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Reductive Pentose Phosphate-Independent CO_2 Fixation in *Rhodobacter sphaeroides* and Evidence that Ribulose Bisphosphate Carboxylase/Oxygenase Activity Serves To Maintain the Redox Balance of the Cell

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Whole-cell CO_2 fixation and ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity were determined in *Rhodobacter sphaeroides* wild-type and mutant strains. There is no obvious difference in the levels of whole-cell CO_2 fixation for the wild type, a form I RubisCO deletion mutant, and a form II RubisCO deletion mutant. No ribulose 1,5-bisphosphate-dependent CO_2 fixation was detected in a form I-form II RubisCO double-deletion mutant (strain 16) or strain 16PHC, a derivative from strain 16 which was selected for the ability to grow photoheterotrophically with CO_2 as an electron acceptor. However, significant levels of whole-cell CO_2 fixation were detected in both strains 16 and 16PHC. Strain 16PHC exhibited CO_2 fixation rates significantly higher than those of strain 16; the rates found for strain 16PHC were 30% of the level found in photoheterotrophically grown wild-type strain HR containing both form I and form II RubisCO and 10% of the level of the wild-type strain grown photolithoautotrophically. Strain 16PHC could not grow photolithoautotrophically in a CO_2-H_2 atmosphere; however, CO_2 fixation catalyzed by photoheterotrophically grown strain 16PHC was repressed by addition of the alternate electron acceptor dimethyl sulfoxide. Dimethyl sulfoxide addition also influenced RubisCO activity under photolithoautotrophic conditions; 40 to 70% of the RubisCO activity was reduced without significantly influencing growth. Strain 16PHC and strain 16 contain nearly equivalent but low levels of pyruvate carboxylase, indicating that CO_2 fixation enzymes other than pyruvate carboxylase contribute to the ability of strain 16PHC to grow with CO_2 as an electron acceptor.

Purple nonsulfur photosynthetic bacteria exhibit great metabolic diversity and are capable of five distinct modes of growth (22). In *Rhodobacter sphaeroides*, carbon dioxide fixation has been intensively investigated, and two different forms of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme of the Calvin reductive pentose phosphate pathway, have been found in this organism. These two catalytically distinct enzymes (12) show low sequence homology (11, 13, 37) and are located on different structural gene operons (10, 11), on diverse genetic elements (30, 31). Previous investigations indicated that the synthesis of the two enzymes may be both independently (15) and interdependently (11, 14) controlled. Form I RubisCO was found to be specifically inactivated by the addition of organic acids or excess CO_2 to photolithoautotrophically grown cells (16). This inactivation has been characterized and was recently shown to be reversible both in vivo and in vitro (38–40). A RubisCO double-deletion mutant strain was isolated and was found to be incapable of growth with CO_2 as an electron acceptor, under photolithoautotrophic or photoheterotrophic growth conditions (5). The inability of the RubisCO deletion strain to grow photoheterotrophically was assumed to be related to the capacity of RubisCO to function as an electron sink to maintain a proper redox balance in photoheterotrophically grown cells (5, 14). This hypothesis was supported by the fact that dimethyl sulfoxide (DMSO), an alternative electron acceptor (22, 44), allowed two different RubisCO double-deletion strains to grow photoheterotrophically (5, 14). Moreover, the *R. sphaeroides* RubisCO-deficient strain is capable of photoheterotrophic growth with reduced substrates in the presence of suitable alternative electron acceptors such as DMSO, fully substantiating the original suggestion that *R. sphaeroides* fixes CO_2 via the Calvin cycle to maintain the cellular redox balance under photoheterotrophic growth conditions (5).

In this study, we have isolated a photoheterotrophically competent strain (strain 16PHC) from the RubisCO deletion strain (strain 16) that has gained the capacity for photoheterotrophic growth with CO_2 as an electron acceptor. We have used strain 16PHC, strain 16, a form I RubisCO deletion mutant, and a form II deletion mutant, along with the wild type, to investigate the physiological and biochemical regulation of CO_2 fixation in *R. sphaeroides*. Strong support for the presence of alternative CO_2 fixation mechanisms was found in this investigation; these studies also implicate RubisCO as playing a major role in metabolism beyond its function in the Calvin reductive phosphate pathway.

MATERIALS AND METHODS

Reagents. All biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Agarose and Gel Bond film were from FMC Corp. (Rockland, Maine). Sodium [^{14}C]bicarbonate (20 mCi/mmol) was from Amersham Corp. (Arlington Heights, Ill.).

