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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 K_m for ribulose 1,5-diphosphate is 83 mM, and for CO_2 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.

Ribulose 1,5-diphosphate carboxylase (3-phosphate-D-glycerate carboxy-lyase (dimerizing) EC 4.1.1.39) catalyzes the formation of 2 moles of 3-P-glyceric acid from 1 mole of CO_2 and 1 mole of ribulose-1,5-di-P. This reaction is ubiquitously distributed among autotrophic organisms, including the photosynthetic and chemosynthetic bacteria, blue-green algae, and higher and lower plants (2–5). In higher plants, the carboxylase is a high molecular weight protein (mol wt 557,000, $s^0_{20,w}$ 21) that makes up as much as 16% of the soluble protein of the green leaf (6). Although the specific activity of the enzyme in extracts of photoautotrophically grown *Rhodospirillum rubrum* is higher than in green plant extracts (7), the *s* value of the carboxylase is only 6.2, indicating a much smaller protein (8). It is immediately evident that these two carboxylases are quite different in molecular structure and possibly in activity. It was therefore of interest to isolate and characterize the carboxylase from this photosynthetic bacterium and to compare the smaller ribulose-1,5-di-P carboxylase of *R. rubrum* with the carboxylase of higher plants.

In *R. rubrum*, modulation of ribose-5-P isomerase activity appears to be important in the control of carbohydrate metabolism (9). Five important metabolites—AMP and ADP, both involved in energy metabolism; citrate, involved in the tricarboxylic acid cycle; ribulose 1,5-diphosphate, an intermediate in the reductive pentose phosphate pathway; and inorganic phosphate—modify the kinetic properties of this enzyme. As part of a study of the control of photosynthetic carbon metabolism in this bacterium, it was important to determine whether the activity of the carboxylase, as well, would be modulated by certain important metabolites. Although levels of ribose-5-P isomerase are not appreciably altered by different growth conditions, ribulose-1,5-di-P carboxylase levels are markedly higher in autotrophically grown cells (7). It seemed possible that control of ribulose-1,5-di-P carboxylase, the central enzyme of the reductive pentose phosphate cycle, might occur both in enzyme induction and in direct modulation of enzyme activity by metabolites.

The enzyme has been purified severalfold from autotrophically cultured *R. rubrum* and appears to be essentially homogeneous. The molecular weight has been estimated, and the optimum pH and kinetics of the reaction have been determined. The carboxylase is inhibited by three important metabolites: citrate, an intermediate of the tricarboxylic acid cycle; inorganic phosphate; and 3-P-glyceric acid, the product of the reaction catalyzed by the carboxylase.

MATERIALS AND METHODS

Growth of Organism—R. rubrum, strain S-1, was cultured on 5 mM $(NH_4)_2SO_4$ under 1% CO_2 in H_2, as previously described (10). Cells were harvested during logarithmic growth, washed twice with 10 mM potassium phosphate, pH 7.0, and stored at −25°.

Ribulose-1,5-di-P Carboxylase Assay—Two different assays were used to measure ribulose-1,5-di-P carboxylase activity.

During purification, ribulose-1,5-di-P carboxylase was assayed at 25° by the spectrophotometric method of Racker (11). Each cuvette contained 50 μmoles of Tris-HCl, pH 8.1; 0.25 μmole of ribulose-1,5-di-P; 0.12 μmole of DPNH; 5 μmoles of GSH; 250 μg of glyceraldehyde-3-P dehydrogenase; 5 μg of phosphoglyceric acid kinase; 12 μmoles of ATP; 10 μmoles of $MgCl_2$; and 75 μmoles of $NaHCO_3$ in a final volume of 1 ml. Reaction was initiated with enzyme and followed by the change in optical density at 340 mμ with a Cary 14 recording spectrophotometer with the use of a sensitive slide-wire. There was usually a lag, from a few seconds to a minute in duration, before a reaction could be detected. Activity was measured only after the reaction had become linear; the same portion of the curve (with

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respect to extent of reaction) was used for all activity measurements.

