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Oxygen Regulation of Ribulose 1,5-Bisphosphate Carboxylase Activity in *Rhodospirillum rubrum*

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The carboxylase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPC/O) decreased when an anaerobic culture of *Rhodospirillum rubrum* was exposed to atmospheric levels of oxygen. From 70 to 80% of the activity was lost within 12 to 24 h. Inactivation was apparent when the enzyme was assayed in situ (in whole cells) and when activity was measured in dialyzed crude extracts. The quantity of enzyme protein, as estimated from sodium dodecyl sulfate-polyacrylamide gels or as quantified immunologically, did not decrease within 24 h of exposure to air. Following extended exposure to aerobic conditions (48 to 72 h), degradation of enzyme occurred. These results indicate that the inactivation of RuBPC/O in *R. rubrum* may be due to an alteration or modification of the preformed enzyme, followed by eventual degradation of the inactive enzyme. When shifted back to anaerobic conditions (under an argon atmosphere), the RuBPC/O activity increased rapidly. This increase appeared to be due to de novo synthesis of enzyme. The increase in activity was not observed when the culture was maintained in the dark or in the absence of a suitable carbon source. Thus, the oxygen-mediated inactivation of RuBPC/O appeared to be due to some form of irreversible modification. The cloned *R. rubrum* RuBPC/O gene, expressed in *Escherichia coli*, yielded functional enzyme that was not affected by oxygen, indicating that inactivation in *R. rubrum* is mediated by a gene product(s) not found in *E. coli*.

Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBPC/O) catalyzes the first reaction of both the reductive and oxidative photosynthetic carbon cycles (10). In the absence of oxygen, RuBPC/O catalyzes the carboxylation and cleavage of RuBP, resulting in the formation of two molecules of 3-phosphoglyceric acid. In the presence of oxygen, RuBPC/O catalyzes the oxygenolysis of RuBP, resulting in the formation of 1 molecule of 3-phosphoglyceric acid and 1 molecule of phosphoglycolate. Usually the phosphoglycolate formed is further metabolized via the glycolate pathway, resulting in the wasteful loss of carbon and nitrogen. The oxygenase function of the enzyme therefore results ultimately in a futile cycle, with loss of CO_2 and enormous energy expenditure (5). The ability of RuBPC/O to catalyze these two distinct reactions necessitates careful metabolic regulation of enzyme activity. *Rhodospirillum rubrum*, like all members of the family *Rhodospirillaceae*, synthesizes high levels of RuBPC/O when grown under anaerobic photosynthetic conditions with butyrate or hydrogen as the electron donor (1, 13, 15, 16, 18). In the present investigation, it is shown that RuBPC/O is metabolically regulated in *R. rubrum* following exposure to oxygen. The evidence suggests that inactivation occurs by some form of alteration or modification of the enzyme when cells are exposed to atmospheric levels of oxygen. The physiological significance of this response is evident and may be related to the need to inactivate RuBPC/O in order to limit the wasteful loss of carbon and nitrogen through photorespiration.

MATERIALS AND METHODS

Organisms and growth conditions. *R. rubrum* S-1 was grown photoheterotrophically in a butyrate-bicarbonate medium as previously described (13). When aeration was required, the culture was transferred to a Roux flask and sparged with air. Darkness was attained by wrapping several layers of aluminum foil around each flask.

R. rubrum was also grown photolithotrophically under an atmosphere of 1.5% CO_2–98.5% H_2 (13). After maximum levels of enzyme were obtained, 0.4% butyrate was added aseptically, and the culture was aerated by sparging with air. Because cultures would occasionally show a sharp initial increase in RuBP carboxylase activity after the aerobic shift, the procedure was modified by first shifting the cultures to argon when butyrate was added. After 1 to 3 h under argon, the culture was shifted to air. This procedure allowed RuBPC/O to reach maximal levels before the cultures were exposed to air.