Organism and growth. *R. sphaeroides* HR, a strain derived from ATCC 17023 (42), was the wild-type strain used in these studies. The form I and form II RubisCO deletion mutants (5) were derived from strain HR and grown photolithoautotrophically in 500-ml bottles containing 300 ml of

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Ormerod medium under a gas mixture of 1.5% carbon dioxide in hydrogen as previously described (15). The medium was supplemented with 15 μg of biotin and 1 mg each of nicotinic acid and thiamine hydrochloride per liter. During photoheterotrophic growth, 30 mM DL-malate was added as a carbon source and the organism was grown as a standing culture in completely filled 500-ml bottles. In all cases, the nitrogen source was 20 mM NH_4Cl. *R. sphaeroides* 16, the RubisCO double-deletion mutant, was grown photoheterotrophically in the presence of DMSO (60 mM) (5). Strain 16PHC, derived from strain 16, was grown photoheterotrophically in the presence or absence of DMSO.

Whole-cell CO_2 fixation assay. Cells grown photolithoautotrophically and photoheterotrophically to late exponential phase were harvested by centrifugation at 5,500 × *g* for 15 min and were washed in Ormerod medium lacking malate. The cells were then resuspended in the same medium to an A_{650} of about 0.6 and kept at 4°C for later use; under these conditions, the cells maintained their CO_2 fixation activity for at least 48 h. For the CO_2 fixation assay, the reaction was carried out in a 25-ml vial sealed with a rubber serum stopper. Each vial contained 4 ml of Ormerod medium, 0.5 ml of cell suspension, and 0.5 ml of sodium [^{14}C]bicarbonate, 0.25 μCi/μmol and 20 mM (final concentration), plus either malate or hydrogen as an electron donor. Each vial was degassed, flushed, and then filled with either argon or hydrogen, depending on the experiment. The vials were then placed into an illuminated, shaking 30°C water bath, and at various times, a 0.5-ml sample was withdrawn from each vial and acidified with 0.2 ml of 100% acetic acid. The acidified samples were set in a hood overnight, and a 0.2-ml sample was subsequently counted as previously described (26).

Labeling and isolation of CO_2 fixation products. *R. sphaeroides* wild type and strains 16 and 16PHC were harvested by centrifugation, washed in Ormerod medium lacking organic carbon, and then resuspended in the same medium to an optical density of about 2.0. The cells were exposed to ^{14}C-labeled sodium bicarbonate (2 mCi/ml) for 30 s, and a methanol-soluble fraction was isolated and spotted on Whatman no. 1 filter paper (20 by 20 cm); two-dimensional paper chromatography was performed according to Shiba et al. (28) and Kandler and Stetter (17). Standards, at a concentration of 5 mM, were chromatographed in both the first and second dimensions.

Whole-cell RubisCO assay. In situ RubisCO activity was measured in permeabilized cells at pH 7.2 as previously described and later modified (34, 38). One unit of RubisCO activity is defined as the amount of enzyme needed to fix 1 μmol of CO_2 per min at 30°C. All assays represent duplicate or triplicate determinations.

Pyruvate carboxylase assay. Cell extracts (100,000 × *g* supernatant fraction) were prepared as previously described (38). Pyruvate carboxylase activity was measured in cell extracts by using a reaction mixture (18) containing Tris-HCl (100 mM, pH 8.0), $MgCl_2$ (5 mM), sodium pyruvate (5 mM), ^{14}C-labeled sodium bicarbonate (50 mM [4 μCi/μmol]), ATP (2.5 mM, and acetyl coenzyme A (100 μM). The reaction was initiated with ATP and quenched with 1 M formic acid. The acid-stable product was counted with a liquid scintillation counter. No significant fixation was obtained in the absence of pyruvate, and as previously demonstrated (23, 24), activity was dependent on low levels of acetyl coenzyme A.

Quantitation of form I and form II RubisCO in crude extracts. The amount of form I and form II RubisCO present in crude extracts was determined by rocket electroimmunoassay as previously described (15). Protein concentrations were determined by the method of Lowry et al. (21) with bovine serum albumin as the standard.