Kinetic constants were measured at 30° by the use of a ^{14}C assay similar to that used by Paulsen and Lane (6). The reaction mixture contained, in a total volume of 2 ml, enzyme, 100 μmoles of Tris-HCl (pH 8.1), 5 μmoles of *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, 2.5 μmoles of 2-mercaptoethanol, 5 μmoles of glutathione, 1.25 μCi of $NaH^{14}CO_3$, $NaH^{12}CO_3$, and ribulose-1,5-di-P. When $NaHCO_3$ was varied, 0.5 μmole of ribulose-1,5-di-P and 200, 100, 40, 20, 14, or 10 μmoles of $NaHCO_3$ were used. When ribulose-1,5-di-P was varied, 150 μmoles of $NaHCO_3$ and 2, 1, 0.2, 0.12, 0.08, or 0.05 μmoles of ribulose-1,5-di-P were used. Enzyme was incubated in the mixture for 5 min without ribulose-1,5-di-P; then the reaction was initiated with ribulose-1,5-di-P. At 30-sec intervals, 350 μl were removed into 2 ml of glacial acetic acid, dried by heating at 50° in a forced-draft oven, taken up in 0.2 ml of water and 5 ml of Bray's solution (12), and counted for 20 min in a liquid scintillation counter (Nuclear-Chicago). The 5-min incubation of the enzyme in the reaction mixture eliminated the lag noted in the spectrophotometric assay. The reaction was linear with time.

Estimates of kinetic constants were made with the use of the computer program of Hanson, Ling, and Havir (13). Mean K_m values were estimated by the use of the reciprocal of the variance as a weighting factor. In the case of competitive inhibition, the variance of K_i was estimated from the equation

$$\mathrm{Var}(\hat{K}_i) = \frac{(iK_p)^2}{(K_m - K_p)^4}\,\mathrm{Var}(\hat{K}_m) + \frac{(iK_m)^2}{(K_m - K_p)^4}\,\mathrm{Var}(\hat{K}_p)$$

where $\hat{K}_m$ is the estimator of K_m, $\hat{K}_p$ is the estimator of K_m in the presence of inhibitor, and i is inhibitor concentration. In the case of noncompetitive inhibition, the variance of K_i was estimated by the formula

$$\mathrm{Var}(\hat{K}_i) \cong \frac{(iV_{max})^2}{(V_{max} - V_p)^4}\,\mathrm{Var}(\hat{V}_p) + \frac{(iV_p)^2}{(V_{max} - V_p)^4}\,\mathrm{Var}(\hat{V}_{max})$$

where i is inhibitor concentration and V_p the value obtained for V_{max} in the presence of inhibitor.

Protein was assayed by the method of Warburg and Christian as described by Layne (14). A Zeiss spectrophotometer was used for optical density measurements.

The sedimentation coefficient of the carboxylase was determined by the method of Martin and Ames (15). The Stokes radius of the carboxylase was determined according to Siegel and Monty (16).

A Radiometer pH meter 4 was used for determination of pH.

TABLE I

Purification of ribulose-1,5-di-P carboxylase from R. rubrum

Step	Protein	Activity[a]	Specific activity[b]	Purification	Recovery
	mg			*-fold*	*%*
225,000 × *g* supernatant	130	38	0.31		
$MnCl_2$; $(NH_4)_2SO_4$ fractionation	57	51	0.9	2.9	>100
Sephadex G-200	14	17	1.2	3.9	44
Hydroxyapatite	0.47	2.3	4.8	15	5.8

[a] Expressed as micromoles of 3-P-glyceric acid formed per min.

[b] Expressed as micromoles of 3-P-glyceric acid formed per min per mg of protein.

Metal-free Experiments—Enzyme, purified through the hydroxyapatite step, was made 1 mM in EDTA (pH 7.6), allowed to stand for 15 min at 0°, and passed through a Sephadex G-25 column (2.5 × 39 cm) in metal-free Tris-HCl, pH 8.1. The Sephadex G-25 (100 to 300 μ) had been soaked for 3 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 ^{14}C assay except that $NaH^{14}CO_3$ was omitted. At 1-min intervals, 100-μl samples were removed into 100 μl of 1 M 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, $NaHCO_3$, and ribulose-1,5-di-P carboxylase were omitted. Identical activity curves were obtained in the presence and absence of added $MgCl_2$ (10 mM). The Mg^{++} concentration was 100 μM in the absence of added $MgCl_2$.