Escherichia coli JM103(pRR2119) was obtained from C. R. Somerville. This strain contains the RuBPC/O gene of *R. rubrum* cloned into the *Bam*HI site of the *lacZ* gene (14). The strain was maintained in tryptone-yeast extract broth containing ampicillin (50 μg/ml) or carbenicillin (200 μg/ml). *E. coli* JM103(pRR2119) was cultured either aerobically or anaerobically (under argon) in 800-ml quantities in tryptone-yeast broth supplemented with 0.5% glycerol, 0.2% fumarate, and either ampicillin or carbenicillin.

Preparation of crude extracts. Cultures were harvested in stationary phase and frozen at −70°C. Crude extracts were prepared by suspending the cells in 1/20 the original volume of TEMMB buffer (20 mM Tris-sulfate [pH 8.0], containing 1 mM EDTA, 10 mM $MgCl_2 \cdot 6H_2O$, 5 mM β-mercaptoethanol, and 50 mM $NaHCO_3$). Lysozome was added to a final concentration of 0.125 mg/ml, and the slurry was incubated at room temperature for 20 min. The cells were broken by sonication in a Branson Sonic Power sonifier (model 350) fitted with a microtip. Usually three 30-s bursts at minimal output were sufficient to break almost all the cells. Whole cells were removed by centrifugation in an Eppendorf microfuge. Membranes were removed by one of two methods. In the first method, crystalline $MgCl_2$ was added to a final concentration of 50 mM, and the extract was

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incubated for exactly 10 min at 50°C and then cooled immediately on ice. Membranes could then be removed by low-speed centrifugation at 10,000 rpm with the SS-34 rotor of the Sorvall centrifuge (18). In the second method, the $MgCl_2$ heat treatment was omitted and membranes were removed by ultracentrifugation at 100,000 × *g* in a 70 Ti rotor for 1 h at 4°C.

Rocket immunoelectrophoresis. Antiserum to native *R. rubrum* RuBPC/O was a gift from R. Quivey. Rocket immunoelectrophoresis was performed by the method of Plumley and Schmidt (12). Antiserum was diluted 1:500 in a solution of agarose (80 mM Tris-acetate, pH 8.6, 40 mM sodium acetate, 1 mM EDTA, 25 mM calcium acetate, 1% agarose). The agarose matrix was prepared on a Gel-bond film (7.5 by 10 cm; FMC Corporation, BioProducts, Rockland, Maine), and 10-$\mu m^3 v$ holes were punched into the agarose. Extracts were diluted so that approximately 100 ng of RuBPC/O could be loaded in each hole. Proteins were electrophoresed overnight at 100 V towards the anode. Purified RuBPC/O from *R. rubrum* was used to construct a standard curve relating the height of the rocket to the concentration of antigen.

Gel electrophoresis. One- and two-dimensional gel electrophoresis was performed as described previously (3, 13).

RuBP carboxylase activity assays. Activity was assayed as described previously (21). The enzyme activity of *E. coli* JM103(pRR2119) was determined in situ by the method described for *R. rubrum*, with cells permeabilized with toluene (17).

RESULTS

Decrease in RuBP carboxylase activity under aerobic conditions. *R. rubrum* was grown photoheterotrophically in butyrate-bicarbonate medium to the stationary phase. After maximum derepression of RuBPC/O synthesis in photoheterotrophically grown cells, the culture was harvested aseptically at room temperature and resuspended in fresh medium. Air was bubbled through the culture for 24 h, beginning at time zero (Fig. 1). RuBP carboxylase activity, as measured in toluenized cells, decreased following transfer to the air atmosphere, resulting in a 70% loss of activity after 12 h (about one doubling time) and 80% loss after 24 h. The culture was subsequently shifted back to anaerobic conditions by bubbling with argon. Carboxylase activity reached the original (pre-O_2) levels about 10 h after anaerobiosis was reestablished and continued to rise, reaching more than twice its original activity within 18 to 24 h.

