Properties of Ribulose-1,5-Diphosphate Carboxylase (Carboxydismutase) From Chinese Cabbage and Photosynthetic Microorganisms

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Abstract. Ribulose-1,5-diphosphate carboxylase (carboxydismutase) was prepared from Chinese Cabbage [Brassica peTsai (Parl.)] and the $K_m$ values and molecular weight were determined. These parameters were found to be in good agreement with values reported for this enzyme from other higher plants. Investigation of carboxydismutase activity from the photosynthetic micro-organisms Chlamydomonas reinhardi (IU 89+), Plectonema boramum (IU 894), and Chromatium strain D showed striking similarity to the higher plant enzyme, when the sedimentation coefficients were compared.

It is now well established that carboxydismutase (ribulose-1,5-diphosphate [RUDP] carboxylase) is the enzyme primarily responsible for photosynthetic "CO$_2$" fixation in many species of plants [(3); for notable exceptions see Slack and Hatch (20) and Evans et al. (6)]. The unique features of the higher plant enzyme which distinguish it from other chloroplast soluble proteins are its high sedimentation coefficient of approximately 18 S and its molecular weight of approximately 500,000. Fraction I protein (a soluble component of higher plant cytoplasm) exhibits these same properties (5,19), and recent evidence strongly supports the conclusion that Fraction I protein and carboxydismutase are identical (23,24).

The experiments of a number of investigators (7,9,17,21) have established the existence of carboxydismutase in a number of photosynthetic microorganisms, including blue-green algae and photosynthetic bacteria. No information relating to the molecular nature of this enzyme was reported in these studies and only recently have physical properties of carboxydismutase from such sources been reported (1,11,14).

It was our purpose, therefore, to characterize carboxydismutase from 3 photosynthetic microorganisms (blue-green alga, green alga, and photosynthetic bacterium) and to compare these properties with those of the higher plant enzyme. In the work described below striking similarities in S values (and in 1 case in $K_m$ values) to the higher plant enzyme were found with all of the photosynthetic microorganisms tested. Further characterization of carboxydismutase from Chinese cabbage, which was the subject of an earlier report (10), is described. The $K_m$ values, S, and molecular weight are in good agreement with values for carboxydismutase from other plants (23,24,26).

Materials and Methods

Carboxydismutase was prepared and purified from Chinese cabbage [Brassica peTsai (Parl.)] using a procedure similar to Trown's (24) except that whole leaves rather than chloroplasts were used as starting material. All operations were carried out at 0 to 5°. The leaves were harvested at various stages of growth, were washed with distilled water, and the midribs were removed. They were then ground in a meat grinder with 2 times their weight of a pH 7.5 extraction buffer (0.2 M tris-SO$_4$, 1 mM GSH, and 1 mM EDTA). This slurry was then transferred to a Waring Blender and blended for 3 minutes at top speed. The homogenate was then filtered through 4 to 8 layers of cheesecloth and the filtrate brought to 35% saturation by addition of solid (NH$_4$)$_2$SO$_4$; the pH was adjusted to approximately 7 with 2 N NH$_4$OH. After centrifugation of this suspension for 20 minutes at 8000 rpm in the Servall GSA rotor, the resulting pellet was discarded; the supernatant was brought to 50% saturation by addition of solid (NH$_4$)$_2$SO$_4$, and the pH adjusted as described. This was cen...
trifuged as described above, and the supernatant discarded. The 35 to 50% \((\text{NH}_4)_2\text{SO}_4\) pellet is crude carboxydismutase. After being dissolved in 0.05 M tris-\(\text{SO}_4\), 1 mM GSH (pH 7.5) the crude enzyme was reprecipitated 3 times by bringing the solution to 50% saturation of \((\text{NH}_4)_2\text{SO}_4\) re-dissolved in 0.05 M tris-\(\text{SO}_4\), 1 mM GSH (pH 7.5) and dialyzed against the same buffer.

This preparation was then purified by chromatography on Sephadex G-200. Generally, a 3 to 5 ml zone containing 200 to 300 mg of protein was layered on a column of dimension 25 cm \(\times\) 100 cm and eluted with a buffer (0.05 M tris-\(\text{SO}_4\), pH 7.4: 0.2 M \((\text{NH}_4)_2\text{SO}_4\); 1 mM EDTA; and 1 mM 2-mercaptoethanol) used by Trown (24). Five ml fractions were collected and assayed for carboxydismutase activity and the absorbancy at 280 \(\mu\)m was determined. On some fractions phosphoribosylisomerase activity was also assayed using the procedure of Axelrod and Jang (2).