Purification of Enzyme—All operations were carried out at 0–4°. 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 × *g*, and the supernatant solution was further centrifuged for 25 min at 225,000 × *g*. Then 0.1 volume of 0.5 M $MnCl_2$ 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 $(NH_4)_2SO_4$ 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 × 30 cm, 40 to 120 μ) 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 × 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° following ammonium sulfate fractionation. The enzyme purified through the hydroxyapatite step was denatured by freezing (varying amounts of activity up to 30% remained). Activity was lost only slowly from solution at 0°, provided that 2-mercaptoethanol or glutathione was present.

Reagents—Barium ribulose-1,5-di-P, ATP, Tris, DPNH, and metal-free $(NH_4)_2SO_4$ 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, glyceraldehyde-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

Matheson Coleman and Bell, and metal-free Tris from Mann. All other reagents were analytical reagent grade.

RESULTS

Ribulose-1,5-di-P carboxylase has been purified 15-fold from autotrophically cultured *R. rubrum*. Analytical ultracentrifugation reveals a major component, $s^0_{20,w}$ 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-di-P 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-di-P carboxylase (6).

The $s^0_{20,w}$ of this ribulose-1,5-di-P carboxylase was estimated to be 6.2 (Fig. 1), relative to the sedimentation of glyceraldehyde-3-P dehydrogenase ($s^0_{20,w}$ 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 *Athiorhodaceae*, *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-P dehydrogenase by filtration on Sephadex G-200 (Fig. 2). The distribution coefficient (K_d) of glyceraldehyde-3-P dehydrogenase 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 A 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 cm³ per g for the partial specific volume (16). The molecular weight of the plant enzyme is 557,000, according to Paulsen and Lane (6).

The optimal pH for the *R. rubrum* carboxylase reaction is 8.1. A sharp pH dependence curve was obtained (Fig. 3). Changing

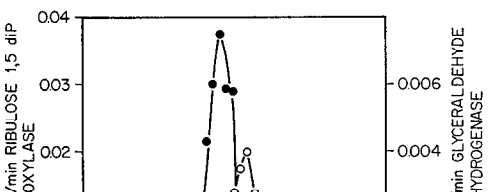

FIG. 1. Determination of s value of ribulose-1,5-di-P carboxylase by sucrose density gradient analysis. The carboxylase, purified through the $(NH_4)_2SO_4$ step, and 0.1 mg of glyceraldehyde-3-P dehydrogenase were dissolved in 100 µl of 2.5% buffered sucrose and layered onto a linear 5 to 20% gradient of sucrose in 10 mM potassium phosphate-10 mM $MgCl_2$, pH 7.6. After 12½ hours centrifugation at 39,000 rpm and 4° in an SW-39 rotor, 37 fractions were collected, diluted with 1 ml of potassium phosphate buffer, and assayed. The $s^0_{20,w}$ of glyceraldehyde-3-P dehydrogenase is 7.7 (18). The s value of the carboxylase is then 6.2.

