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RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM PARSLEY

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SUMMARY

Ribulose-1,5-bisphosphate carboxylase/oxygenase from parsley leaves was purified by Sepharose 6B gel filtration at pH 8.3 as a single, colorless peak containing both activities. Approximately 0.2 g atom copper per mole enzyme was detected by atomic absorption spectroscopy, but this copper was not detectable by EPR spectrometry.

INTRODUCTION

Since the discovery of the oxygenase activity of ribulose-P_2 carboxylase/oxygenase (E.C. 4.1.1.39), much evidence has been accumulated which indicates that both the carboxylase and oxygenase activities are due to one enzyme. Therefore, the recent report (1) that the two activities could be separated by gel filtration on Sepharose 6B at pH 8.3, and that the oxygenase fraction was blue and contained copper required further investigation. Small and varying amounts of copper have been reported to be asociated with the A_8B_8 enzyme from spinach leaves: 1 g atom/mole (2), 0.11 to 0.14 g atom/mole (3), 0.2 g atom/mole (4), and in this report 0.18 g atom in the enzyme from parsley. An EPR spectrum of Cu(II) bound to the spinach enzyme has been published (2). This paper presents unsuccessful attempts to separate the oxygenase from the carboxylase activities by the procedure of Brändén (1). The specific activity of ribulose-P_2 carboxylase/oxygenase prepared from parsley was similar to that obtained from spinach or tobacco leaves.

MATERIALS AND METHODS

Ribulose-P_2 carboxylase/oxygenase was prepared from the leaves of parsley (from local grocery store) according to Brändén (1). One hundred g of

Abbreviations: Ribulose-P_2 for ribulose-1,5-bisphosphate.

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tissue was ground in a Waring blender at high speed for 60 s with 200 ml of 50 mM Tris-HCl at pH 8.3 and 4°. This buffer was prepared in 2 ways for different experiments; in one the pH was adjusted at 4°, the other at room temperature (22-24°) according to Brändén (personal communication). The homogenate was filtered through several layers of cheese cloth and centrifuged at 11,000xg for 30 min. The supernatant was filtered through Miracloth and solid $(NH_4)_2SO_4$ was added slowly, with constant stirring, to 30% saturation (16.4 g/100 ml). The suspension was stirred for 15 min, and stood for 45 min at 4° before centrifuging at 11,000xg for 40 min. This precipitate was discarded, and the supernatant was made 50% saturated with solid $(NH_4)_2SO_4$ (11.8 g/100 ml) to precipitate the enzyme. The stirring, standing, and centrifugation were repeated. The 30-50% $(NH_4)_2SO_4$ precipitate was resuspended in 25 ml or less of buffer. In one case the buffer was 50 mM Tris, pH 8.3 (adjusted at 4°), and in the second case the buffer was 5 mM Tris, adjusted at room temperature to pH 8.3. Usually the resuspended and redissolved precipitate was clarified by centrifugation at 25,000xg for 15 min. The supernatant was filtered through a Sephadex G-25 (medium), 6 x 25 cm column, which had been previously equilibrated with the resuspension buffer. The peak fractions, as determined by absorbance at 280 nm, were pooled and concentrated to 5-7 ml with a PM 30 membrane in an Amicon ultrafiltration chamber, pressurized with argon at 60 psi. The concentrate was chromatographed on a Sepharose 6B column (2.6 x 90 cm) equilibrated with the same resuspension buffer. All steps were carried out at 4°. The entire procedure was repeated with 2 mM β-mercaptoethanol in all buffers. The spinach enzyme was prepared by our usual procedure (5).

Ribulose-P_2 was prepared enzymatically (6). Sephadex G-25 and Sepharose 6B were from Pharmacia Fine Chemicals, $NaH^{14}CO_3$ from Amersham and other reagents from Sigma.

The enzyme was activated by adjusting preparations to 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ and incubating them at 30° for at least 10 min prior to assaying. The carboxylase assays were run at 30° for 1 min and initiated by the addition of 10 µl aliquots of activated enzyme to 240 µl of 100 mM N,N-bis-(2-hydroxyethyl)glycine (Sigma, Bicine) at pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$, 1 mM dithiothreitol, and 0.5 mM ribulose-P_2. The ^{14}C acid stable product was measured by scintillation counting. The oxygenase assay was based on the ribulose-P_2 dependent oxygen uptake in a Rank Brothers oxygen electrode. The reactions were normally initiated by the addition of 20 µl of activated enzyme to 480 µl of 100 mM N,N-bis(2-hydroxyethyl)glycine at pH 8.2, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM ribulose-P_2. The reactions were monitored for 50-60s at 30°. Assays were also run according to Brändén (1) by initiating with ribulose-P_2, after a 5 to 10 min incubation of the enzyme in the assay buffer in the oxygen electrode chamber.

