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Deduced Amino Acid Sequence, Functional Expression, and Unique Enzymatic Properties of the Form I and Form II Ribulose Bisphosphate Carboxylase/Oxygenase from the Chemoautotrophic Bacterium *Thiobacillus denitrificans*

JULIA M. HERNANDEZ,[1] STEFANIE H. BAKER,[2] STANLEY C. LORBACH,[2] JESSUP M. SHIVELY,[2] AND F. ROBERT TABITA[1,3,4]*

The Ohio State Biochemistry[1] and Plant Molecular Biology/Biotechnology[3] Programs and the Department of Microbiology,[4] The Ohio State University, Columbus, Ohio 43210-1292, and the Department of Biological Sciences, Clemson University, Clemson, South Carolina 29634[2]

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The *cbbL cbbS* and *cbbM* genes of *Thiobacillus denitrificans*, encoding form I and form II ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), respectively, were found to complement a RubisCO-negative mutant of *Rhodobacter sphaeroides* to autotrophic growth. Endogenous *T. denitrificans* promoters were shown to function in *R. sphaeroides*, resulting in high levels of *cbbL cbbS* and *cbbM* expression in the *R. sphaeroides* host. This expression system provided high levels of both *T. denitrificans* enzymes, each of which was highly purified. The deduced amino acid sequence of the form I enzyme indicated that the large subunit was closely homologous to previously sequenced form I RubisCO enzymes from sulfur-oxidizing bacteria. The form I *T. denitrificans* enzyme possessed a very low substrate specificity factor and did not exhibit fallover, and yet this enzyme showed a poor ability to recover from incubation with ribulose 1,5-bisphosphate. The deduced amino acid sequence of the form II *T. denitrificans* enzyme resembled those of other form II RubisCO enzymes. The substrate specificity factor was characteristically low, and the lack of fallover and the inhibition by ribulose 1,5-bisphosphate were similar to those of form II RubisCO obtained from nonsulfur purple bacteria. Both form I and form II RubisCO from *T. denitrificans* possessed high K_{CO_2} values, suggesting that this organism might suffer in environments containing low levels of dissolved CO_2. These studies present the initial description of the kinetic properties of form I and form II RubisCO from a chemoautotrophic bacterium that synthesizes both types of enzyme.

Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) is one of the two unique enzymes of the reductive pentose phosphate pathway, or Calvin-Bassham-Benson cycle. In this pathway, RubisCO functions to catalyze the actual CO_2 assimilatory step to convert RuBP and CO_2 into two molecules of 3-phosphoglyceric acid via a six-carbon carboxylated intermediate. RubisCO also may act as an internal monooxygenase, resulting in the formation of one molecule each of 3-phosphoglycerate and 2-phosphoglycolate, the latter of which is converted to glycolate by phosphoglycolate phosphatase and further metabolized via the photorespiratory pathway. These two reactions follow a sequential and ordered mechanism in which enzyme-bound RuBP is converted to an enediolate which then reacts with CO_2 or O_2 (15, 38). In most cases, RubisCO is a hexadecamer with a native molecular weight of about 550,000 and is composed of two kinds of subunits, eight large catalytic and eight small subunits with molecular weights of about 56,000 and 15,000, respectively. This type of RubisCO is called form I (or type I) and is present in nearly all eukaryotic photosynthetic organisms and virtually all bacteria that use the Calvin cycle to fix CO_2 (50). A second type, form II (or type II), which is composed of only large (M_r, ~56,000) subunits has also been described. The number of subunits in form II enzymes varies with the organism and ranges from two in *Rhodospirillum rubrum* (55) to eight as *Rhodopseudomonas palustris* (50). Extensive structural (44) and catalytic/mutagenesis studies (15, 16) have made the *R. rubrum* enzyme the paradigm for structure-function studies of RubisCO. Other form II RubisCO enzymes have been found in organisms that also express the form I enzyme. Both forms were originally found in *Rhodobacter sphaeroides*, from which form I and form II RubisCO enzymes with different molecular weights and catalytic properties were isolated (12). A single gene encoding the form II enzyme was cloned (34, 40) and sequenced (57), and the deduced amino acid sequence showed that form II RubisCO is highly homologous to the *R. rubrum* enzyme and that it contained several highly conserved regions among the L_8S_8 and L_2 enzymes (10). Other photosynthetic bacteria were also found to contain two forms of RubisCO (51). Recently, evidence for separate genes encoding the two forms of RubisCO was established in such aerobic and facultatively anaerobic chemoautotrophic bacteria as *Thiobacillus denitrificans* (6), *Thiobacillus intermedius* (49), and *Hydrogenovibrio marinus* (3). Since these organisms are classified in different evolutionary branches and they normally are found in environments that are vastly different from those in which nonsulfur purple bacteria are found, it was reasoned that analysis of the RubisCO enzymes from these organisms might provide further insights into how structure is related to function. For example, recent studies indicate that form I RubisCO of marine non-