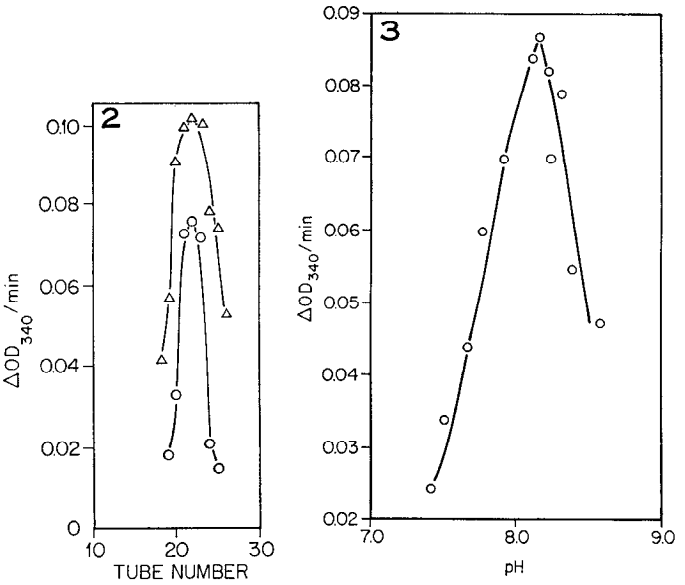

FIG. 2 (*left*). Determination of Stokes radius of ribulose-1,5-di-P carboxylase from *R. rubrum* by exclusion from Sephadex G-200. Ribulose-1,5-di-P carboxylase purified through the $(NH_4)_2SO_4$ precipitation step and 0.1 mg of glyceraldehyde-3-P dehydrogenase were layered onto a Sephadex G-200 column. Fractions of 50 drops (4.5 ml) were collected. The exclusion volume of the column was 650 drops (tube 13). The Stokes radius of ribulose-1,5-di-P carboxylase is 47 A (see text). ○, glyceraldehyde-3-P dehydrogenase; △, ribulose-1,5-di-P carboxylase.

FIG. 3 (*right*). Dependence of ribulose-1,5-di-P carboxylase on pH. The spectrophotometric assay was used. The pH of the mixture was measured at 25° immediately after activity determination.

the concentration of ribulose-1,5-di-P 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-di-P is 0.083 ± 0.007 mM, and for CO_2 it is 65 ± 13 mM. For the spinach enzyme, the K_m for ribulose-1,5-di-P is 0.12 mM; for CO_2, 22 mM; and for Mg^{++}, 1.1 mM (6). Addition of $MgCl_2$ to 10 mM levels in the assay for ribulose-1,5-di-P 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). $(NH_4)_2SO_4$ did not affect the bacterial enzyme, although the plant enzyme is inhibited by $(NH_4)_2SO_4$ (6). Citrate is competitive with respect to ribulose-1,5-di-P. 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-di-P and noncompetitive with respect to CO_2 (Table II). Inorganic phosphate is noncompetitive against either substrate (Table II). Excess ribulose-1,5-di-P (0.7 mM) inhibits the spinach enzyme (19), but 1.35 mM ribulose-1,5-di-P has no effect on the activity of the bacterial carboxylase.

TABLE II

Effect of three metabolites on activity of R. rubrum ribulose-1,5-di-P carboxylase

Compound	Concentrations used	Varied substrate	Inhibition	K_i
	mM			mM
Citrate	0.1, 0.3, 1.0	Ribulose-1,5-di-P	Competitive	0.047 ± 0.013
		CO_2	Mixed	$V_p > V_{max}$; $K_p > K_m$
3-P-Glycerate	0.3, 0.7, 1.0	Ribulose-1,5-di-P	Competitive	0.019 ± 0.004
		CO_2	Noncompetitive	0.28 ± 0.13
Phosphate	10, 25	Ribulose-1,5-di-P	Noncompetitive	23 ± 2.6
	10, 25	CO_2	Noncompetitive	13.6 ± 3.5

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 *s* value (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 are related proteins and thus whether there is a possibility that the one protein evolved from the other.

Like the plant enzyme, the bacterial enzyme has a high K_m for CO_2. Levels of the carboxylase are 10-fold higher than 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 CO_2. It should be kept in mind that the intracellular levels of CO_2 have not been determined in higher green plants or in photosynthetic bacteria. "CO_2" is used here to denote total CO_2 concentration, which would include hydrated CO_2, H_2CO_3, HCO_3^-, and $CO_3^=$. Cleland (20) has pointed out that K_m values are often indicative of the intracellular concentrations of substrates. If this is true for the carboxylase, then the CO_2 levels in *R. rubrum* and in the green plant chloroplast are about 10^{-2} M.

R. rubrum ribose-5-P isomerase, the first enzyme in the sequence of reactions leading from ribose-5-P and CO_2 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, by 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 utilizable 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 CO_2. In the presence of utilizable carbon compounds, citrate may be formed; ribulose-1,5-di-P carboxylase activity may then be negatively modulated; reduction of CO_2 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 CO_2 fixation (24). A similar fluctuation in 3-P-glyceric acid levels in *R. rubrum* could serve to shut off the reductive pentose phosphate cycle through the effect on the affinity of the carboxylase for ribulose-1,5-di-P. 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, utilizable 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 CO_2. 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. CO_2 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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