Roles of RubisCO and the RubisCO-Like Protein in 5-Methylthioadenosine Metabolism in the Nonsulfur Purple Bacterium *Rhodospirillum rubrum*† by Jaya Singh1,2,3 and F. Robert Tabita1,2,3*+ 

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Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme of the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway, catalyzes the assimilation of atmospheric CO₂ into organic matter. The discovery of a new family of enzymes, the RubisCO-like proteins (RLPs), has revealed structural homologs of RubisCO. RLPs are unable to catalyze CO₂ fixation. RLPs from Chlorobaculum tepidum, *Bacillus subtilis*, *Geobacillus kaustophilus*, and Microcystis aeruginosa have been shown to participate in sulfur metabolism. Whereas the precise function of C. tepidum RLP is unknown, the *B. subtilis*, *G. kaustophilus*, and *M. aeruginosa* RLPs function as tautomeras/enolases in a methionine salvage pathway (MSP). Here, we show that the form II RubisCO enzyme from the nonsulfur purple bacterium *Rhodospirillum rubrum* is also able to function as an enolase *in vivo* as part of an MSP, but only under anaerobic conditions. However, unlike *B. subtilis* RLP, *R. rubrum* RLP does not catalyze the enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate. Instead, under aerobic growth conditions, *R. rubrum* RLP employs another intermediate of the MSP, 5-methylthioribulose-1-phosphate, as a substrate, resulting in the formation of different products. To further determine the interrelationship between RubisCOs and RLPs (and the potential integration of cellular carbon and sulfur metabolism), the functional roles of both RubisCO and RLP have been examined *in vivo* via the use of specific knockouts and complementation studies of *R. rubrum*. The presence of functional, yet separate, MSPs in *R. rubrum* under both aerobic (chemoheterotrophic) and anaerobic (photoheterotrophic) growth conditions has not been observed previously in any organism. Moreover, the aerobic and anaerobic sulfur salvage pathways appear to be differentially controlled, with novel and previously undescribed steps apparent for sulfur salvage in this organism.

RubisCOs, the RLPs are unable to carry out CO₂/O₂ fixation because their sequences contain dissimilar residues at positions analogous to RubisCO’s active-site residues (25). The structures of the *Geobacillus kaustophilus* and *Chlorobaculum tepidum* RLPs have now been solved, and there are indeed differences between the tertiary structures of these two proteins and the bonafide RubisCO enzymes (14, 17, 25). Moreover, distinct patterns of active-site residue identities among the different clades of the RLP lineage suggest that these subgroups of RLPs are likely to utilize different substrates and perform dissimilar reactions (23, 25, 26).

Previous studies performed with the *Chlorobaculum tepidum* RLP (of the IV-Photo group) gave the first indication that the RLPs may be involved in some aspect of sulfur metabolism (12, 13). This was later substantiated when the precise function was established for the *Bacillus subtilis* (2), *Microcystis aeruginosa* (4), and *Geobacillus kaustophilus* (14) RLPs of the IV-YkrW group. All three proteins catalyze a tautomerase/enolase reaction of a methionine salvage pathway (MSP) in which the substrate 2,3-diketo-5-methylthiopentyl-1-phosphate (DK-MTP 1P) is converted to 2-hydroxy-3-keto-5-thiomethylpent-1-ene 1-phosphate (HK-MTP 1P) (Fig. 2). This reaction is very reminiscent of the enolization of RuBP catalyzed by RubisCO. Moreover, form II RubisCO from *Rhodospirillum rubrum* was shown to complement an RLP mutant strain of *B. subtilis*, with the ability to catalyze the identical tautomerase/enolase reaction (2). Interestingly, in addition to the presence of a form II
MSP. Several genes were indeed identified to encode homologs of known enzymes that participate in a conventional catalyzed by this RLP suggests that lose-5-phosphate, at a 3:1 ratio (15) (Fig. 2). The novel reaction for additional genes in the R. rubrum genome that might be functions largely unknown.

