Photosynthesis in *Rhodospirillum rubrum*

IV. ISOLATION AND CHARACTERIZATION OF RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE*

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SUMMARY

Ribulose 1,5-diphosphate carboxylase has been isolated from autotrophically cultured *Rhodospirillum rubrum*. The molecular weight is 120,000. The *Km* for ribulose 1,5-diphosphate is 83 mM, and for CO₂ is 59 mM. The enzyme is inhibited by three important metabolites: citrate, an intermediate of the tricarboxylic acid cycle; inorganic phosphate; and 3-phosphoglyceric acid, the product of the reaction catalyzed by the carboxylase. Both the levels and the activity of ribulose 1,5-diphosphate carboxylase are apparently subject to metabolic control in this facultative photoautotroph.

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Metabolic experiments—Enzyme, purified through the hydroxypatite step, was made 1 mM in EDTA (pH 7.6), allowed to stand for 13 min at 0°C, and passed through a Sephadex G-25 column (2.5 X 30 cm) in metal-free Tris-HCl, pH 8.1. The Sephadex G-25 (100 to 300 μm) had been packed for 5 days in 1 mM EDTA and washed with metal-free Tris-HCl. All glassware used in the experiment was rinsed with fuming nitric acid. The components of the reaction mixture were the same as those used in the 14C assay except that NaH14CO3 was omitted. At 1-min intervals, 100-μl samples were removed into 100 μl of 2 mM potassium phosphate, pH 7.5, in a boiling water bath. After 1 min, samples were removed, chilled, and assayed for 3-P-glyceric acid by the use of the usual components of the spectrophotometric assay system, except that ribulose-1,5-di-P, NaHCO3, and ribulose-1,5-di-P carboxylase were omitted. Identical activity curves were obtained in the presence and absence of added MgCl2 (10 mM). The Mg2+ concentration was 100 μM in the absence of added MgCl2.

Purification of enzyme—All operations were carried out at 0-4°C. Cells, fresh or frozen, were suspended in sufficient 10 mM potassium phosphate (pH 7.6) to give 20 to 40 mg of protein per ml, and released from 12,000 p.s.i. through a French pressure cell. The extract was centrifuged for 10 min at 10,000 x g, and the supernatant solution was further centrifuged for 25 min at 225,000 x g. Then 0.1 volume of 0.5 M McIlvaine was added to the supernatant, the solution was allowed to stand for 3 hours, and the precipitated nucleic acids were removed by centrifugation for 10 min at 10,000 × g. Solid, metal-free (NH4)2SO4 was added to the supernatant solution, and the fraction precipitating between 40 and 55% saturation was collected by centrifugation for 10 min at 10,000 × g. The precipitate was made to 2 ml with 10 mM sulfonate buffer (pH 7.6) containing 0.5 mM dithiothreitol and was layered onto a Sephadex G-200 column (2.5 X 30 cm, 40 to 120 μl) that had been equilibrated with 10 mM sulfonate buffer (pH 7.6) and 0.5 mM dithiothreitol. Tubes containing peak enzyme activity were combined and placed on a hydroxyapatite column (2.2 X 1.9 cm). The carboxylase was eluted with 3 mM potassium phosphate (0.5 mM dithiothreitol, pH 7.6). The results of a typical purification are given in Table I. Except in the hydroxyapatite step, 5 mM 2-mercaptoethanol could be substituted for 0.5 mM dithiothreitol; the enzyme seemed somewhat more stable in the presence of 2-mercaptoethanol.

Activity was retained in frozen crude extracts (225,000 × g supernatant) for several months and for several days at 0°C following ammonium sulfate fractionation. The enzyme purified through the hydroxyapatite step was denatured by freezing (varying amounts of activity up to 80% remained). Activity was lost only slowly from solution at 0°C, provided that 2-mercaptoethanol or glutathione was present.

Reagents—Barium ribulose-1,5-di-P, ATP, Tris, DPNH, and metal-free (NH4)2SO4 were obtained from Sigma. Sodium ribulose-1,5-di-P was prepared as previously described (7). Concentration of ribulose-1,5-di-P was determined by the use of excess purified ribulose-1,5-di-P carboxylase and limiting ribulose-1,5-di-P in the spectrophotometric assay. Glutathione, glyeerolaldehyde-3-P dehydrogenase, 3-phosphoglyceric acid kinase, and hydroxyapatite powder were obtained from Calbiochem. Sephadex G-200 was a product of Pharmacia. Serva DEAE-cellulose was obtained from Gallard-Schlesinger Manufacturing Corporation, New York. 2-mercaptoethanol from...
Ribulose-1,5-diphosphate carboxylase has been purified 15-fold from autotrophically cultured *R. rubrum*. Analytical ultracentrifugation reveals a major component, $s_{20,w} = 6.2$, and a minor component, of lower $s$, which amounts to not more than 10% of the protein in the solution. It is evident that a large part of the soluble protein in autotrophically cultured *R. rubrum* consists of ribulose-1,5-diphosphate carboxylase. The specific activity of the enzyme in some crude extracts is high enough to account for 15% of the soluble protein, and in this respect this bacterium resembles the higher plant; up to 16% of the soluble protein of the green leaf consists of ribulose-1,5-diphosphate carboxylase (6).