RESULTS

Isolation of *R. sphaeroides* 16PHC. Strain 16PHC was initially isolated when *R. sphaeroides* 16, previously grown aerobically on complex (PYE) medium, was inoculated into malate minimal medium and allowed to incubate in the light for approximately 2 months. Upon examination of extracts from the malate-grown culture, the strain was found to have no RubisCO activity or traces of form I or form II RubisCO antigen. This isolate, designated *R. sphaeroides* 16PHC (for photoheterotrophic competent), retained the photoheterotrophic growth phenotype after the strain was subcultured under nonphotosynthetic aerobic conditions on complex media. Strain 16PHC thus appears to be a true genetic variant, since photoheterotrophic growth on malate occurs with lag times that are typical for photosynthetic bacterial strains precultured aerobically and then transferred to photoheterotrophic conditions. Reisolation of a photoheterotrophically competent variant from the parental *R. sphaeroides* 16 was made by incubating *R. sphaeroides* 16 under the same photosynthetic growth conditions on minimal malate liquid medium lacking DMSO, in the presence of trimethoprim and kanamycin to maintain selection of the RubisCO-negative phenotype. Cultures started with single colonies from PYE complex medium plates resulted in the isolation of a strain of *R. sphaeroides* 16 for each colony tested. Growth was observed in the sealed test tubes after an incubation period of about 6 weeks. During the incubation period, cultures were monitored for the first sign of tubidity to determine a minimum time in which the photoheterotrophically competent phenotype would develop. The culture medium remained virtually clear for most of the incubation period except for cell clumps from the initial inoculum. After a 5- to 6-week period, a sudden onset of growth occurred with a doubling time of 7.7 h. After streak purification, photoheterotrophic isolates were again cultured to confirm the original RubisCO-negative phenotype. All strain 16PHC isolates lacked RubisCO entirely and remained incapable of photolithoautotrophic growth under a CO_2-H_2 atmosphere and could not grow photoheterotrophically in a butyrate-bicarbonate medium. Southern blots, using restricted DNA from both strains 16 and 16PHC, gave identical and expected patterns when probed with internal sequences of the form I and form II RubisCO genes of *R. sphaeroides* (5), confirming that the original double RubisCO mutation has been maintained in strain 16PHC. The *R. sphaeroides* 16PHC phenotype appears very similar to that of a RubisCO deletion strain of *Rhodospirillum rubrum* (strain I-19) (6, 7), in that both strains are able to grow photoheterotrophically on malate with CO_2 as an electron acceptor (Fig. 1). *R. sphaeroides* 16PHC was able to attain densities (600 nm) of over 2.0 when grown on malate with CO_2 as the electron acceptor.

Physiological analysis of whole-cell CO_2 fixation in *R. sphaeroides* strains. Previous experiments have shown that whole-cell RubisCO assays yielded considerably more RubisCO activity than did in vitro assays (38–40). One possible explanation offered was that RubisCO might exist in some form of complex or organized unit within the permeabilized cells used for whole-cell measurements, such that the enzyme could more efficiently fix CO_2. Although the whole-cell RubisCO assays presumably more closely ap-

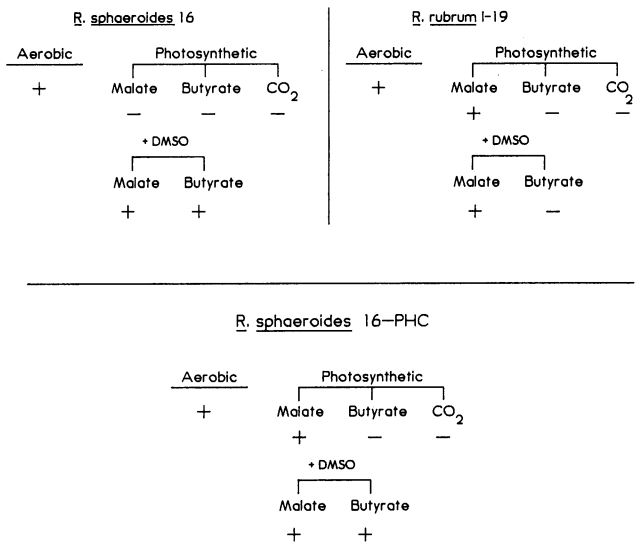

FIG. 1. Phenotypes of RubisCO deletion strains. Aerobic growth was tested by using a mineral salts malate medium. Photosynthetic growth was determined with CO_2 or DMSO (+DMSO) as the electron acceptor and malate, butyrate, or CO_2 as the carbon source; malate, butyrate, and hydrogen, respectively, served as electron donors. +, growth, −, no growth.

proximate the intracellular environment, such measurements may not totally reflect whole-cell CO_2 fixation per se. To approach this question, we analyzed the ability of cells cultured under a variety of conditions to catalyze both whole-cell CO_2 fixation and whole-cell RuBP-dependent CO_2 fixation. Not unexpectedly, photolithoautotrophically grown cells most effectively used hydrogen as an electron donor to support whole-cell CO_2 fixation, with about twice the level obtained for cells grown with malate as an electron donor (Fig. 2A). Interestingly, when cells were grown photoheterotrophically, malate was the preferred electron donor (Fig. 2B). When these experiments were extended to various mutants of *R. sphaeroides*, it was found that the whole-cell CO_2 fixation rate was higher than the whole-cell RubisCO activity in cells grown photoheterotrophically with malate as an electron donor (Table 1). For photolithoautotrophically grown cells, the CO_2 fixation rate and the level of whole-cell RubisCO were nearly the same when malate was used as the electron donor. However, there was a 100% increase in whole-cell CO_2 fixation when hydrogen was used as the electron donor (data not shown). Interestingly, the whole-cell CO_2 fixation rate of photoheterotrophically grown cells was considerably higher than the whole-cell RubisCO activity. From these results (Table 1), it appeared that form I RubisCO activity was more affected than form II RubisCO activity during photoheterotrophic growth, in agreement with previous demonstrations of inhibition of form I RubisCO activity by metabolism of organic substrates (16, 38). However, there are no obvious differences in the levels of whole-cell CO_2 fixation. DMSO, an alternative to CO_2 as an electron acceptor, has been used to probe the role of RubisCO in photoheterotrophically grown cells (5, 14). The addition of DMSO barely influenced CO_2 fixation of the wild type and the form I deletion mutant, but it had an obvious deleterious effect on CO_2 fixation of the form II deletion mutant. After exposure of the wild-type strain to DMSO for 10 days, there was a decrease in CO_2 fixation to about 10%