FIG. 1. Summary of the different classes of RubisCO found in nature so far (25). Forms I, II, and III catalyze bonafide CO₂/O₂ fixation reactions by using RuBP as the substrate. Form IV RubisCO (RLP) does not catalyze RuBP-dependent CO₂/O₂ fixation and is divided into six known clades (25), with only representatives of the type IV-YkrW and IV-DeepYkrW subgroups shown to catalyze defined, yet distinct, reactions (Fig. 2).

RubisCO gene (cbbM), the genome of R. rubrum also encodes an RLP that clusters with the IV-DeepYkrW group (25). The function of this protein was recently determined, and it was shown to catalyze a distinct reaction that uses 5-methylthioribulose-1-phosphate as the substrate (15). Via an unprecedented 1,3-proton transfer, with two successive 1,2-proton transfers from its substrate, R. rubrum RLP catalyzes the formation of two products, respectively.

The presence of an RLP-encoding gene triggered the search for additional genes in the R. rubrum genome that might be homologs of known enzymes that participate in a conventional MSP. Several genes were indeed identified to encode homologs of MSP enzymes. However, to this point there is no experimental evidence for the existence of a functional MSP (21) in R. rubrum. Thus, in this study, we sought to determine the role of the RLP and RubisCO protein in sulfur salvage since each protein catalyzes different reactions and RubisCO is known to be synthesized only under anaerobic conditions (6, 7). Moreover, it is well appreciated that R. rubrum possesses a versatile metabolic capacity and is able to grow under both anaerobic and aerobic conditions, using a variety of carbon sources. The involvement of RLP and RubisCO in sulfur salvage was thus determined and found to be associated with aerobic and anaerobic metabolism, respectively.

MATERIALS AND METHODS

Bacterial strains and growth conditions. R. rubrum strains used in the current study are Str-2 (wild type; a spontaneous streptomycin-resistant derivative of strain S1 [ATCC 11170]) and I19A (cbbM mutant; a form I RubisCO disruption strain [10]). PYE complex medium consisting of 0.3% peptone, 0.3% yeast extract, 10% Ormbero’s basal salts, and 15 μg of biotin per liter was used for aerobic chemoheterotrophic growth of R. rubrum in conjugation experiments. Ormbero’s medium (OM) (19), containing xta-malate as the carbon source, was used for all phototrophinocrotrophic growth experiments and was also used as the defined medium under aerobic chemoheterotrophic growth conditions. MTA (5-methylthioadenosine)-dependent growth was achieved with sulfur-depleted OM, prepared by replacing the sulfate salts with equimolar amounts of chloride salts. Antibiotics used for selection of R. rubrum mutants and transconjugants were kanamycin (50 μg ml⁻¹), gentamicin (10 μg ml⁻¹), tetracycline (36 μg ml⁻¹), and streptomycin (50 μg ml⁻¹).

Escherichia coli strain DH5α (Invitrogen) was used as the host strain for all the cloning procedures; strain SM-10 was used as the donor strain in the conjugation experiments (22). E. coli cultures were grown in Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl (wt/vol). Antibiotics used for plasmid selection in E. coli were ampicillin at 100 μg ml⁻¹, kanamycin at 50 μg ml⁻¹, gentamicin at 15 μg ml⁻¹, erythromycin at 30 μg ml⁻¹, and chloramphenicol at 30 μg ml⁻¹. Antibiotics and media components were purchased from either Sigma or Fisher. A list of all strains and plasmids used in this study is provided (Table 1).

MTA-dependent growth of R. rubrum. Single colonies were used to inoculate culture tubes containing Ormbero’s malate minimal medium under aerobic conditions at 30°C with shaking at 200 rpm. Growth proceeded until the mid-exponential phase (A₆₆₀nm = 0.6 to 0.8). Cells were harvested by centrifuging cultures at 12,000 × g for 3 min; cell pellets were washed three times with sulfur-depleted medium and then resuspended in the same medium. Washed cells were inoculated into sulfur-depleted malate minimal medium supplemented with MTA. As a negative control in all the experiments, cells were also inoculated into sulfur-depleted medium lacking any exogenous sulfur source. Anaerobic phototrophinocrotrophic MTA-dependent growth was accomplished by performing the same procedure described above, using cells grown chemoheterotrophically and then made anaerobic inside an anaerobic chamber (Coy Labs, Grass Lake, MI) that maintained an atmosphere of 2.5 to 3% hydrogen and balance nitrogen. Anaerobic media were prepared under a 100% nitrogen atmosphere and dispensed (10 ml per