* Corresponding author. Mailing address: Department of Microbiology, The Ohio State University, 484 W. 12th Ave., Columbus, OH 43210-1292. Phone: (614) 292-4297. Fax: (614) 292-6337. Electronic mail address: Tabita.1@osu.edu.

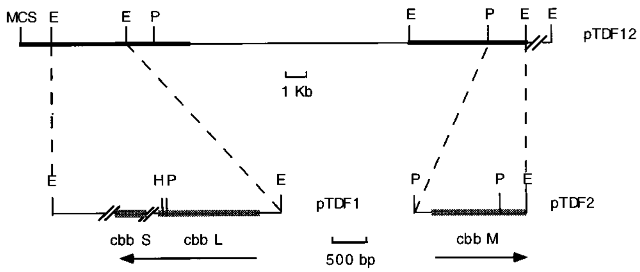

FIG. 1. Map of the *Eco*RI inserts in plasmids pTDF1, pTDF2, and pTDF12. MCS refers to the multiple cloning site of pLARF5. The thick lines indicate areas that have been completely mapped. The shaded boxes represent genes; the arrows indicate the directions of transcription of the genes. E, *Eco*RI; P, *Pst*I; H, *Hin*dIII.

green algae has an extremely high specificity factor (or τ value); presumably, the enzyme from these organisms evolved this property, since such organisms have no capability to metabolize phosphoglycolate, the product of the oxygenase reaction of RubisCO (42). As τ provides a measure of the ability of RubisCO to discriminate between CO_2 and O_2, considerable effort has been expended by many laboratories to ascertain the structural determinants and mechanism by which CO_2 and O_2 specificity is conferred (15, 16). Inasmuch as the specificity of RubisCO may evolve according to the environment in which the host organism is found (42), it was deemed particularly cogent to examine the properties of both form I and form II RubisCO from *T. denitrificans*, a chemoautotrophic bacterium capable of both aerobic and anaerobic CO_2 fixation. Certainly, for form II RubisCO, only the enzyme from *R. rubrum* and *R. sphaeroides* has been thoroughly studied; thus, examination of the properties of form II RubisCO from an organism capable of aerobic CO_2-dependent growth would be especially interesting.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Plasmids pTDF1, pTDF2, and pTDF12, which carry *cbbL cbbS* (form I), *cbbM* (form II), or both the *cbbL cbbS* and *cbbM* genes, respectively (Fig. 1), were isolated from a pLAFR5 genomic DNA library of *T. denitrificans* by hybridizing colony lifts with heterologous *cbbL* and *cbbM* probes (6). The *cbbL cbbS* and *cbbM* genes are oriented in opposite directions and are separated by about 17 kb. The three cosmid clones encompass approximately 53 kb of DNA; other Calvin cycle (*cbb*) genes were not detected in this region. The three cosmid clones were transformed into *Escherichia coli* S17-1 (45) for delivery into *R. sphaeroides* RubisCO deletion strain 16 (7). *E. coli* strains were grown at 37°C in Luria-Bertani medium (31) containing tetracycline at 12.5 μg/ml for the LE392 cultures and streptomycin at 30 μg/ml for S17-1. Cultures of *R. sphaeroides* 16 complemented with each of the three constructs were grown under aerobic conditions in a peptone-yeast extract (PYE) medium (58) or photosynthetically in Ormerod's medium (37). Photoheterotrophic cultures were grown in 0.4% malate in 9- or 22-ml screw-cap tubes or in 400-ml bottles bubbled with argon. Photoautotrophic cultures were grown in 400-ml bottles bubbled with 1.5% CO_2 and 98.5% H_2, as previously described (20). For *R. sphaeroides* cultures grown in PYE, antibiotics were added as follows: tetracycline, 12.5 μg/ml; streptomycin, 30 μg/ml; trimethoprim, 250 μg/ml; and kanamycin, 50 μg/ml. For those grown photosynthetically, trimethoprim at 200 μg/ml and kanamycin at 25 μg/ml were added.