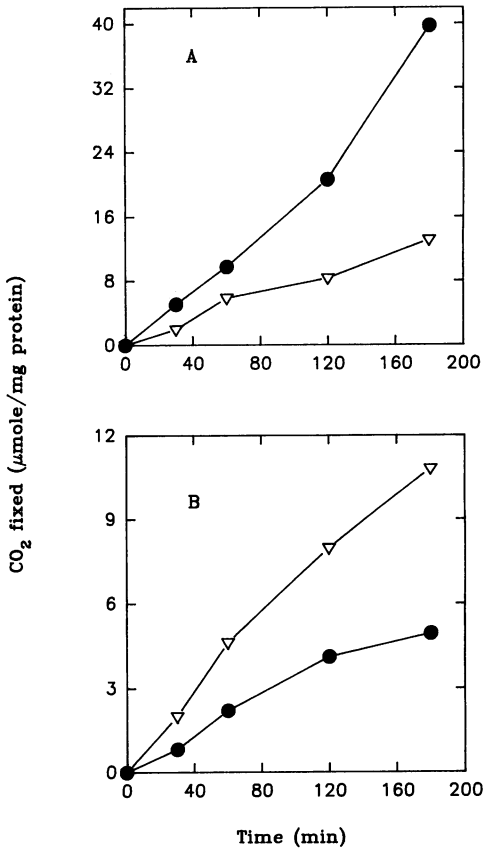

FIG. 2. (A) Whole-cell CO_2 fixation of *R. sphaeroides* HR previously grown photolithoautotrophically in a 1.5% CO_2–H_2 atmosphere. CO_2 fixation assays were performed with hydrogen (●) or malate (▽) as the electron donor. (B) CO_2 fixation in cells grown photoheterotrophically in a mineral salts malate medium with CO_2 as the electron acceptor. CO_2 fixation assays were performed with hydrogen (●) or malate (▽) as the electron donor. Assay conditions are described in Materials and Methods.

of the level of cells grown in the absence of DMSO, even though RubisCO activity remained essentially the same.

Strain 16, a RubisCO double-deletion mutant, is incapable of photolithoautotrophic or photoheterotrophic growth with CO_2 as the electron acceptor (5). If this strain was supplemented with DMSO, it grew photoheterotrophically with approximately the same rate as did the wild type (Table 2). Strain 16PHC, derived from strain 16, was selected for its capacity for photoheterotrophic growth with CO_2 as an electron acceptor. This strain had a doubling time substantially greater than that of the wild-type strain in both large cultures (Table 2) and test tube cultures (data not shown). Strain 16 is unable to grow photolithoautotrophically (5), and of course both strain 16 and strain 16PHC did not exhibit RubisCO activity (Table 1) or synthesize either the form I or form II RubisCO protein (5, 6). A significant level of whole-cell CO_2 fixation, however, was demonstrated for both strains, particularly strain 16PHC (Table 1).

CO_2 fixation in strain 16PHC is elevated and repressible. We initiated experiments to describe the physiological properties of strain 16PHC. The rate of whole-cell CO_2 fixation of strain 16PHC was obviously higher than in strain 16 (Table 1) but was considerably less than in the form II deletion strain, which synthesizes copious amounts of form I RubisCO (Fig.

TABLE 1. Whole-cell CO_2 fixation and whole-cell RubisCO activities in *R. sphaeroides* wild-type and mutant strains grown under different physiological conditions[a]

Growth mode	Strain	CO_2 fixed[b] (nmol/min/mg of protein)	RubisCO activity (nmol/min/mg of protein)
Photolithoautotrophic[c]	HR	76.9	92.9
	Form I deletion	85.1	62.9
	Form II deletion	66.3	73.1
Photoheterotrophic[d]	HR	67.7	11.4
	Form I deletion	42.9	8.6
	Form II deletion	41.1	2.0
	16PHC	15.0	0
Photoheterotrophic with DMSO[e]	HR	62.0	9.5
	Form I deletion	41.7	9.2
	Form II deletion	12.6	7.3
	16	5.6	0
Photoheterotrophic with DMSO[f]	HR	4.9	14.3

[a] All assays represent the averages of duplicate determinations.
[b] In all cases, malate was used as the electron donor.
[c] Cells were cultured in an atmosphere of 1.5 CO_2–98.5% H_2.
[d] Cells were cultured in a medium containing 30 mM DL-malate.
[e] Cells were cultured in a medium containing 30 mM DL-malate and 60 mM DMSO.
[f] Cells were cultured for 10 days in 30 mM DL-malate–60 mM DMSO.