FIG. 2. Distinct reactions catalyzed by type IV-YkrW (A) and type IV-DeepYkrW (B) classes of form IV RubisCO/RLP, exemplified by the proteins from B. subtilis and R. rubrum, respectively.
mid DNA from both E. coli growth experiments. Gentamicin, which are commercially available as sulfate salts, were not used for sulfur source, or without any sulfur source (negative control). The concentration of sulfur in the light at 27°C in a growth chamber (Environment Growth Chambers, Chagrin over the stopper (Bellco Glass Inc., Vineland, NJ). Anaerobic cultures were grown in the light at 27°C in a growth chamber (Environm

**TABLE 1. Bacterial strains and plasmids used in this study**

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<th>Strains</th>
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Molecular biology protocols. Genomic DNA was purified using the Wizard genomic DNA purification kit (Promega). Southern blot analysis and PCRIs were carried out using standard protocols (3, 18). All the genes were amplified from the genomic DNA of the respective organisms by performing PCR using either Taq DNA polymerase (Invitrogen) or Pfu DNA polymerase (Stratagene). Plasmid DNA from both E. coli and R. rubrum cells was isolated using a plasmid miniprep kit (Qiagen).

Inactivation of the rlpA (RLP) gene. The R. rubrum rlpA gene was amplified from genomic DNA by PCR with Pfu DNA polymerase (Invitrogen) by using primers 5′-CGAGGAGGCAGGATCGCGCCATCGG-3′ and 5′-GGGCCCCCTG CAGGAGGATCGGACTCC-3′. The PCR-amplified rlpA region was cloned into the pCR-Blunt II-TOPO vector (Invitrogen), resulting in plasmid pTBRrRLP. The rlpA gene was disrupted after insertion of an XmnI- and AfeI-digested genta

Inactivation of the 5-methylthioribose-1-phosphate isomerase (mtrI) gene. The mtrI gene was amplified from the genomic DNA of R. rubrum by using primers 5′-GGG GAACATATGTCGAGGCGATCGGCGCCATCGG-3′ and 5′-GGGCCCCCTG CAGGAGGATCGGACTCC-3′. The PCR-amplified mtrI region was cloned into the pCR-Blunt II-TOPO vector (Invitrogen), resulting in plasmid pTBRrMTRI. The mtrI gene was disrupted by inserting an Accl- and AfeI-digested gentami

Cloning for complementation studies. Plasmid pPR was constructed by cloning the putative promoter region of the R. rubrum rlpA gene into pRK415 (Table 1). The promoter region was amplified by PCR using a forward primer incorporating the NdeI site (5′-GGGCTGGTGAATATAGACGGTCCCGG-3′) and a reverse primer incorporating the BamHI site (5′-GGGCTGGTGAATATAGACGGTCCCGG-3′). The PCR product was digested with NdeI and BamHI and cloned into the corresponding sites in pRK415. This resulted in plasmid pRPS-MCS3, which was used for expressing various genes under the direction of the RLP promoter for all complementation studies. All the Rubisco and RLP genes, except the B. subtulis RLP gene, were amplified from the genomic DNA of R. rubrum by using primers 5′-GGG GAACATATGTCGAGGCGATCGGCGCCATCGG-3′ and 5′-GGGCCCCCTG CAGGAGGATCGGACTCC-3′. The PCR-amplified mtrI region was cloned into the pCR-Blunt II-TOPO vector (Invitrogen), resulting in plasmid pTBRrMTRI. The mtrI gene was disrupted by inserting an Accl- and AfeI-digested gentamicin resistance cassette into Accl- and SrfI-digested pPR. The disrupted gene was subcloned into the suicide vector pSUP202. The disrupted gene fragment was digested with EcoRI and inserted into the EcoRI site of pSUP202; this resulted in the formation of plasmid pSUP-MPlgM. Plasmid pSUP-MPlgM was transferred to wild-type R. rubrum by conjugation; transconjugants were selected for gentamicin resistance. The genotype of the mtrI disruption strain was confirmed by Southern blot analysis.
Bacterial conjugation and selection of transconjugants. Conjugation was performed by biparental matings. *R. rubrum* recipient strains were grown for 3 to 4 days in PYE (complex) medium to the late exponential or early stationary phase (optical density at 660 nm [OD$_{660}$] = 1.2 to 1.5); the cells were then diluted 1:10 and grown for 1 to 2 days until mid- to late exponential phase (OD$_{660}$ ~ 0.9 to 1.2). *E. coli* strain SM-10 was used as the donor strain for the matings. Overnight cultures of *E. coli* grown in LB medium with the appropriate antibiotics were diluted 1:10 in LB (without antibiotic) and incubated at 37°C with shaking at 220 rpm for 2 h. Matings were set up by combining recipient cells (1.0 ml) with donor cells (1.0 ml) in an Eppendorf tube and centrifuging the cells for 4 min at 13,600 × g in a microcentrifuge. This mating mixture pellet was resuspended in 30 µl of PYE medium, and the resuspension was spotted onto a PYE medium plate. Control plates containing either recipient cells only or donor cells with an empty plasmid (pPRK) without any insert were prepared as described above and included in each conjugation experiment. The mating PYE plates were incubated in the dark at 30°C overnight.

