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RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE AND OXYGENASE FROM GREEN PLANTS ARE TWO DIFFERENT ENZYMES

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SUMMARY: Ribulose-1,5-diphosphate oxygenase is shown to be an enzyme different from ribulose-1,5-diphosphate carboxylase. The enzymes can be separated from each other by a preparation method employing gel filtration at a pH of 8.3 which is higher than that previously used. The oxygenase activity is correlated with a blue color and a characteristic Cu EPR signal. This indicates that the oxygenase is a copper-containing enzyme.

An inhibition of the biosynthesis of glycolate leads to a lower photorespiratory rate (1). This may increase plant productivity, and several attempts have been made to find such inhibitors (2). In 1971 and 1973 it was reported that RuP_2* carboxylase (EC 4.1.1.39) besides fixing CO_2 also worked as an oxygenase producing phosphoglycolate (3,4), although it could not be excluded that a minor contaminant was responsible for the oxygenase activity (2). Experiments in vivo and with the isolated enzyme showed that the amount of phosphoglycolate formed was sufficient to account for the photorespiratory rate (4,5). Therefore, this enzyme has attracted much attention (6).

However, it seemed difficult to achieve an inhibition of the oxygenase function without impairing the desired carboxylase activity since CO_2 and O_2 were assumed to bind to the

*Abbreviations used: RuP_2 = D-ribose-1,5-diphosphate
TRIS = Tris(hydroxymethyl)-aminomethan

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same site (7).

A new approach to this problem is made possible by the findings in this paper. It is shown that the RuP_2 carboxylase and oxygenase activities are associated with two different enzymes. Spectroscopic studies indicate that the oxygenase contains copper, a result that might help in the search for specific inhibitors.

MATERIALS AND METHODS: RuP_2 carboxylase and oxygenase from parsley were prepared using TRIS-HCl buffer at pH 7.4 or 8.3, containing 2 mM β-mercaptoethanol. Fresh parsley (200 g) and 400 ml of 50 mM buffer was turmixed in a Waring blendor at highest speed for 60 s. The suspension was filtered through 4 layers of cheese cloth and centrifuged for 10 min at 28 000 x g. Solid $(NH_4)_2SO_4$ was added to the supernatant, and the precipitate obtained at 30 - 50% saturation was dissolved in 50 ml of 5 mM buffer. The solution was applied to a Sephadex G-25 column (8 x 50 cm) equilibrated with the same buffer. The eluted protein fractions were concentrated in an Amicon ultrafiltration cell, supplied with a PM 30 filter. The concentrate (10 ml) was applied to a Sepharose 6B column (3.5 x 100 cm) equilibrated with the same buffer and eluted with a rate of 30 ml/h. Preparations were also performed in which β-mercaptoethanol was omitted either in the last gel filtration step or in the whole procedure. In addition, RuP_2 carboxylase and oxygenase were prepared from sweet pepper at pH 8.3 without β-mercaptoethanol.

RuP_2, NADH, ATP, dithiothreitol and glyceraldehyde-3--phosphate dehydrogenase were purchased from Sigma Chemical Co. Sephadex G-25 and Sepharose 6B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Phosphoglycerate kinase from bakers' yeast was prepared as described by Arvidsson *et al.* (9) and was a generous gift from T. Hjelmgren. All other chemicals were of analytic grade. Buffers were prepared from deionized distilled water.

RuP_2 oxygenase activity was determined at pH 8.3 from the oxygen consumption measured with a Clark electrode. The enzyme was activated as described by Lorimer *et al.* (10), and 100 µl of the enzyme was added to 2.0 ml of 50 mM TRIS-HCl buffer, containing 20 mM $MgCl_2$. After about 10 min the rate of oxygen consumption was constant, and 100 µl of 5 mM RuP_2 was added. The increase in the rate of oxygen consumption immediately after the addition was noted. RuP_2 carboxylase activity was measured according to the method of Racker (11). Activated enzyme was added to 50 mM TRIS-HCl buffer at pH 8.3, containing 20 mM $NaHCO_3$, 20 mM $MgCl_2$, 1 mM NADH, 1 mM ATP, 1 mM dithiothreitol, 0.25 mM RuP_2, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase.