A Varian Model 175 Spectrophotometer was used for the atomic absorption spectroscopy using copper sulfate as a standard. EPR spectra were recorded at 77K with a Varian E-4 spectrometer operating at 9.22 GHz; microwave power, 20 mwatts; modulation frequency, 100 KHz; modulation amplitude, 5 gauss; field set, 2900 gauss; field sweep, 2000 gauss; time constant, 0.1 sec; instrumental gain, 2×10^3.

RESULTS AND DISCUSSION

Ribulose-P_2 carboxylase and ribulose-P_2 oxygenase activity from parsley leaves chromatographed as a single peak on Sepharose 6B at 4° in 50 mM Tris

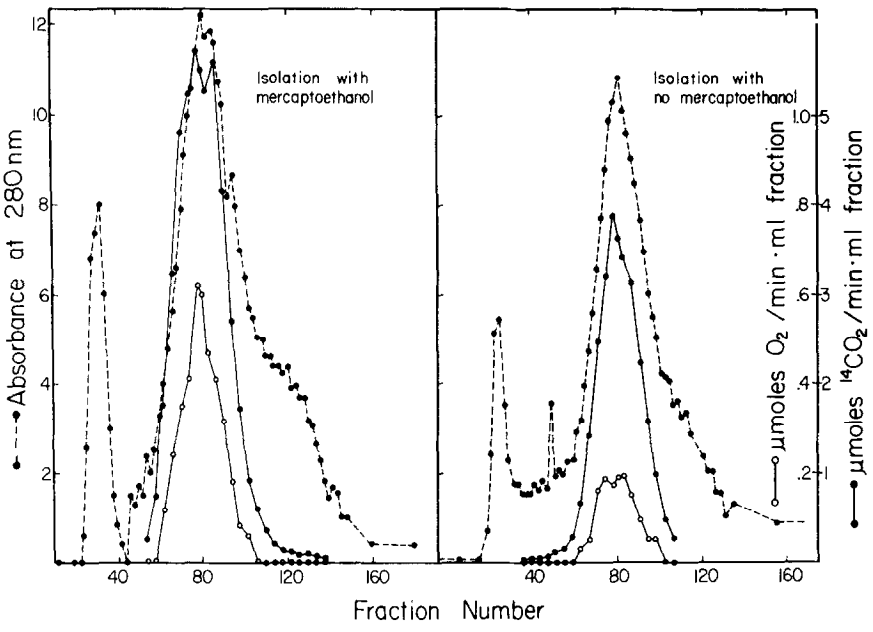

Figure 1. Separation of ribulose-P_2 carboxylase/oxygenase from parsley leaves on a sepharose 6B column. The buffer was 50 mM Tris adjusted to pH 8.3 at 4°.

buffer at pH 8.3 (Fig. 1). Similar results were obtained with or without 2 mM β-mercaptoethanol. These procedures and results are similar to those previously observed for enzyme for spinach or tobacco leaves (5), as well as parsley (Brändén, personal communication). To separate the two activities Brändén used 5 mM Tris, adjusted to pH 8.3 at room temperature, for the Sepharose 6B filtration step. This modification of the procedure with or without β-mercaptoethanol, did not result in the separation of the carboxylase/oxygenase activities in our experiments (Fig. 2). When the clarification step preceding the G-25 column was omitted (Methods), to bring the present procedure in line with that in reference 1, a shoulder absorbing at 280 nm, presumably nucleic acids, preceded the enzyme activity off the Sepharose 6B column (Fig. 2). This omission had no influence on the subsequent distribution of the carboxylase and oxygenase activities.