Following mating, cells from each plate were resuspended in 1 ml of PYE medium. Dilutions of 10$^{-1}$ to 10$^{-4}$ were plated onto PYE medium plates containing the appropriate antibiotics. The *R. rubrum* wild-type strain is resistant to streptomycin. Streptomycin was used as a counterselection for *E. coli* whenever wild-type *R. rubrum* was the recipient strain. Kanamycin and gentamicin were also used for counterselection when the RLP/RubisCO double disruption strain was used as the recipient. Selection was accomplished in all experiments by incubating plates in the dark at 30°C until colonies appeared (6 to 10 days). Colonies were grown in PYE or Ormerod’s malate medium broth, supplemented with the appropriate antibiotics, and used for further manipulations.

RESULTS

Correlation between the presence of RLP and a functional MSP. If an organism is grown on MTA as the sole sulfur source, needed sulfur-containing amino acids must be synthesized as a result of MTA metabolism via some type of sulfur salvage pathway or MSP. It was previously shown that both *R. rubrum* and *Rhodopseudomonas palustris* are capable of growth by using MTA as the sole sulfur source under aerobic chemoheterotrophic growth conditions, whereas neither *Rhodobacter sphaeroides* nor *Rhodobacter capsulatus* was able to metabolize MTA (25). All four organisms were able to grow on media containing methionine as the sole sulfur source, showing that *R. capsulatus* and *R. sphaeroides* do not lack the ability to metabolize methionine. Based on the abilities to metabolize MTA, it could thus be concluded that *R. rubrum* and *R. palustris* must have a mechanism to salvage sulfur from MTA, presumably via some form of MSP. Using *B. subtilis* as a paradigm, in order for *R. rubrum* and *R. palustris* to grow using MTA as the sole sulfur source, an enolase/tautomerase that would catalyze the conversion of DK-MTP 1P to HK-MTP 1P would be required as part of the MSP (21). This ability to use MTA correlated with the presence of one or more RLP genes in the genome of each of these organisms, genes which are not found in *R. capsulatus* or *R. sphaeroides* (25). Thus, our working hypothesis was that *R. capsulatus* and *R. sphaeroides* lacked any means to convert MTA to methionine, while both *R. rubrum* and *R. palustris* possess this metabolic capability by virtue of possessing RLP genes.