Ascorbate oxidase activity was measured as described by Dawson *et al.* (12).

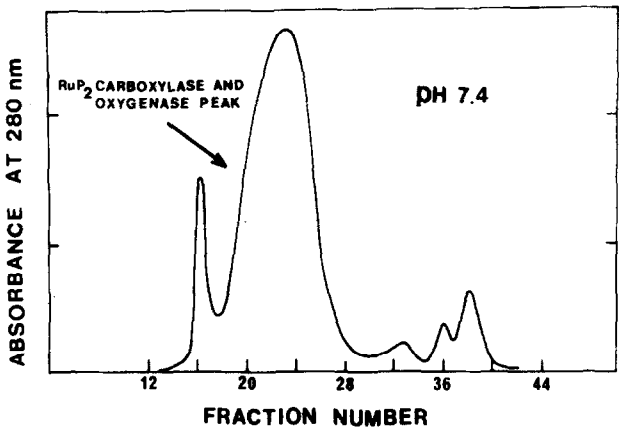

Fig. 1. Sepharose 6B gel filtration at pH 7.4 in the preparation of RuP_2 carboxylase and oxygenase from parsley. The buffer contained 2 mM β-mercaptoethanol. The absorbance at 280 nm is given in arbitrary units.

Total copper was determined with 2,2'-biquinoline (13). EPR spectra were recorded at 9.2 GHz and 77 K in a Varian E-3 spectrometer, and optical spectra were recorded in a Beckman Acta M IV spectrophotometer in a 1 cm cuvette.

RESULTS: For a preparation performed at pH 7.4 the elution pattern in the last gel filtration step is shown in Fig. 1. The RuP_2 carboxylase and oxygenase activities appeared in the same main peak in agreement with earlier reports (4). However, if the pH of the preparation was raised to 8.3, the RuP_2 carboxylase and oxygenase activities separated and a distinct shoulder was seen on the protein peak (Fig. 2).

If β-mercaptoethanol was omitted in the preparation the fractions in the shoulder in Fig. 2 were light green-blue and had the optical spectrum shown in Fig. 3. At pH 7.4 the same color was distributed over the entire main peak.

The EPR spectrum of the RuP_2 carboxylase and oxygenase peak from a preparation made at pH 7.4 in absence of β-mercaptoethanol consists of a mixture of several signals

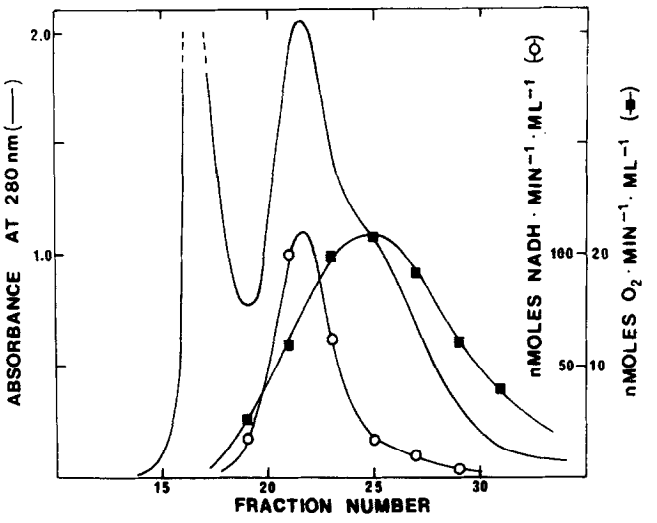

Fig. 2. Separation of the RuP_2 carboxylase and oxygenase activities from parsley on a Sepharose 6B column at pH 8.3. The buffer contained 2 mM β-mercaptoethanol.

but is mainly due to copper (Fig. 4A). However, the fractions in the shoulder obtained at pH 8.3 exhibit a cleaner spectrum (Fig. 4B) which is composed of two copper signals with about the same intensity and with hyperfine splitting constants 6.2 and 13.3 mT, respectively.