Crude extracts were prepared from an anaerobic culture, an oxygen-treated culture, and an oxygen-treated culture which had been incubated under argon until enzyme activities had recovered. For this experiment, *R. rubrum* was cultured photoheterotrophically on the butyrate-bicarbonate medium to the stationary phase (A_{650}, ≥3), at which time RuBPC/O synthesis was maximally derepressed (13). A portion of the culture was harvested and washed in TEMMB buffer, and the pellet was frozen for subsequent preparation of crude extracts. The remainder of the culture was washed at room temperature in fresh butyrate-bicarbonate medium, transferred to a Roux flask, and bubbled with argon for 2 h before oxygen exposure was begun. The culture was bubbled for 36 h with air, and then a portion was harvested and washed, and the cells were frozen. The remainder of the culture was returned to anaerobic conditions until enzyme activity recovered. The loss of RuBP carboxylase activity, determined by in situ assay of toluene-treated whole cells

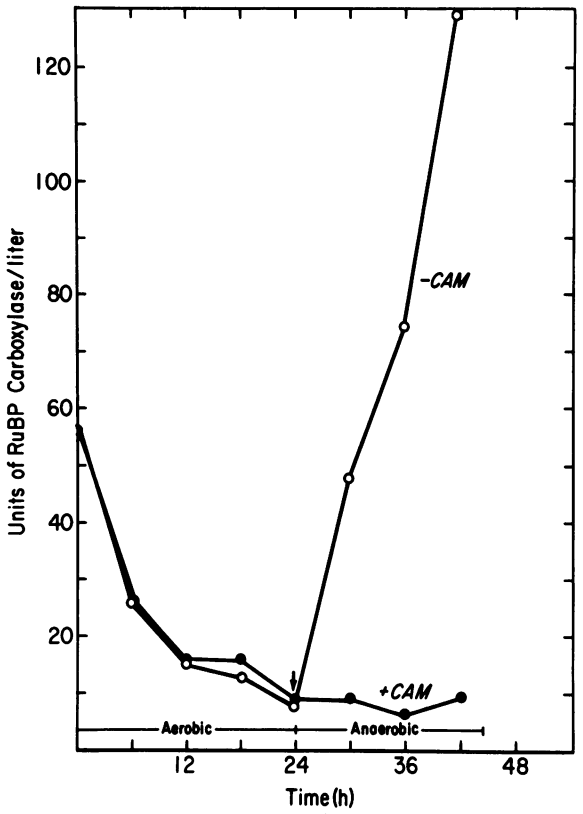

FIG. 1. Decrease in RuBP carboxylase activity in response to air and anaerobic recovery in the presence and absence of chloramphenicol (CAM). *R. rubrum* was grown anaerobically under photosynthetic conditions in butyrate-bicarbonate medium until enzyme synthesis was derepressed. The cells were then washed and resuspended in fresh medium and bubbled with air. After 24 h of exposure to air, the cultures were shifted back to anaerobic conditions by being bubbled with argon. Chloramphenicol (final concentration, 80 μg/ml) was added to one of the cultures at the time of the shift back to argon (●). The open circles (○) represent a control culture to which no chloramphenicol was added. Samples were taken periodically to determine RuBP carboxylase activity by the whole-cell assay (17).

(Fig. 1), paralleled the loss of activity measured in crude extracts prepared from these cells (Table 1). Dialysis of the crude extracts against TEMMB buffer did not affect the activity, indicating that the loss of RuBP carboxylase activity was not due to some loosely bound, allosteric inhibitor of the enzyme.