Role of RLP and RubisCO in MTA-dependent growth of *R. rubrum*. The RLP gene was disrupted after insertion of a gentamicin gene cassette within the coding sequence via homologous recombination; the genotype of this strain was confirmed after Southern blot analysis (data not shown). The rlpA gene was disrupted both in the wild type as well as in a form II RubisCO disruption (*cbbM* mutant) background. Compared to their respective parent strains, single rlpA disruption and RLP/RubisCO double-disruption strains did not show any apparent phenotypic difference when grown under either phototrophic or chemoheterotrophic conditions by using ammonium sulfate, a readily assimilable sulfur source. The rlpA disruption strain of *R. rubrum* was incapable of using MTA as the sole sulfur source under aerobic growth conditions (Fig. 3A), indicating that RLP is required for me-
tabolizing MTA. The form II RubisCO disruption strain (cbbM mutant) was able to grow using MTA as the sole sulfur source under these conditions. As expected, the RubisCO/RLP double disruption strain (cbbM rlpA mutant) was unable to carry out aerobic MTA-dependent growth (Fig. 3A).

Because RubisCO expression and function in *R. rubrum* require anaerobiosis (6), we decided to first test for the presence of a functional MSP under anaerobic phototrophospherocrop growth conditions with the wild-type strain. As shown in Fig. 3B, the wild type is able to utilize MTA (Fig. 3B). This was surprising because the known MSP of *B. subtilis* has an oxygen-requiring enzymatic step (21). Further analysis showed that the *R. rubrum* rlpA mutant strain, which cannot metabolize MTA as a sulfur source under aerobic chemoheterotrophic growth conditions (Fig. 3A), was able to grow in the same medium under anaerobic phototrophospherocrop growth conditions (Fig. 3B). It was also observed that the *R. rubrum* cbbM mutant (form II Rubisco deletion) strain, which still had a functional RLP, was barely able to grow on MTA media under anaerobic conditions, indicating the potential involvement of Rubisco (instead of RLP) in MTA metabolism under these growth conditions (Fig. 3B). The Rubisco/RLP double disruption strain (cbbM rlpA mutant) was unable to metabolize MTA under anaerobic phototrophospherocrop conditions, further confirming the requirement of a functional Rubisco under these growth conditions (Fig. 3B).

**Complementation of *R. rubrum* cbbM rlpA strain under aerobtic growth conditions.** Because of its inability to metabolize MTA under either aerobic or anaerobic growth conditions, the cbbM rlpA strain was used for complementation experiments. Different RLPS and Rubiscos were tested for their ability to support MTA-dependent growth in the cbbM rlpA strain by expressing them on plasmid pPRR, which is a derivative of pRK415 (see Materials and Methods). Plasmid pPRR was constructed by cloning the presumptive upstream promoter region of the rlpA gene from *R. rubrum* into pRK415 (10) (Table 1); if this sequence did, in fact, contain the *R. rubrum* rlpA promoter, it would ensure similar levels of transcription for all the different genes. Genes encoding the form I and form II Rubisco enzymes from *R. palustris* and the RLPS from *R. rubrum* (RrRLP), *C. tepidum* (CtRLP), *B. subtilis* (BsRLP), and *R. palustris* (RpRLP1) were tested for their ability to support MTA-dependent growth under aerobic growth conditions. As expected, the *R. rubrum* rlpA gene, when expressed on the plasmid, was able to complement for MTA-dependent growth of the cbbM rlpA mutant strain, indicating that a promoter sequence was present within the upstream region that was used to construct the expression plasmid. *B. subtilis* ykrW (*mtnW*) was able to partially rescue the MTA-dependent growth phenotype, whereas none of the other genes were able to complement for MTA-dependent growth (Table 2; see also Fig. S1 in the supplemental material).

**Complementation of the cbbM rlpA disruption strain under anaerobic growth conditions.** When expressed on a plasmid (pRRS-RcbbM), the *R. rubrum* form II Rubisco (cbbM) gene was able to rescue the MTA-dependent growth phenotype of the cbbM rlpA strain under anaerobic growth conditions (Fig. 3C), further confirming the results observed with the rlpA mutant strain. The *R. palustris* form II Rubisco (RpCbbM), which resembles RrCbbM, was also able to support MTA-dependent growth under anaerobic conditions in the cbbM rlpA strain of *R. rubrum* (Fig. 3D).