3) (4). The elevated CO_2 fixation in strain 16PHC (relative to strain 16) was repressed by the addition of DMSO to the culture (Fig. 4A); if DMSO was subsequently removed from the culture, strain 16PHC showed a time-dependent increase in its CO_2 fixation capacity (Fig. 4B). The removal of DMSO from strain 16 did not result in any increase in CO_2 fixation (data not shown), consistent with the inability of this strain to grow with CO_2 as the electron acceptor (5). The highest rate of CO_2 fixation for strain 16PHC was about 28.6 nmol/min/mg of protein, which is about 30% of the level of the wild-type strain growing photoheterotrophically or about 10% of the rate for photolithoautotrophically growing wild-type cells. The observed level of CO_2 fixation exhibited by strain 16PHC cannot support photolithoautotrophic growth of this strain, even when DMSO was added to the cultures (data not shown). Besides form I and form II RubisCO, another CO_2-fixing enzyme, pyruvate carboxylase, has been previously demonstrated in *R. sphaeroides* (23, 24). In *R. sphaeroides*, pyruvate carboxylase activity was found to be acetyl coenzyme A dependent (23, 24) and was present in the wild-type and mutant strains at levels of 5.6 to 7.9 nmol of CO_2 fixed per min per mg of protein. It would appear that this enzyme exists constitutively in *R. sphaeroides* since its activity did not vary in photoheterotrophically or photolithoautotrophically grown wild-type cells (data not shown). No phosphoenolpyruvate carboxylase was detected, in agreement with previous studies (23), and our efforts have thus far failed to detect CO_2 fixation enzymes of the reductive tricarboxylic acid cycle, α-ketoglutarate synthase and pyruvate synthase, as well as ATP-dependent citrate lyase under conditions in which these enzymes were readily demonstrable in extracts from *Chlorobium limicola* (41). Finally, preliminary experiments designed to determine the labeled products of short-term $^{14}CO_2$ fixation have indicated there is a significant different pattern of fixation for photoheterotrophically cultured (malate-grown) wild-type and strain 16PHC cells (41).

TABLE 2. Growth responses of *R. sphaeroides* wild-type and RubisCO deletion strains[a]

Strain	Doubling time (h)[b]	
	Without DMSO	With DMSO
HR	6.5	6.0
Form I deletion	7.0	7.5
Form II deletion	12.0	12.5
16	NG[c]	5.3
16PHC	14.0	13.5

[a] All strains were grown photoheterotrophically in 300-ml cultures with malate as the electron donor.
[b] Results of duplicate determinations.
[c] NG, no growth.

Effect of DMSO on CO_2 fixation and RubisCO synthesis under photolithoautotrophic growth conditions. RubisCO is often referred to as the most abundant protein on earth (1); in plants, algae, and photosynthetic bacteria, it constitutes up to 50% of the soluble protein (5, 26, 33). There has been

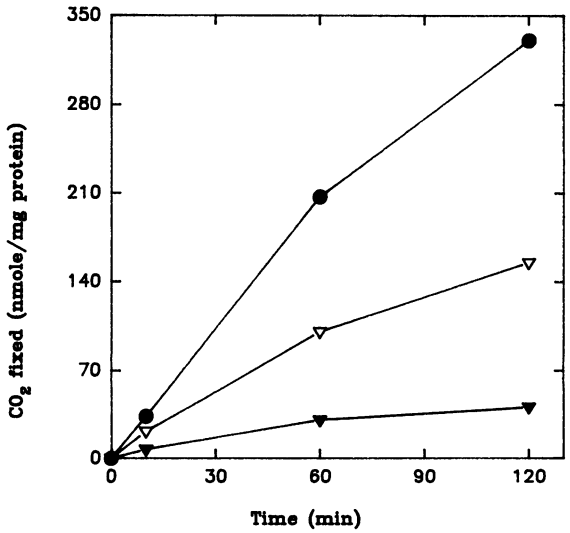

FIG. 3. Comparison of whole-cell CO_2 fixation of the form II deletion mutant (●), strain 16PHC (▽), and strain 16 (▼). Malate was used as the electron donor in all cases.