If the fractions in the shoulder seen in Fig. 2 were pooled and applied to a Sepharose 6B column in absence of β-mercaptoethanol the ratio between the RuP_2 oxygenase activity and the intensity of the copper signal seen in Fig. 4C is constant.

If ascorbate, dithiothreitol or β-mercaptoethanol was added to the samples of Fig. 3 and 4B the color and the narrow hyperfine signal disappeared within 30 s, while the other copper signal (Fig. 4C) was reduced more slowly with a half time of 5 - 10 min at 25° C. The addition of 1 mM RuP_2 resulted in a very slow disappearance of the color

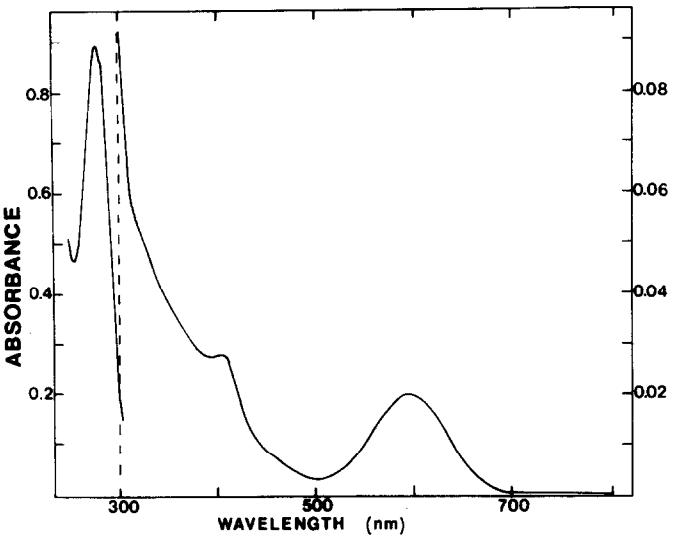

Fig. 3. Optical spectrum of the green-blue fractions from parsley eluted from a Sepharose 6B column at pH 8.3 and having high RuP_2 oxygenase activity. No β-mercaptoethanol was present in this preparation.

and the narrow hyperfine signal only.

The total amount of copper in the pooled fractions having RuP_2 carboxylase and oxygenase activity was about 0.2 g-atoms per mole of RuP_2 carboxylase, based on the previously given extinction coefficient at 280 nm for the spinach protein (4). The EPR spectrum in Fig. 4B corresponds to 20% of the total copper.

The molecular weight of the RuP_2 oxygenase was estimated to be 200,000 - 300,000 from the elution pattern on the Sepharose 6B column.

The shoulder in Fig. 2 includes a contaminant which constitutes more than half of the protein and which can be removed by $(NH_4)_2SO_4$ precipitation without affecting the spectroscopic properties and the oxygenase activity.