Quantification of RuBPC/O enzyme protein as a function of catalytic activity. Under the conditions described for the experiment depicted in Fig. 1, cell division and growth continued under aerobic conditions in the butyrate-bicarbonate medium. Thus, the loss in specific activity might be

TABLE 1. Comparison of RuBP carboxylase activity in toluenized cells with activity in crude extracts

Cell sample	In situ activity (U/g)	Activity remaining (%)	In vitro activity (U/mg of protein)	Activity remaining (%)
Anaerobic	23.0	100	0.498	100
O_2 treated	4.2	18	0.116	23.3
Recovered	30.7	133	0.690	138

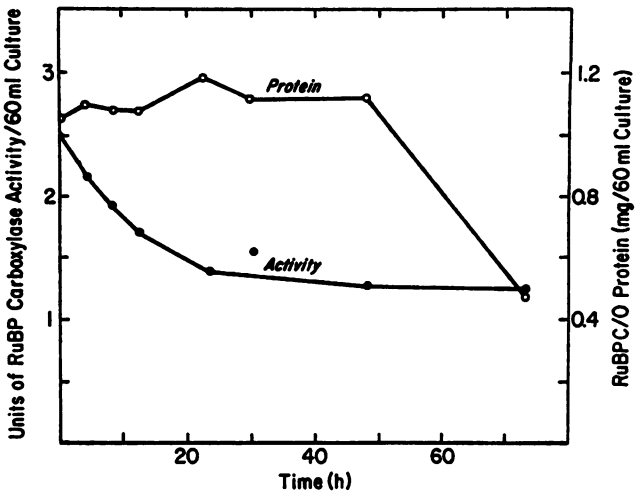

FIG. 2. Comparison of RuBP carboxylase activity with quantity of RuBPC/O protein in crude extracts. Growth of *R. rubrum*, preparation of crude extracts, and enzyme assays were performed as described in Materials and Methods. RuBPC/O protein in the crude extract was quantified by rocket immunoelectrophoresis (3).

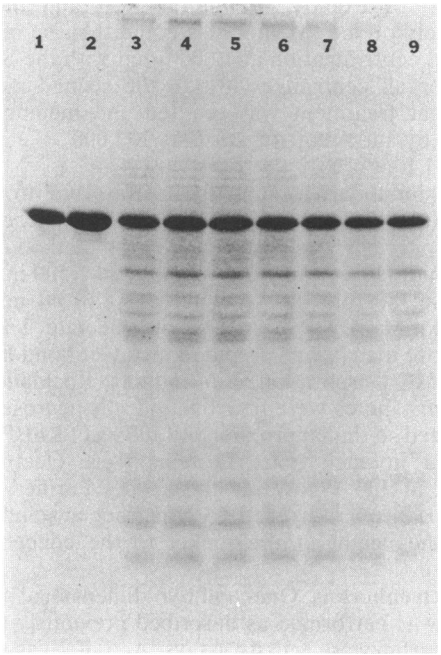

FIG. 3. SDS slab gel electrophoresis of crude extracts of *R. rubrum* cells exposed to air. Extracts were prepared from the soluble fraction of disrupted cells as described in Materials and Methods; approximately the same amount of protein was applied in lanes 3 to 9. Lanes 1 and 2 (from the left) contain RuBPC/O purified from *R. rubrum*. Lanes 3 to 9 contain extracts prepared from *R. rubrum* cells exposed to air for (lane 3) 0 h (2.40 U/60 ml of culture), (lane 4) 4 h (2.14 U/60 ml), (lane 5) 8 h (1.90 U/60 ml), (lane 6) 12 h (1.68 U/60 ml), (lane 7) 24 h (1.36 U/60 ml), (lane 8) 36 h (1.54 U/60 ml), or (lane 9) 48 h (1.26 U/60 ml).

due in part to cessation of enzyme synthesis and subsequent dilution of the enzyme. In a separate experiment, conditions were modified so that growth did not resume during exposure to the aerobic environment. This was done by taking advantage of the previously observed long adaptation period required by photolithotrophically grown cells before they are capable of aerobic growth with butyrate (L. S. Cook and F. R. Tabita, unpublished results). *R. rubrum* was thus grown photolithotrophically in an atmosphere of 1.5% CO_2–98.5% H_2 until enzyme synthesis was derepressed (13). The culture was shifted to air at time zero, at which time sterile butyrate was added to a final concentration of 0.4% (vol/vol). Crude extracts were prepared from the culture throughout the course of inactivation. At each time point (Fig. 2), 60 ml of culture was harvested, and crude extracts were prepared as described in Materials and Methods. In this system, where there is little culture growth, loss of activity was somewhat less dramatic (Fig. 2); typically, 45 to 65% of the activity was lost within 24 h.