Previously, a mutation in an essential residue (Ile-164) of *R. rubrum* CbbM was shown to retain only 1% of the wild-type level of activity (5). Mutant *R. palustris* CbbM constructs which had point mutations in the same Rubisco active-site residue were available in the laboratory. This residue, Ile-165 in *R. palustris* CbbM, was substituted with different residues, and some of the resultant mutants were severely compromised with regard to their ability to fix CO2. When introduced into a Rubisco deletion strain of *Rhodobacter capsulatus* (20), the wild-type RpCbbM was able to complement for Rubisco function, whereas an I165T mutant was unable to do so (S. Satagopan and F. R. Tabita, unpublished results). In order to determine if this residue, which is critical for Rubisco function, is also required for alleviating the MTA phenotype of the cbbM rlpA strain, the pRRS-MCS-I165T construct was conjugated into the cbbM rlpA strain of *R. rubrum*. The resultant strain was able to grow on MTA medium under anaerobic phototrophospherocrop conditions (Fig. 3D), indicating that this Rubisco-compromised mutant protein was functional with regard to MTA metabolism (summarized in Table S2 in the supplemental material).

**Disruption of the *R. rubrum* 5-methylthioribose-1-phosphate isomerase (*mtrI*) gene.** The *mtrI* gene that encodes 5-methylthioribose-1-phosphate isomerase catalyzes the reaction that results in the formation of 5-methylthio-ribulose-1-phosphate (MTRu 1P). Homologs of this enzyme are present in both *R. rubrum* (Rp 0360) and *R. palustris* (RP4 4820) (Fig. 4). Although *R. rubrum*’s RLP and Rubisco appear to have functional differences when grown under aerobic and anaerobic conditions, it is possible that the *mtrI* homolog catalyzes a reaction that would be critical for MTA metabolism under both aerobic and anaerobic conditions. To test this possibility, the *mtrI* gene was disrupted by insertion of a gentamicin cassette into the open reading frame, using a strategy that is similar to the generation of the rlpA disruption strain. The resultant mutant was tested for its ability to metabolize MTA. It was unable to support MTA-dependent growth under either aerobic chemoheterotrophic or anaerobic phototrophospherocrop conditions (see Fig. S2A and S2B, respectively, in the supplemental material). These results provided further evidence for the necessity of some form of

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* + growth; –, no growth; ND, not determined. The genes expressed on plasmids for complementation are underlined.
MTA metabolic pathway under both aerobic and anaerobic growth conditions. Furthermore, the mtrI gene product catalyzes a reaction that appears to be critical to this pathway under both aerobic and anaerobic conditions.

**DISCUSSION**

MTA is a by-product of spermidine biosynthesis, as well as acyl homoserine lactone and ethylene biosynthesis. In most organisms, including plants and humans, MTA is converted back to methionine by an MSP (21). Whereas the RLPs of the IV-YkrW group catalyze enolization (tautomeration) of DK-MTP 1P as part of the MSP (2, 4, 11, 14) (Fig. 4), it is surprising that the form II RubisCO (CbbM) from *R. rubrum* could also function as a DK-MTP 1P tautomerase/enolase in vitro and could complement for the loss of RLP function in *B. subtilis* (2). Curiously, *R. rubrum* RLP uses a different substrate and was shown to catalyze a novel isomerization reaction.
whereby 5-methylthio-d-ribulose 1-phosphate is converted to a 3:1 mixture of 1-methylthio-xylulose 5-phosphate and 1-methylthioribulose 5-phosphate (15) (Fig. 2). These findings suggested that both RLP and Rubisco may have different physiological roles relative to MTA metabolism in _R. rubrum_. We show here that MTA may serve as the sole sulfur source under both aerobic and anaerobic growth conditions. Although the presence of an MSP under anaerobic growth conditions has long been speculated, there has been no evidence reported thus far. Because of the presence of an oxygen-requiring dioxygenase step in currently constituted MSP schemes (Fig. 4) (21), it is not possible for this traditional pathway to function in the absence of oxygen in either _R. rubrum_ or _R. palustris_, both of which have been shown to metabolize MTA under these conditions (23). Moreover, it appears that the _mtr_ gene is functional and required for both aerobic and anaerobic MTA metabolism. It is thus possible that the product of this reaction is ultimately metabolized into methionine via a mechanism which bypasses the dioxygenase step of the current MSP paradigm (21). It is interesting that homologs of genes whose products would catalyze the reactions beyond the RLP-catalyzed step are absent within the genomes of both _R. rubrum_ and _R. palustris_, suggesting that the aerobic part of this pathway may also function differently from the current _B. subtilis_ MSP paradigm (Fig. 4).

