passed through a layer of nylon mesh (50 μm) to remove them from residual segments, which were re-digested a further 2 times with new digestion media with the same composition as in the first digestion. Digested leaf tissues (enriched in veins) were extracted with 3% perchloric acid as mentioned in Section 2. The effect of the extract on the activity of spinach RuBisCO is shown in Fig. 2. The whole-leaf extract of midday leaves, the control, caused little inhibition. The extract of vein-enriched tissues inhibited enzyme activity more than the extract of whole predawn leaves. Since three digestions liberated over 70% of leaf chlorophyll (data not shown), the ratio of the veins to mesophyll cells must have increased in the final digested tissues. These results suggest that CA1P was concentrated in the veins.

### Table 2

<table>
<thead>
<tr>
<th>Activity of RuBisCO from chloroplasts of midday and predawn leaves of Phaseolus vulgaris</th>
<th>RuBisCO activity (μmol CO₂ mg⁻¹ Chl h⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Direct assay</td>
</tr>
<tr>
<td>Midday leaves</td>
<td></td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>ND</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>207.7 ± 0.7</td>
</tr>
<tr>
<td>Predawn leaves</td>
<td></td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>35.9 ± 5.1</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>58.6 ± 1.7</td>
</tr>
</tbody>
</table>

*The assay mixture included 0.1% Triton X-100. Values are means ± S.D. of the activity of six experiments. ND, not determined.
mechanism and the enzyme had no chance to encounter CA1P until the leaf powder had been put into the preincubation mixture devoid of RuBP or was extracted by alkaline buffer containing MgCl₂.

The observed absence of inhibition of RuBisCO activity by CA1P in the predawn leaves may suggest that CA1P was absent from chloroplasts or CA1P did not interact with RuBisCO in chloroplasts. Isolated chloroplasts and protoplasts did not show inhibition after incubation. This result may indicate that CA1P was not present in the chloroplasts. The activities recovered in the chloroplast and protoplast fractions from the predawn leaves were much lower than those from the midday leaves. Illumination of the leaf segments in the digestion mixture is essential for isolation of undamaged protoplasts. The activities recovered in the chloroplast and protoplast fractions from the predawn leaves were much lower than those from the midday leaves. Illumination of the leaf segments in the digestion mixture is essential for isolation of undamaged protoplasts from Phaseolus leaves [25]. However, the segments from the predawn leaves were digested in complete darkness in this experiment to avoid any degradation of CA1P. This caused the lower recovery of the enzyme activity. The lower activity was not due to the binding of CA1P to the enzyme, since the catalytic activity measured on the basis of CABP-binding sites was 3.6-3.8 s⁻¹ site⁻¹. These results indicate that CA1P resides neither in chloroplasts nor in mesophyll cells in Phaseolus leaves. This conclusion is inconsistent with the results reported recently by Seemann’s group [27]. They isolated leaf chloroplastic, cytoplasmic, and vacuolar materials by the non-aqueous fractionation method to conclude that CA1P and CA1P phosphatase did occur in chloroplasts [27]. It has also been reported that CA1P phosphatase can occur in both cytoplasm and chloroplasts of tobacco leaves [15]. However, the whole leaves were ground in liquid N₂ or an isotonic medium. It is hard to distinguish the subcellular fractions from the tiny vein network from those from the mesophyll cells of the leaves in these methods, as has been claimed in the case of the subcellular distribution of glutamine synthetase I in rice leaves [28].

A possible location for CA1P in Phaseolus leaves was the veins. The veins are vascular bundles composed of xylem and phloem tissues. The xylem is a water-conducting tissue and the phloem, composed of sieve elements and companion cells, is a food-conducting tissue. Phloem tissues are the site of glutamine synthetase I in rice leaves [28]. Carboxyarabinitol accumulates in the vacuoles in mesophyll cells [29]. The exact function of CA1P in the veins and the mechanism of the transfer of the carbon skeleton of carboxyarabinitol from the veins to the mesophyll vacuole are interesting, but it was not our concern in the present study.

Low concentrations (25-50 mM) of ammonium sulfate hinder the binding of exogenous CA1P to purified RuBisCO and the enzyme in leaf extracts [12]. Accordingly, inclusion of the sulfate in this range prevents RuBisCO from artifactual binding of CA1P to the enzyme during extraction. This observation provided the basis for experiments to demonstrate that CA1P in the complex with RuBisCO in the leaf extract in the presence of ammonium sulfate was the entity that exactly bound to the enzyme in the predawn leaves. However, there is no information on how sulfate performs in the interaction of RuBisCO and endogenous CA1P.

We do not need to divide the higher plants into two groups based on the regulatory mechanism of RuBisCO. Phaseolus leaves contained CA1P in the organ where RuBisCO does not function. 70% of RuBisCO in the predawn leaves showed the activity. The remaining 30% was not in the complex with CA1P, but decarbamylated since CA1P was not in the mesophyll cells. This range of activation may be explained by the effect of inorganic phosphate on the activation [22]. The repeatedly observed higher activation of RuBisCO in the dark in spinach, radish, and wheat, which do not synthesize CA1P, must also be due to a function of inorganic phosphate as has been found in the light [22]. RuBisCO itself may be highly activated in the day and in the night but cannot function in CO₂ fixation in the night because of a lack of the other substrate RuBP.

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