Carboxydismutase activity was assayed according to a modification of the procedure of Trown (24) in a system of volume 0.20 ml containing the following components: tris-\(\text{Cl}\), pH 8.0, 0.1 M RUDP, 0.5 mM; \(\text{NaH}^+\text{CO}_3\), 50 mM; GSH, 1 mM; EDTA, 50 \(\mu\)m; \(\text{MgCl}_2\), 10 mM and varying amounts of protein, usually approximately 10 \(\mu\)g. After incubation at 25° the reaction was terminated by addition of 0.05 ml of 6 N CH\(_3\)COOH. The reaction mixture was plated on a planchet, dried in a 100° oven, and counted in a Beckman “Low Beta” gas flow counter at an efficiency of approximately 20%. All results are uncorrected for counting efficiency, except where otherwise noted. Appropriate controls lacking the substrate RUDP were also performed.

Protein concentrations were determined by the Lowry method (12) using bovine serum albumin (BSA) or lysozyme as standards. In the case of purified preparations, the concentration was determined from the absorbancy at 280 \(\mu\)m assuming the extinction coefficient to be the same as that determined by Trown (24) for spinach carboxydismutase. i.e., at a concentration of

\[
1 \text{ mg/ml} = 1.41.
\]

The \(K_m\) values were determined by varying the concentration of a substrate over a 10 to 100 fold range while maintaining the other substrate at saturating concentration (0.5 mM for RUDP and 0.05 M for NaHCO\(_3\)). Computer analyses of the data were performed which fitted a least square line to the slope-intercept form of the Lineweaver-Burk equation. We thank Dr. D. McMahon for performing these analyses.

Polyacrylamide gel electrophoresis was performed on Canalo apparatus according to the methods of Davis (4), with slight modification. No spacer gel was used; the sample containing 30 \(\mu\)g of protein in 20 \(\mu\)l of 0.05 M tris-\(\text{SO}_4\), 1 mM mercaptoethanol, 0.1 M EDTA, 20% sucrose was layered directly over the analyzer gel with a micropipette. The 7 and one-half percent gel was run in the tris-glycine buffer (pH 8.9) of Davis (4). The 3% gel was run in 0.05 M tris-\(\text{SO}_4\), 1 mM mercaptoethanol, 0.1 M EDTA (pH 7.5). The gels were formed in tubes of inner diameter 5 mm. After electrophoresis, the gels were stained with 1% naphthol blue-black in 7% acetic acid for 1 to 2 hours, and then destained electrophoretically at 15 mA per gel.

Sedimentation equilibrium and sedimentation velocity experiments were performed in a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics and electronic speed control. Measurement of fringe displacement in sedimentation equilibrium experiments were made with a Nikon 6C microcomparator.

Glycerol gradient centrifugation was performed by layering 1 ml of soluble protein on a 5 to 30% glycerol gradient in 0.05 M tris-\(\text{SO}_4\), 1 mM GSH (pH 7.5) and centrifuging for 20 to 24 hours at 0 to 4° at 24,000 rpm in the SW 25.1 rotor in the Spinco Model L or L-2 centrifuge. One ml fractions were collected and assayed for carboxydismutase as described and the absorbancy at 280 \(\mu\)m determined.

Wild type \(C. \text{ reinhardtii} \) (IU89+) was grown under constant illumination on a rotary shaker in the high salt minimal (HSM) medium of Sueoka (22). \(Plectonema \text{ boryanum} \) (IU594) was grown using techniques described by Luftig and Haselkorn (13). \(Chlamydomonas \text{ strain D} \) was kindly supplied (as frozen cells) by Dr. M. Kamen and was grown autochrophically or heterotrophically as previously described (8). IU etc. designates the Indiana University Algal culture collection number.

Crude soluble protein extracts from these organisms were prepared from log phase cells using the following procedure. After harvesting by centrifugation, the cells were washed once with 0.05 M tris-\(\text{SO}_4\) (pH 7.4), and weighed. After resuspension in 2 ml of tris buffer per g wet weight of cells, the cells were broken by sonication at 1 minute intervals for a total of 10 minutes at setting No. 5 of a Bronson sonifier at 4°. Cell breakage was monitored by microscopic examination. The crude lysate was centrifuged at 12,000 rpm in the SS 34 rotor of the Servall centrifuge for 10 minutes and the pellet discarded. The supernatant from this centrifugation was used as the crude enzyme extract in the experiments described.