Brändén's oxygenase assays were plagued by a substantial endogenous rate of oxygen consumption which was not dependent on the presence of

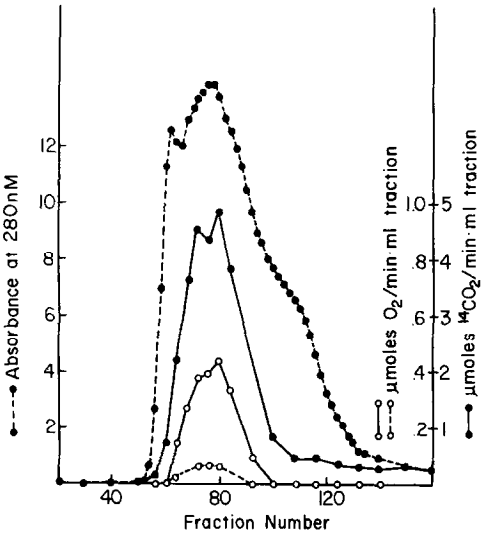

Figure 2. Separation of ribulose-P_2 carboxylase/oxygenase from parsley leaves on a sepharose 6B column. The buffer was 5 mM Tris adjusted to pH 8.3 at 22°. The oxygenase assays were initiated by the addition of activated enzyme to the assay mix containing ribulose-P_2 (o—o) or alternatively by the addition of ribulose-P_2 to oxygenase preincubated in the assay mix for 10 min (o- -o).

ribulose-P_2. This forced him to use a 5-10 min preincubation of the enzyme in the assay buffer before initiation of the reaction with ribulose-P_2. With partially purified enzyme preparations from spinach, tobacco, and now parsley, we did not observe an endogenous rate of O_2 uptake. Brändén (personal communication) has attributed his endogenous rate to oxidation of the dithiothreitol in the activating and assay buffers. Copper has been reported to accelerate the oxidation of dithiothreitol (7), but addition of small amounts of Cu(II) to our system did not produce a significantly faster endogenous rate, so we are at loss to explain his endogenous rate in the assay.

In our experiments ribulose-P_2 oxygenase assays were initiated by addition of an aliquot of the concentrated, activated enzyme into an assay buffer without $NaHCO_3$. An initially linear, substrate and enzyme dependent rate of O_2 uptake was measured over the first 30 s. Without substrate or before adding enzyme the endogenous rate was zero. If a

purified enzyme from spinach leaves was first activated by preincubation with 10 mM $NaHCO_3$ and $MgCl_2$, and then assayed for oxygenase activity at various times after its addition to the assay buffer, rapid inactivation occurred with a half time of approximately 1 min (data not shown). This rapid decline in activity has been analyzed by Laing and Christeller (8) and Lorimer et al. (9) and attributed to a dissociation of the active enzyme-CO_2-Mg^{2+} complex in the final assay mixture which contained only 0.4 mM $NaHCO_3$ from carryover in the aliquot of activated enzyme in 10 mM $NaHCO_3$. Performance of the oxygenase assay in the manner described by Brändén (1) resulted in much lower activites due to dissociation of the activated complex. Thus 10 min after dilution of the activated enzyme from parsley into the assay buffer, the oxygenase activity was severely reduced (o--o in Fig. 2). Nevertheless, this small amount of remaining ribulose-P_2 oxygenase coincided with the ribulose-P_2 carboxylase peak from the Sepharose 6B column.

Additional observations by Brändén (1) were a blue color in the fractions with oxygenase activity and a strong EPR signal characteristic of "blue" oxidases. Our atomic absorption spectroscopy revealed trace amounts of copper, 0.18 g atom/mole enzyme, in the peak fractions (assuming a molecular weight of 550,000) and a similar amount of copper per mg protein in the shoulder fractions. No EPR signal characteristic of copper was found in any of the Sepharose 6B column fractions with carboxylase/oxygenase activity that contained approximately 10 μM copper by atomic absorption, even though the limits of detection in the EPR experiments were estimated to be between 1-2 μM for Cu(II). Addition of 1 mM ferricyanide to convert Cu(I) to Cu(II) was without effect on the EPR spectrum. Thus it seems that any Cu(I) present in the protein samples was inaccessible to ferricyanide or that the copper was EPR inactive. Cu(II) (10 μM) was added to fractions from Fig. 2, but it had no effect on the rate or on the location of the oxygenase activity.

In conclusion, ribulose-P_2 carboxylase/oxygenase from parsley leaves seems to be a single protein similar to that from spinach and tobacco leaves. Brändén's (1) reported separation of these two activities has not been confirmed. We also emphasize that in the ribulose-P_2 oxygenase assay, the rate must be determined quickly before the enzyme is inactivated in the absence of CO_2. The difference in the copper content of the preparations may be in the source of parsley. In view of the instability of ribulose-P_2 (10), a nonspecific copper-protein complex might also oxidize this substrate. Until the mechanism of action and the cofactor requirement for ribulose-P_2 oxygenase are known, a continuing concern about copper in the enzyme can be expected.

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