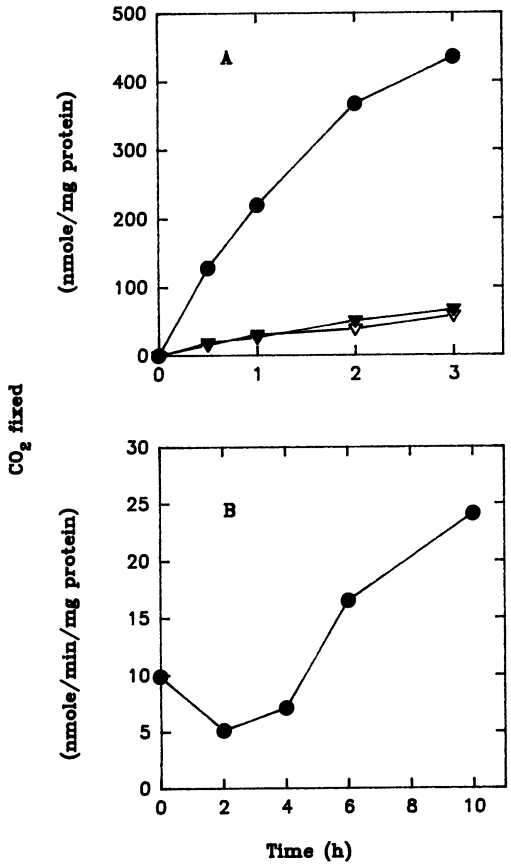

FIG. 4. Control of whole-cell CO_2 fixation in *R. sphaeroides* 16PHC. (A) Repression in the presence of DMSO (60 mM) of the wild type (strain HR) (●), strain 16PHC (▼), and strain 16 (▽); (B) induction of whole-cell CO_2 fixation in strain 16PHC after the removal of DMSO. Cells were cultured to early stationary phase under photoheterotrophic growth conditions in the presence of DMSO (4), harvested, and then washed with Ormerod medium in the absence of malate; CO_2 fixation rates were determined at 30°C in medium containing malate. In panel B, cells were cultured to early stationary phase in the presence of DMSO, washed with Ormerod medium in the absence of malate, and then incubated in malate medium lacking DMSO. At the indicated times, cells were withdrawn and assayed for whole-cell CO_2 fixation ability.

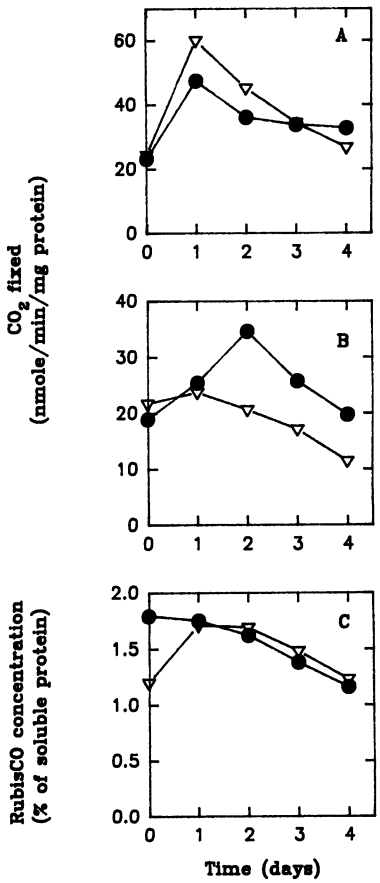

FIG. 5. Effect of DMSO on whole cell CO_2 fixation (A), whole-cell form I RubisCO activity (B), and form I RubisCO synthesis (C) in the form II deletion mutant of *R. sphaeroides*. Cells were grown photolithoautotrophically, washed, and resuspended in malate medium in the absence (●) or presence (▽) of 60 mM DMSO.

much speculation about the reason for this protein's abundance, including the fact that it possesses such poor catalytic capacity (low k_{cat}). In purple nonsulfur photosynthetic bacteria, it has been demonstrated that the addition of DMSO effectively replaces the function of RubisCO under photoheterotrophic growth conditions, in support of the idea that CO_2 may be important as an electron sink during metabolism (8, 14, 32, 36). However, it is not clear whether this suggested function for CO_2 fixation might apply under photolithoautotrophic growth conditions. We therefore determined the effect of DMSO addition on CO_2 fixation, RubisCO activity, and RubisCO synthesis in the form I and form II deletion mutants (Fig. 5 and 6). It is apparent that both strains were influenced and that the activity of the form II RubisCO enzyme (in the form I deletion mutant) was influenced most severely (Fig. 6). The addition of DMSO partially affected CO_2 fixation in the form II RubisCO-containing strain; however, there was a substantial loss of form II RubisCO activity which greatly exceeded the decrease in RubisCO protein (Fig. 6). In the form II deletion mutant, there was a much diminished effect on CO_2 fixation and whole-cell form I RubisCO activity (Fig. 5).