Results

Chinese Cabbage Carboxydismutase. The first series of experiments described elucidate some of the properties of carboxydismutase from Chinese cabbage leaves. The protein used in these experiments was purified using a procedure similar to Trown’s (24) with the exception that only 1 Sephadex G-200 step was used. It was found in agree-
ment with Trown that all of the carboxydismutase eluted in the void volume and was coincident with a 280 nm absorbancy peak. The fraction containing highest enzyme activity (specific activity of 0.031 
\( \mu \)mole NaHCO₃ fixed per min per mg protein) showed a single symmetrical peak in the analytical ultracentrifuge with an \( S_{20,w} = 17.0 \) at a concentration of 5 mg/ml. The ratio \( A_{280}/A_{400} \) for this preparation was 1.82. Assay for phosphoriboisomerase indicated 0.2 % (w/w) of this enzyme or \( \approx 1 % \) of the total protein on a molar basis assuming a molecular weight of 100,000 for the isomerase and 500,000 for carboxydismutase. These characteristics are similar to those of plant carboxydismutases described by others (23, 24, 26).

Figure 1 shows the pattern obtained upon electrophoresis of this preparation on polyacrylamide gel at 2 pH values. In both gels a major component is seen to migrate towards the anode, followed by a minor component. The position of the minor component is similar to that described for the dimer of carboxydismutase by Ridley et al. (18) and by Trown (24).

It was of interest to compare the molecular weight of this preparation with those previously described by Trown (24) for spinach carboxydismutase and by Thornber et al. (23) for the spinach beet enzyme. For this purpose the high speed sedimentation equilibrium method of Yphantis (27) was used.

**Figure 1.** Polyacrylamide gel electrophoresis patterns of Sephadex purified carboxydismutase from Chinese cabbage. The left-hand 7 and one-half percent gel was run at pH 8.9, the right-hand 3 % gel run at pH 7.5. In both gels the anode was at the bottom of gel. The samples were applied directly to the top of each gel, and electrophoresis was carried out for approximately 60 minutes at 5 ma per gel at room temperature.

**Figure 2.** Sedimentation equilibrium plot for Sephadex purified carboxydismutase from Chinese cabbage. The data were plotted from photographs taken 24 hours after attainment of equilibrium. Initial protein concentration was 0.16 mg/ml, the speed 9000 rpm, the temperature 22.2°C. The slope of 1.0456, \( v = 0.730 \) ml/g, \( R = 8.31 \times 10^9 \) ergs deg⁻¹ mole⁻¹, and \( \rho = 1.010 \), was used in calculation of \( M_e = 511,000 \) daltons.

In figure 2 is shown a plot of log \( \Delta y \) vs \( x^2 \) for the preparation of carboxydismutase described above. The slope \( d \log \Delta y/dx^2 = 1.0456 \) was used to calculate the weight average molecular weight (\( M_w \)) by use of the derivative form of the sedimentation equilibrium equation:

\[
\frac{1}{M_w} = \frac{2}{RT} \frac{d \ln \Delta y}{(1 - \frac{v}{\rho})\omega^2 dx^2}
\]  

(1)

where \( \Delta y \) is the vertical fringe displacement and is proportional to \( c \) at some point in the cell.

Using \( v = 0.730 \) ml/g determined for spinach carboxydismutase by Trown (24), \( M_w = 511,000 \) is obtained for Chinese cabbage carboxydismutase. This value is in good agreement with Trown's (24) value of 515,000 for spinach carboxydismutase, but is somewhat lower than the value of 565,000 determined for the spinach beet enzyme by Thornber et al. (23).

As another means of comparison the \( K_m \) values for the Chinese cabbage enzyme were determined.
The values were determined over a hundred-fold range of substrate concentration. The rates were determined previously to be in the linear range with respect to [E]. The following values were obtained from Lineweaver-Burk plots: \( K_m \) (RUDP) = \((1.0 \pm 0.1) \times 10^{-4}\) M, and \( K_m \) (NaHCO_3) = \(0.03 \pm 0.01\) M. These values are in good agreement with the values obtained for spinach carboxydismutase by Weissbach et al. (26) of \(2.5 \times 10^{-4}\) M for RUDP and \(1.1 \times 10^{-2}\) M for NaHCO_3.

Algal Carboxydismutase. The next series of experiments describes some properties of carboxydismutase in crude extracts of some photosynthetic microorganisms. It was decided that the simplest method of comparison to the higher plant enzyme would be measurement of \( S \) by the use of a glycerol gradient. In the case of Chromatium a somewhat more extensive study was carried out. In the glycerol gradient experiments the 18 S region was established by centrifugation of purified Chinese cabbage enzyme under identical conditions.