We have shown that the RLP disruption strain of _R. rubrum_ is incapable of MTA-dependent growth under aerobic growth conditions. Although an intact _cbbM_ gene is present in this strain, it is barely expressed under aerobic growth conditions (6). Further, _R. rubrum_ cells that do contain exogenously expressed Rubisco were found to oxidatively inactivate and then subsequently degrade this protein under aerobic conditions (6, 7). It was thus not surprising that the _rlpA_-disruption strain failed to metabolize MTA in the presence of oxygen. The inability of form I (CbbLS) and form II (CbbM) Rubisco to support aerobic MTA-dependent growth of the _cbbM rlpA_ mutant strain of _R. rubrum_ (Table 2; see also Fig. S1 in the supplementary material) may also be attributed to either poor gene expression or oxidative inactivation of the proteins or may perhaps be due to the fact that these proteins are inherently unable to catalyze the RLP-type reaction. In contrast, the results from complementation to aerobic MTA-dependent growth with different RLP genes in the _cbbM rlpA_ strain (Table 2) seem to be reflective of the differences between the putative active-site residue sets used by different subgroups of RLPs. It may also be attributable to the differences in other structural features (17, 25). Only _B. subtilis ykrW_ (_mtnW_) showed some ability to complement the _cbbM rlpA_ strain under these conditions. Inasmuch as _B. subtilis_ and _R. rubrum_ RLPs catalyze reactions that involve different substrates, it is apparent that the products of both reactions are somehow incorporated into a pathway that allows for aerobic MTA-dependent growth.

Both the _R. rubrum_ and _R. palustris cbbM_ genes were able to support MTA-dependent growth of the _cbbM rlpA_ strain when expressed on a broad-host-range plasmid under anaerobic phototrophic growth conditions. This is undoubtedly due to the fact that _R. rubrum_ form II Rubisco (CbbM) can catalyze the DK-MTP 1P reaction (2) (and by extension, so does _R. palustris_ CbbM). Based on knowledge of the structure and reaction mechanism employed by Rubisco, it is apparent that both substrates interact at the same active site (2, 14, 17, 25). Interestingly, the Rubisco active-site mutant ( _R. palustris cbbM_ I165T mutant), which is compromised in its ability to fix CO₂, is able to complement for the MTA phenotype. This indicates that active-site residues required for Rubisco function are not necessarily equivalent for the enolase/tautomerase reaction of the anaerobic MSP catalyzed by form II Rubisco. Residue Ile-165 is in van der Waals contact with two other Rubisco active-site residues, K191 and D193, and magnesium ions (5). Clearly, as shown by the _in vivo_ complementation studies, the interaction of these residues may not be critical for binding of the substrate DK-MTP 1P used in MTA metabolism.

Most importantly, it appears that Rubisco catalyzes two separate reactions in _R. rubrum_ and thus appears to participate in both carbon (via the CBB CO₂ assimilatory cycle) and sulfur (via an MSP) metabolism. Clearly, MTA-dependent growth in _R. rubrum_ requires RLP under aerobic growth conditions, but the organism obligatorily requires Rubisco to grow with MTA as the sole sulfur source under anaerobic growth conditions. These findings suggest that the genes and at least one of the proteins required for MTA-dependent growth are likely to be differentially regulated in _R. rubrum_, and this scenario is probably the same for _R. palustris_ as well. Clearly, these studies are indicative of the plasticity of Rubisco’s active site to function in a physiologically relevant fashion in two separate and important pathways. Further work on the mechanism of the reaction catalyzed by Rubisco in anaerobic MTA metabolism will help in understanding how the different residues interact with diverse substrates that are turned over by the Rubisco active site.

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