DISCUSSION

RubisCO catalyzes CO_2 fixation in most photosynthetic and chemolithoautotrophic organisms, and since RubisCO is usually the predominant CO_2 fixation enzyme (33), it is often used as an indicator of CO_2 fixation by these organisms. In previous experiments, we found that the whole-cell RubisCO activity in permeabilized *R. sphaeroides* cells was substantially higher than the activity determined in vitro (38–40). In the current investigation, we have observed that determinations of whole-cell in situ RubisCO activity greatly underestimated total whole-cell CO_2 fixation (Table 1). Since it has been previously demonstrated that 3-phosphoglyceric acid may not be the sole initial product of CO_2 fixation by purple nonsulfur bacteria, the current results and past studies indicate that alternative, non-RuBP-dependent pathways of CO_2 fixation may be physiologically significant (6, 19, 23, 24, 29, 41, 43). It is also apparent that cells grown under different physiological conditions prefer different electron donors to support CO_2 fixation (Fig. 1). Photoheterotrophically grown *R. sphaeroides* exhibited 30% of the CO_2 fixation capacity of photolithoautotrophically grown cells

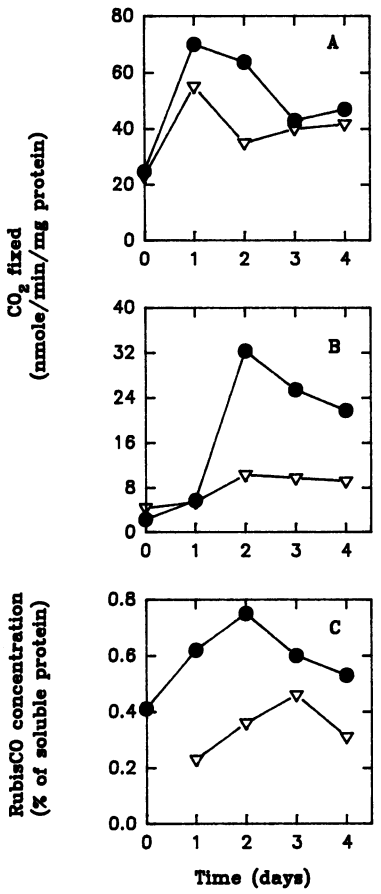

FIG. 6. Effect of DMSO on whole-cell CO_2 fixation (A), whole-cell form II RubisCO activity (B), and form II RubisCO synthesis (C) in the form I deletion mutant of *R. sphaeroides*. Cells were grown photolithoautotrophically, washed, and resuspended in malate medium in the absence (●) or presence (▽) of 60 mM DMSO.

(Fig. 2A); this is very close to the CO_2 fixation level of photolithoautotrophically grown cells incubated with malate as the electron donor. However, whole-cell RubisCO assays indicated that RubisCO activity in photoheterotrophically grown cells was only about 10% of the level found in photolithoautotrophically grown cells (Fig. 2B), suggesting that CO_2 fixation occurs mainly through alternative CO_2 fixation pathway(s) in photoheterotrophically grown cells. Several carboxylating enzymes have been detected in various photosynthetic bacteria (2, 9, 19, 23, 24), although thus far only pyruvate carboxylase and phosphoenolpyruvate carboxykinase have been demonstrated in *R. sphaeroides* (23, 24). Since *R. sphaeroides* cannot use phosphoenolpyruvate carboxykinase to produce oxaloacetate from phosphoenolpyruvate, and there is no phosphoenolpyruvate carboxylase and phosphoenolpyruvate synthase in this organism (23), *R. sphaeroides* must use pyruvate carboxylase to supply required C_4 dicarboxylic acids when grown on certain substrates.

It was originally suggested by Lascelles (20) that RubisCO synthesized in photoheterotrophically grown cells may be employed to maintain a proper intracellular redox balance; this hypothesis has been invoked in more recent studies as well (14). In *R. sphaeroides*, there are two structurally and catalytically distinct RubisCO enzymes, form I and II (12); the latter is similar to the enzyme found in *R. rubrum* (35). When the artificial electron acceptor DMSO was added to cultures grown photoheterotrophically, it was apparent that strains which contain only form I RubisCO showed decreased total CO_2 fixation but not RubisCO activity. Likewise, if the wild-type strain was incubated with DMSO for 10 days, the level of CO_2 fixation was greatly reduced, whereas RubisCO activity did not change appreciably (Table 1). Again, these results indicate that CO_2 fixation pathways other than the Calvin cycle are functional and respond to the addition of DMSO. Strain 16PHC, derived from the form I and form II RubisCO double-deletion mutant (strain 16), obviously does not possess any RubisCO activity; surprisingly, however, whole-cell CO_2 fixation was reduced to a very low level when cells were grown photoheterotrophically in the presence of DMSO (Fig. 2A). This finding may suggest that the alternative CO_2 fixation pathway, even though it dominates over the RubisCO pathway in photoheterotrophically grown wild-type cells, might require the presence of RubisCO to operate effectively. Whether this is a polar effect at the genetic level or the result of some physiological interaction remains to be determined.