The $s_{20,w}$ of the ribulose-1,5-diphosphate carboxylase was estimated to be 6.2 (Fig. 1), relative to the sedimentation of glyceraldehyde-3-phosphate dehydrogenase (3.7, 7.7) (18). The $s$ value obtained by analytical ultracentrifugation is identical with that obtained by density gradient analysis. The *R. rubrum* enzyme has the lowest $s$ value found thus far for a protein with carboxylase activity. Two other members of the *A. thiochristum* family, *Rhodopseudomonas palustris* and *spheroides*, have carboxylases with $s$ values of 12 and 14.5, respectively; all other forms thus far examined, including the purple sulfur photosynthetic bacterium *Chromatium*, have carboxylases with $s$ values between 18 and 21 (8).

The carboxylase could not be separated from added rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by filtration on Sephadex G-200 (Fig. 2). The distribution coefficient ($K_d$) of the carboxylase on Sephadex G-200 is 0.31 (17). The $K_d$ of the carboxylase must then be 0.31. By the method of Siegel and Monty (16), a value of 47 Å for the Stokes radius of the carboxylase is obtained.

The molecular weight can be estimated to be 120,000 from the sedimentation coefficient and Stokes radius, with 0.725 cm$^2$/s per g for the partial specific volume (16). The molecular weight of the plant enzyme is 557,000, according to Paulus and Lano (6).

The optimal pH for the *R. rubrum* carboxylase reaction is 8.1. A sharp pH dependence curve was obtained (Fig. 3). Changing the concentration of ribulose-1,5-diphosphate or of CO$_2$ did not affect the pH optimum. The pH optimum of the bacterial enzyme is close to, or the same as, that of the chloroplast enzyme (19), but the pH dependence curve for the chloroplast enzyme is broader than that for the bacterial enzyme.

**Kinetic Parameters**—The $K_m$ for ribulose-1,5-diphosphate is 0.083 ± 0.007 mM, and for CO$_2$ it is 05 ± 13 mM. For the spinach enzyme, the $K_m$ for ribulose-1,5-diphosphate is 0.12 mM; for CO$_2$, 22 mM; and for Mg$^{2+}$, 1.1 mM (6). Addition of Mg$^{2+}$ to 10 mM levels in the assay for ribulose-1,5-diphosphate carboxylase had no effect on the activity of the bacterial enzyme, even after prior treatment of the enzyme with EDTA. Unlike the carboxylase from higher plants, the enzyme from *R. rubrum* does not require high levels of a divalent cation for activity.

**Inhibitors**—Arsenate inhibited the *R. rubrum* enzyme 70% under the standard (spectrophotometric) assay conditions. It also inhibits the activity of the chloroplast enzyme (6, 19). (NH$_4$)$_2$SO$_4$ did not affect the bacterial enzyme, although the plant enzyme is inhibited by (NH$_4$)$_2$SO$_4$ (6). Citrate is competitive with respect to ribulose-1,5-diphosphate. Mixed-type inhibition kinetics were observed when CO$_2$ levels were varied (Table II). The product of the reaction catalyzed by the enzyme, 3-P-glyceric acid, is competitive with respect to ribulose-1,5-diphosphate and non-competitive with respect to CO$_2$ (Table II). Inorganic phosphate is non-competitive against either substrate (Table II).

Excess ribulose-1,5-diphosphate (0.7 mM) inhibits the spinach enzyme (19), but 1.35 mM ribulose-1,5-diphosphate has no effect on the activity of the bacterial carboxylase.
### Discussion

Ribulose-1,5-di-P carboxylase from *R. rubrum* is markedly smaller than the enzyme from other species. The finding of a carboxylase of intermediate size (and hence, probably, of intermediate size) in the rhodopseudomonads suggests that the carboxylase with the large *s* value of 21 may have evolved in the photosynthetic bacteria from the small carboxylase in *R. rubrum*. We do not know whether the enzyme from *R. rubrum* and that from the green plant related proteins and thus whether there is a possibility that the same protein evolved from the other.