Figure 3 shows a glycerol gradient fractionation of a crude extract prepared from C. reinhardtii (IU 89+) grown on HSM. All of the carboxydismutase activity sediments as a narrow zone in the region of the gradient corresponding to 16 to 18 S and is coincident with a 280 \(\mu\) absorbancy peak. After treatment of the extract with EDTA and pancreatic RNase a component with \(S_{280},w = 16\) is observed in the model E analytical ultracentrifuge. Having established that the \( S \) of carboxydismutase in this eucaryote is 16 to 18 S, it was instructive to perform the same experiment with a blue-green alga, a procaryote whose cellular morphology resembles that of a higher plant chloroplast. The other procaryote chosen for experimentation was Chromatium strain D, well studied in bacterial photosynthesis.

A crude extract was prepared from log phase cells of P. boryanum (IU 594), a filamentous blue-green alga. After centrifugation on a 5 to 30 % glycerol gradient and assay for carboxydismutase activity as described in Materials and Methods, the results obtained are presented in figure 4. The enzyme activity is seen to sediment with an \( S = 16 \) to 18 and this component accounts for all of the activity in the gradient. The large 280 \(\mu\) absorbancy peak is probably due to phycoeyanin, since a blue band was also observed in this region of the gradient. As in the case of C. reinhardtii the \( S \) of the carboxydismutase activity is quite similar to that of higher plants.

Bacterial Carboxydismutase. We turn next to the carboxydismutase from Chromatium strain D. In initial experiments crude extracts were prepared from autotrophically and heterotrophically grown cells. Typical values obtained for the specific activity of carboxydismutase in these extracts were as follows: autotrophically-grown: \(0.087 \mu\)moles HCO_3 fixed per mg protein per 10 minutes at 29\(^\circ\); heterotrophically-grown: \(0.048\) units per mg; these differences are in agreement with the work of...
Table I. Fractionation of Chromatium Crude Extract With (NH₄)₂SO₄

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.087</td>
</tr>
<tr>
<td>0-20 % (NH₄)₂SO₄</td>
<td>0.031</td>
</tr>
<tr>
<td>20-40 %</td>
<td>0.021</td>
</tr>
<tr>
<td>40-60 %</td>
<td>0.083</td>
</tr>
<tr>
<td>60-80 %</td>
<td>0.51</td>
</tr>
</tbody>
</table>

¹ μmoles HCO₃⁻ fixed/mg protein/10 min, 29⁰.

Fuller et al. (8). The following experiments were performed with autotrophically-grown cells.

Table I shows the results of (NH₄)₂SO₄ fractionation. The highest specific activity is seen to precipitate in the 60 to 80 % saturated fraction. When a 50 to 80 % (NH₄)₂SO₄ fraction is subjected to glycerol gradient centrifugation the result shown in figure 5 is obtained. All of the carboxydismutase is found to sediment in the 16 to 18 S region and is coincident with a 280 μ absorbancy peak. The fractions containing the peak of activity were concentrated by precipitation with 80 % (NH₄)₂SO₄, redissolved in 0.05 M tris SO₄, pH 7.5, and dialyzed against the same buffer. This preparation shows a typical protein spectrum with λ_max = 278 μμ and a λ_min = 250 μμ and A_280/A_260 = 1.50. Gel electrophoresis at pH 9 showed the presence of 1 major band and 5 to 6 minor bands migrating toward the anode. Table II summarizes the purification of the enzyme used for the determination of Kₘ values. The low recovery could be due to inactivation of the enzyme during purification or to the existence of forms of the enzyme which do not precipitate in this (NH₄)₂SO₄ fraction. If these forms exist, they do not differ greatly in size since all of the carboxydismutase activity in a crude extract also sediments in the 16 to 18 S region of a 5 to 30 % glycerol gradient.

Table II. Purification of Chromatium Strain D Enzyme

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein</th>
<th>Total units¹</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>440 mg</td>
<td>33.0 units</td>
<td>0.075</td>
</tr>
<tr>
<td>50-80 % (NH₄)₂SO₄</td>
<td>49.2</td>
<td>14.9</td>
<td>0.30</td>
</tr>
<tr>
<td>5-30 % Grad. + 80 % (NH₄)₂SO₄</td>
<td>2 mg</td>
<td>16 units</td>
<td>0.78</td>
</tr>
</tbody>
</table>

¹ 1 Unit = 1 μmole per 10 min. at 29⁰.