Since RuBP carboxylase activity measured in crude extracts paralleled the activity measured in situ in toluenized whole cells (Table 1), the amount of RuBPC/O protein present in the crude extracts was quantified by rocket immunoelectrophoresis (3). The loss of enzyme units observed in cultures exposed to oxygen did not result from a loss of RuBPC/O protein in crude extracts derived from those cultures. The crude extract showed a 46% loss of activity after the culture was exposed to air for 24 h (Fig. 2). In another experiment, 62% of the activity was lost after 24 h. In both cases, however, the quantity of RuBPC/O protein, as measured immunologically, remained constant during this time span (Fig. 2). Crude extracts prepared from a culture of *R. rubrum* exposed to air for prolonged times (48 to 72 h) exhibited a marked decrease in the quantity of RuBPC/O protein. The specific activity of the enzyme (units per milligram of protein, determined immunologically) decreased from an initial 2.29 U of RuBPC/O per mg at time zero (pre-O_2) to 1.02 U of RuBPC/O per mg at 48 h and then began to rise again to 2.88 U/mg at 72 h, as the amount of total protein diminished. Apparently, *R. rubrum* responded to exposure to air by inactivating RuBPC/O and subsequently degraded not only the inactivated protein, but also other intracellular proteins.

To confirm the immunological studies and to ensure that the cross-reactive material in rocket immunoelectrophoresis was intact enzyme, crude extracts prepared at the time points shown in Fig. 2 were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 3). There was no visible decrease in the amount of protein in crude extracts prepared from a culture exposed to air for up to 48 h, although 46 to 65% of the carboxylase activity had been lost by this time. The crude extract prepared after 72 h of exposure to air did exhibit a large decrease in RuBPC/O protein which approximated the decrease seen by rocket immunoelectrophoresis (data not shown).

Crude extracts were also prepared from an anaerobically grown culture of *R. rubrum* (in situ whole-cell RuBPC/O activity, 1.8 U/60 ml of culture) and an anaerobic culture exposed to air for 22 h (in situ activity, 0.96 U/60 ml). RuBPC/O activity in crude extracts from these cells was 1.2 and 0.4 U/ml, respectively. The crude extracts were subsequently analyzed by two-dimensional gel electrophoresis as described in Materials and Methods (data not shown). There was no significant decrease in the size of the RuBPC/O spot in crude extracts with active or inactive RuBPC/O, nor was the shape of the spot visibly altered.

Reversibility of aerobic inactivation. As discussed previously, when an oxygen-treated culture was shifted back to anaerobiosis, the RuBP carboxylase activity increased rapidly, reaching 175 to 240% of its original activity within 18 to 24 h. Aerobic inactivation of RuBP carboxylase occurred in the presence of chloramphenicol, indicating that protein

synthesis was not required for the loss of activity (data not shown). However, when chloramphenicol was added to the culture before it was returned to anaerobic conditions, the activity did not increase; therefore, protein synthesis was required to restore the activity under anaerobic conditions (Fig. 1). Likewise, aerobic inactivation of RuBP carboxylase occurred in the dark, indicating that light was not required for the loss of activity (data not shown). However, the anaerobic reactivation was light dependent, indicating that photosynthesis was required for the increase in activity seen under anaerobic conditions. (Note that *R. rubrum* cannot grow anaerobically in the dark in the butyrate-bicarbonate medium.)