Like the plant enzyme, the bacterial enzyme has a high *Kₘ* for CO₂. Levels of the two preceding enzymes in the reductive pentose phosphate cycle (ribose-5-P isomerase and ribulose-5-P kinase) in *R. rubrum* (7). The high carboxylase levels may compensate for the apparent low affinity for CO₂. It should be kept in mind that the intracellular levels of CO₂ have not been determined in higher green plants or in photosynthetic bacteria. “CO₂” is used here to denote total CO₂ concentration, which would include hydrated CO₂, H₂CO₃, HCO₃⁻, and CO₃²⁻. Cleland (20) has pointed out that *Kₘ* values are often indicative of the intracellular concentrations of substrates. If this is true for the carboxylase, then the CO₂ levels in *R. rubrum* and in the green plant chloroplast are about 10⁻³ M.

*R. rubrum* ribose-5-P isomerase, the first enzyme in the sequence of reactions leading from ribose-5-P and CO₂ to 2 moles of 3-P-glyceric acid, is inhibited by the following five metabolites: citrate, AMP, ADP, inorganic phosphate, and ribulose-1,5-di-P. Like the isomerase, the carboxylase is also inhibited by citrate, inorganic phosphate, and, in addition, by an intermediate of the reductive pentose phosphate cycle (and of the Embden-Meyerhof pathway), 3-P-glyceric acid.

Citrate is competitive with respect to ribulose-1,5-di-P. The inhibition by citrate might be due to chelation of an essential metal cofactor required only in very low amounts or to some structural similarity between citrate and ribulose-1,5-di-P; regardless of the mode of inhibition, it is clear that citrate is a potent inhibitor of this enzyme in *R. rubrum* and that citrate can control the activity of the carboxylase as well as of the isomerase (9). Differences in endogenous citrate levels have been reported for photoheterotrophically cultured *R. rubrum* (21, 22). Clearly intracellular citrate levels are subject to fluctuation. High citrate levels may reflect high levels of utilisable reduced carbon sources. It is to the advantage of the organism to utilize preformed carbon compounds rather than to expend energy and reducing power in the reductive assimilation of CO₂. In the presence of utilisable carbon compounds, citrate may be formed; ribulose-1,5-di-P carboxylase activity may then be negatively modulated; reduction of CO₂ inhibited; and utilization of the reduced carbon source, favored. Citrate, by inhibiting both the carboxylase and the isomerase, can damp the operation of the reductive pentose phosphate cycle and the energy thus conserved can be used for other purposes. An important effector in other systems (23), citrate would appear to be important also in the control of the reductive pentose phosphate cycle in the photosynthetic bacterium, *R. rubrum*.

A potent inhibitor of the carboxylase, 3-P-glyceric acid, is both the product of this reaction and an intermediate in the Embden-Meyerhof pathway. In the green alga *Chlorella*, there is an instantaneous burst of 3-P-glyceric acid when light is removed during photosynthetic CO₂ fixation (24). A similar fluctuation in 3-P-glyceric acid levels in *R. rubrum* could serve to shut off the reductive pentose phosphate cycle. High levels of 3-P-glyceric acid might also indicate the operation of the Embden-Meyerhof pathway in the direction of gluconeogenesis when high levels of exogenous, utilisable carbon compounds are available.

It may be significant that both citrate and 3-P-glyceric acid are competitive with ribulose-1,5-di-P and not with CO₂. Ribulose-1,5-di-P levels might be expected to vary as a response to internal conditions within the bacterium. Indeed, ribulose-1,5-di-P is a potent inhibitor of another enzyme of the reductive pentose phosphate pathway, ribose-5-P isomerase (9), which suggests that ribulose-1,5-P levels do fluctuate. CO₂ levels, on the other hand, would probably be subject to control from external sources and would not reflect internal metabolic flux. Citrate and 3-P-glycerate, by virtue of being competitive with ribulose-1,5-di-P, would exaggerate the effect of changes of intracellular ribulose-1,5-di-P levels on the activity of the enzyme.

At relatively high levels, phosphate is an inhibitor of the carboxylase and is noncompetitive with either substrate. The isomerase, likewise, is inhibited by phosphate (9). Unfortunately, the normal levels of free phosphate in this bacterium have not been determined. It is possible that high phosphate levels signal a lack of available energy for the operation of the reductive pentose phosphate cycle. On the other hand, phosphate is often a seemingly nonspecific inhibitor of enzyme-catalyzed reactions, and this may be true here.

We have previously shown that ribulose-1,5-di-P carboxylase levels are under metabolic control in *R. rubrum*. The activity of this enzyme appears to be subject to metabolic control as well.

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