The Kₘ values were determined in a range where ν was directly proportional to [E]. Figures 6 and 7 present the data in Lineweaver-Burk form for the 10-fold purified chromatium enzyme. The following values were obtained: Kₘ (RUDP) = (4.0 ± 1.0) × 10⁻⁴ M; Kₘ (NaHCO₃) = (9.0 ± 5.0) × 10⁻¹ M. These values are of the same order of magnitude as those for R. rubrum determined by Anderson et al. (1). This enzyme is quite similar in the properties studied to that of C. reinhardii, P. boryanum, and higher plants (Chinese cabbage, spinach beet, spinach) but is quite different with respect to size from the enzyme isolated from R. rubrum.

Discussion

The procedure used to purify carboxydismutase from Chinese cabbage yielded a preparation relatively free of phosphoribosomerase and of carboxydismutase dimer. The Kₘ values for RUDP and NaHCO₃ of (1.0 ± 0.1) × 10⁻¹ M and 0.03 ± 0.01 M respectively are in good agreement with values reported for this enzyme from other higher plant sources (26). The molecular weight of the Chinese cabbage enzyme of 511,000 is in good agreement with the value of 515,000 for the spinach enzyme as reported by Trown (24). It differs by about 10 % from the value of 565,000 determined for the spinach beet enzyme by Thornber et al. (18, 23).

The similarities in physical properties of carboxydismutases from higher plants are not surprising in light of the early work on Fraction I protein by Wildman and co-workers. These investigators showed the existence of Fraction I protein (19) in
the cytoplasm of a number of higher plants. Dorner et al. (5) showed the existence of this protein not only in higher plants but in every major phylum. More recently van Regenmortel (25) has prepared antisera to Fraction I protein from marrow and has tested soluble protein extracts from a variety of higher plants for reaction with this antiserum. He demonstrated reaction of 33 higher plant extracts with marrow anti-Fraction I serum, indicating wide spread occurrence and similarity of this protein in higher plant species.

Studies on carboxydismutase within a given species using varieties of *Mimulus cardinalis* adapted to growth at different altitudes have been performed by McMahon (15, 16). Although the *Km* values for NaHCO₃ vary, preliminary evidence from Sephadex gel chromatography suggests the same molecular weight for all the enzymes tested. This molecular weight is similar to that of other well studied higher plant carboxydismutases (approx. 500,000). It is clear then that all of the higher plants studied exhibit properties for carboxydismutase which are strikingly similar, i.e. S ≈ 18 and molecular weight ≈ 500,000.

Since there was little evidence to suggest that the same situation would appear in the photosynthetic microorganisms it was quite interesting to find that all of the organisms tested (*C. reinhardtii, P. boryanum*, and *Chromatium*) showed a 16 to 18 S carboxydismutase activity. Assuming a globular molecule (which preliminary electron microscopic examination of *C. reinhardtii* carboxydismutase substantiates) the molecular weight of carboxydismutase in these organisms would be approximately 500,000 as in the case of the higher plant enzyme. Preliminary experiments using antiserum prepared to Chinese cabbage carboxydismutase also indicate some similarities between the higher plant enzyme and the enzyme from these sources.

Unlike the situation in higher plants and in the photosynthetic microorganisms studied here, notable differences exist in the size of carboxydismutase from autotrophic and heterotrophic bacteria. While this work reports an 18 S enzyme from *Chromatium*, Anderson et al. (1) have reported a 6 S (MW ≈ 110,000) enzyme from *Rhodospirillum rubrum*. Very recent reports indicate a 500,000 molecular weight enzyme from *Thiobacillus neapolitanus* (14) while in *Hydrogenomas facilis* the molecular weight is approximately 200,000 (11).

While no conclusions can be drawn as to the size distribution of carboxydismutase in the algal and photosynthetic (and autotrophic) bacteria, the enzymes from these sources will provide useful material for the study of the structure of this enzyme. Studies of a greater number of algae will provide an interesting approach to the evolution of CO₂ fixation in photosynthetic organisms.

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**Fig. 6.** (left) Lineweaver-Burk plot for NaHCO₃ using *Chromatium* carboxydismutase. Four μg of protein were used in the carboxydismutase assay; *v* is expressed as cpm fixed per 10 minutes at 29°C.

**Fig. 7.** (right) Lineweaver-Burk plot for RUDP using *Chromatium* carboxydismutase. Four μg of protein were used in the carboxydismutase assay; *v* is expressed as cpm fixed/10 minutes at 29°C.
Acknowledgments

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Literature Cited