The effects of nitrogen and carbon limitation on the oxygen-mediated loss of RuBPC/O activity and the anaerobic recovery of enzyme activity were also investigated. Presumably, protein synthesis would be inhibited under conditions of carbon or nitrogen starvation. *R. rubrum* was grown photolithotrophically under an atmosphere of 1.5% CO_2–98.5% H_2 until RuBPC/O synthesis was derepressed. This culture was harvested and divided into four parts, each of which was washed and resuspended in fresh butyrate-bicarbonate medium with the following modifications: (i) NH_4^+, HCO_3^-, and butyrate omitted; (ii) NH_4^+ omitted, HCO_3^- and butyrate added; (iii) NH_4^+ and HCO_3^- omitted, butyrate added; and (iv) NH_4^+, HCO_3^-, and butyrate added. All four cultures were exposed to air for 11 h and then shifted back to argon for 10 h. RuBPC/O activities, measured in toluenized whole cells, decreased in all cases when cells were exposed to air, regardless of the presence or absence of carbon and nitrogen sources. In contrast to the aerobic inactivation, recovery of enzyme activity under anaerobic conditions did not occur unless the culture was provided with HCO_3^- (results not shown). Growth in the butyrate medium is possible only in the presence of HCO_3^-, with CO_2 presumably acting as an obligatory electron acceptor under these conditions (19). Enzyme activity increased following return to the argon atmosphere in a culture which contained all the components of the medium and in a culture which lacked a source of exogenous fixed nitrogen. However, this increase in activity was inhibited when bicarbonate was omitted from the medium, regardless of the presence or absence of added butyrate.

The results of experiments in which protein synthesis was inhibited by chloramphenicol, darkness, or a lack of a suitable carbon source and electron acceptor were in agreement. The oxygen-mediated loss of activity was independent of protein synthesis; however, protein synthesis or normal cellular metabolism appeared to be obligatory for the recovery of enzyme activity under anaerobic conditions.

Effects of O_2 on activity of *R. rubrum* RuBP carboxylase expressed in *E. coli*. The RuBPC/O gene of *R. rubrum* has been cloned and placed under control of the *E. coli lac* promoter (14). Induction of *lac* transcription by isopropyl-thiogalactoside (IPTG) results in expression of high levels of functional RuBPC/O. This strain, *E. coli* JM103(pRR2119), harboring the *R. rubrum* RuBPC/O gene in plasmid pRR2119, was grown anaerobically under argon in tryptone-yeast broth containing 0.5% glycerol and 0.2% fumarate as the electron acceptor (Fig. 4). During exponential growth, IPTG was added to a final concentration of 1.0 mM. The culture was incubated for 4 h under argon and then shifted to aerobic conditions by replacing the argon with air. *E. coli* JM103(pRR2119) did not display the aerobic inactivation of RuBPC/O that was seen in *R. rubrum*.