Strain 16PHC, which was derived from the RubisCO double-deletion mutant, can grow photoheterotrophically on malate with CO_2 as the electron acceptor, albeit with a generation time significantly longer than that of the wild-type (Table 2). The CO_2 fixation rate shown by this strain was significantly elevated compared with that of strain 16, which cannot grow photoheterotrophically unless an alternative electron acceptor such as DMSO is added to the culture. The increased CO_2 fixation ability exhibited by strain 16PHC could be repressed by the addition of DMSO, and after the removal of DMSO from the culture, CO_2 fixation ability greatly increased to a level of up to 28.6 nmol of CO_2 fixed per min per mg of protein. This is about 30% of the CO_2 fixation rate of the wild type, which contains both form I and form II RubisCO. Obviously, however, this level of CO_2 fixation for strain 16PHC is not sufficient to support photolithoautotrophic growth in an H_2-CO_2 atmosphere. Perhaps the alternative CO_2 fixation pathway(s) does not provide a source of intermediates which are critical for cell growth; alternatively, the cell may not have the ability to balance the redox potential generated by growth in the 1.5% CO_2–98.5% H_2 atmosphere in the absence of RubisCO.

It has been reported that inactivation of one of the structural genes of nitrogenase in *R. capsulatus* led to the activation of a normally silent gene that replaced the function of the inactivated gene (27). Moreover, mutations in the structural genes encoding cytochrome c_2 in *R. capsulatus* (3) and *R. sphaeroides* (25) result in the biosynthesis and utilization of alternative electron carriers and electron transfer pathways in cytochrome c_2-deficient strains of both organisms. Since strain 16PHC has a stable phenotype, it will be interesting to elucidate the molecular basis for the physiological change exhibited by this strain. Preliminary indications support the activation of a silent gene(s) and are derived from the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis (41). Preliminary investigations have also indicated that the primary products of CO_2 fixation differ in strain PHC and the wild type when both are cultured in a malate medium in which CO_2 is the final electron acceptor (41). Identification of these products, along with elucidation of the enzymatic steps involved, is the subject of ongoing investigations.

It has been hypothesized that autotrophically grown cells

require high levels of RubisCO to compensate for the notoriously low catalytic turnover of this enzyme. Certainly increases in catalytic efficiency of the plant enzyme might lead to increased agricultural productivity (1). The experiments illustrated in Fig. 5 and 6 indicate that considerable amounts of RubisCO in the cell may be utilized to maintain the redox potential of the cell, which is greatly affected by the photosynthetic activity of the organism. DMSO, an alternative electron acceptor, could decrease both the form I and form II RubisCO activity from 40 to 70% in mid-exponential-phase cells without influencing the generation time substantially (Fig. 5B and 6B); indeed, form II RubisCO was influenced more severely than form I RubisCO, which is in accordance with the suggestion that form II RubisCO functions more in a redox-balancing role (5, 14). In addition, it should be stressed that DMSO does not influence the synthesis of form I RubisCO (Fig. 5C), yet the activity of the enzyme was obviously reduced, suggesting that there is some modification or alteration of the enzyme. Whether this inactivation is related to the organic acid-induced or nitrogen starvation-induced RubisCO inactivation previously investigated (16, 38–40) is not clear at this time. However, it is apparent that the regulation of RubisCO activity is closely related to the energy metabolism of the cell, and efforts designed to improve the efficiency of CO_2 fixation should take possible imbalances in the redox potential into consideration.

Pyruvate carboxylase activity was detected in all the strains derived from *R. sphaeroides* HR; however, it does not appear that pyruvate carboxylase could account for the rather substantial level of whole-cell CO_2 fixation detected in strain 16PHC relative to strain 16. In this connection, Payne and Morris (23) suggested that *R. sphaeroides* can synthesize C_4 dicarboxylic acids by a novel pathway which does not involve pyruvate carboxylase under certain growth conditions. We are currently attempting to use a combined biochemical and genetic approach to identify an alternative CO_2 fixation pathway(s) in photosynthetic bacteria, and we hope to elucidate factors which regulate the expression of both the Calvin cycle and alternative CO_2 fixation schemes. A primary goal is to determine the precise relationship of enhanced CO_2 fixation and the ability of strain 16PHC to grow with CO_2 as an electron acceptor under phototrophic growth conditions.

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