The relative ascorbate oxidase activity of the fractions

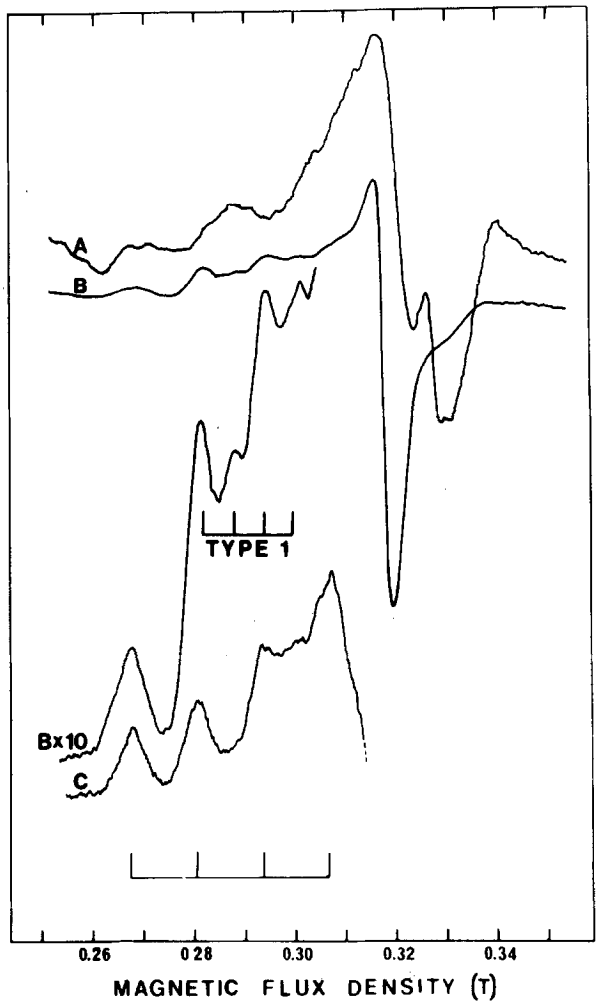

Fig. 4. EPR spectra at 77 K and 9.20 GHz of fractions eluted from a Sepharose 6B column in the preparation of RuP_2 carboxylase and oxygenase from parsley. No β-mercaptoethanol was present in the preparation.
(A) The main RuP_2 carboxylase and oxygenase peak obtained at pH 7.4.
(B) The green-blue fractions eluted at pH 8.3.
(C) Same as (B) but frozen 30 s after the addition of 20 mM ascorbate.
The modulation amplitude was 1 mT, and the gain settings were 1×10^5, 3.2×10^4 and 3.2×10^5 for (A), (B) and (C), respectively. The EPR parameters in (B) are for Type 1 Cu^{2+} $g_{\parallel}$ = 2.242 and $A_{\parallel}$ = 6.2 mT and for the other type of copper $g_{\parallel}$ = 2.269 and $A_{\parallel}$ = 13.3 mT.

with high RuP_2 oxygenase activity (Fig. 2) based on the copper EPR spectrum (Fig. 4B) was less than 3% of that of ascorbate oxidase.

In the preparations from sweet pepper the same characteristic green-blue protein component was obtained with a similar EPR spectrum as that of the protein from parsley.

DISCUSSION: From Fig. 2 it is evident that RuP_2 carboxylase and RuP_2 oxygenase are two different enzymes, which can be separated at high pH. In retrospect, there were several indications in earlier reports for the presence of two different enzymes. Storing of the protein prepared at lower pH caused a decrease in the carboxylase activity, while the oxygenase activity remained constant (4). The ratio between the oxygenase and carboxylase activities increased with the fraction number in the preparation (see Fig. 2 in ref. 4). These findings are now easily explained in terms of two different enzymes.

The blue color and the signal with the narrow hyperfine splitting which disappeared on reduction are both most likely originating from a so called Type 1 Cu^{2+} similar to the Type 1 Cu^{2+} found in the "blue" oxidases (14). The other signal has a hyperfine splitting constant which is intermediate to those of Types 1 and 2 Cu^{2+} in the "blue" oxidases (14).

However, both signals and the color followed the RuP_2 oxygenase activity which strongly suggests that the RuP_2 oxygenase contains copper like many other oxygenases (8). Although the amount of copper is small compared to that of the RuP_2 carboxylase, the oxygenase would still be present in considerable amounts in the chloroplasts of green plants.

For future work it is necessary to purify the RuP_2 oxygenase and more firmly establish the amount and properties

of its Cu. The results obtained so far indicate that specific, strongly binding inhibitors to the enzyme can be found which do not disturb the RuP_2 carboxylase activity. The photorespiration might thus be decreased and plant productivity increased.

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