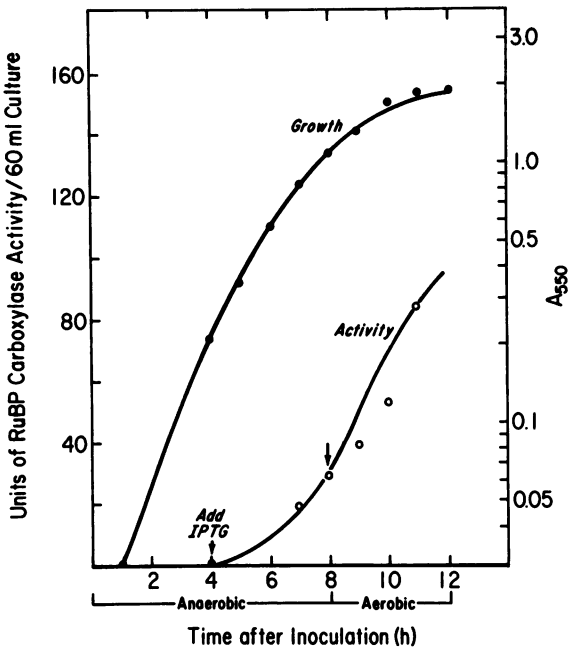

FIG. 4. Effects of air on the *R. rubrum* RuBP carboxylase expressed in *E. coli* JM103(pRR2119). *E. coli* JM103(pRR2119) was grown in tryptone-yeast-glycerol-fumarate broth with ampicillin (50 μg/ml). Anaerobiosis was maintained by bubbling argon through the culture. IPTG was added (final concentration, 0.1 mM) during exponential growth. Four hours after the addition of IPTG, the culture was shifted to aerobic conditions (indicated by the arrow). Samples were taken periodically from the culture, and the RuBP carboxylase activities in toluene-treated cells were determined.

DISCUSSION

RuBP carboxylase activity diminished when an anaerobic culture of *R. rubrum* was transferred to aerobic conditions. As much as 80% of the RuBP carboxylase activity may be lost within one generation of growth. The loss of activity preceded the loss of the protein that cross-reacted immunologically with RuBPC/O, indicating that the enzyme was first inactivated and subsequently degraded. The loss of activity was apparently due to an irreversible chemical alteration or modification of the enzyme rather than specific inhibition of catalytic activity, since dialysis of the crude extract did not restore catalytic activity. *E. coli* JM103(pRR2119), which contains the *R. rubrum* gene for RuPBC/O, expressed fully functional enzyme under anerobic conditions, indicating that the oxygen-mediated inactivation of RuBPC/O in *R. rubrum* requires a gene product(s) that was not cloned into *E. coli* or that *E. coli* possesses some method for coping with oxygen not found in *R. rubrum*. Alternatively, RuBPC/O inactivation requires some small molecule that is present in *R. rubrum* but not in *E. coli*. Inasmuch as both organisms are facultative anaerobes and enteric bacteria have been shown to inactivate glutamine synthetase by a similar oxygen-dependent process (8), it would appear that the first explanation is the most likely.

The use of molecular oxygen to regulate RuBPC/O activity in photosynthetic bacteria is reasonable, since these organisms are capable of high rates of anaerobic CO_2 fixation. *R. rubrum*, in particular, has a difficult time of aerobic chemolithotrophic growth unless the O_2 concentration is lowered below air levels. It is also well established that CO_2 assimilation is inhibited by the presence of O_2 (4, 19), and it has

been proposed that the photoassimilation of CO_2 and respiration compete for a common source of reductant, presumably a reduced pyridine nucleotide (4). An additional factor to be considered is that RuBPC/O is a bifunctional enzyme, catalyzing the oxygenolysis of RuBP. As a result of the oxygenase activity, phosphoglycolate is produced (11), which, when further metabolized via the glycine-serine pathway, results in the loss of cellular carbon and nitrogen. Unless a method exists for posttranslational regulation of RuBPC/O activity, cells growing in an aerobic atmosphere and carrying high levels of RuBPC/O protein would be at a distinct disadvantage due to the potential loss of significant amounts of cellular carbon. The oxygen-mediated control of RuBPC/O in anaerobic photosynthetic bacteria is certainly reminiscent of the oxidative modification of glutamine synthetase in enteric bacteria (6–8). Other studies of the RuBPC/O inactivation system in *R. rubrum* have been presented (2).

The *Rhodospirillaceae* show an unusual amount of nutritional versatility (9, 16, 19). These organisms are able to grow (i) aerobically as chemoheterotrophs in the dark, (ii) aerobically as chemolithoautotrophs with H_2 as the source of energy, O_2 as the electron acceptor, and CO_2 as the carbon source, (iii) anaerobically as photoautotrophs on CO_2 and H_2, (iv) anaerobically as photoheterotrophs with organic carbon sources, and (v) fermentatively on sugars. Such metabolic versatility presumably requires careful regulation of the activity of the primary enzyme of CO_2 assimilation.

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