DNA manipulations and conjugation techniques. Plasmids were isolated by the rapid alkaline extraction procedure (2). Transformations of *E. coli* with plasmid DNA were done by standard procedures. Mobilization of plasmids to *R. sphaeroides* was done as previously described (58), except that 0.5 volume of donor was used; filters were resuspended in 2 ml of phosphate buffer, and 100 μl of this suspension was plated onto selective medium.

DNA sequencing. Three plasmids were used for the sequencing of *cbbL cbbS*: pTDFIE, which carries a 3.5-kb *Eco*RI fragment in pUC18, and subclones pTDFIHE1.7 and pTDFIHE1.8, both containing *Hin*dIII-*Eco*RI fragments in pT7/T3α18 (6) (Fig. 1). Two plasmids were used for the sequencing of *cbbM*: pTDFIIE, which contains a 4.7-kb *Eco*RI fragment, and pTDFIIP, which contains a 1.3-kb *Pst*I fragment; both fragments were cloned in pUC18 (6) (Fig. 1). Oligonucleotide primer walking was used to generate complete double-stranded sequences with overlaps for both genes. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, Iowa). Automated sequencing was accomplished with an ABI Taq DyeDeoxy Cycle sequencing kit, a Perkin-Elmer Cetus DNA thermal cycler, and an ABI 373a DNA sequencer.

Preparation of crude cell extracts. Cells grown photolithoautotrophically were harvested at early stationary phase by centrifugation at 4°C. The cells were washed once in TEM buffer (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM β-mercaptoethanol) and stored at −70°C. Cell pellets were resuspended in 20 ml (1 g of cells per ml) of 1 mM phenylmethanesulfonyl fluoride in TEM buffer. Cells were lysed by passing them twice through a French pressure cell at 15,000 lb/in^2. Cell debris and unbroken cells were removed by centrifugation at 20,000 × *g* and 4°C. Then, 1 M $MgCl_2$ was added to the supernatant to a final concentration of 50 mM, and the extract was incubated at 50°C for 10 min. After the extract was cooled in an ice bath for 10 min, denatured proteins and additional debris were removed by centrifugation at 20,000 × *g* and 4°C for 10 min (12). Extracts were stored at −70°C if they were not used immediately.

Purification of RubisCO enzymes from *T. denitrificans*. The synthesis of the *T. denitrificans* RubisCO enzymes in *R. sphaeroides* was initially detected by immunoblotting extracts and probing with antibodies to form II RubisCO from *R. sphaeroides* and form I RubisCO from the cyanobacterium *Synechococcus* sp. strain PCC 6301. Crude extracts were divided into volumes containing 40 to 50 mg of protein and loaded onto 0.2 to 0.8 M linear sucrose gradients prepared in 40-ml polyallomar centrifuge tubes. Gradients were centrifuged in a Beckman SW28 swinging-bucket rotor at 20,000 rpm and 4°C for 20 h (56). Fractions (1.0 ml) were collected and assayed for carboxylase activity. Active fractions were pooled and concentrated with Centriprep30 concentrators (Amicon Inc., Beverly, Mass.). In earlier preparations, an ammonium sulfate fractionation step (45 to 90% saturation) was included before the sucrose gradients. Eventually, this step was omitted in order to obtain better yields. The protein solution was subsequently fractionated by fast protein liquid chromatography with a model GP-250 gradient programmer (Pharmacia LKB Biotechnology Inc., Milwaukee, Wis.) and a Mono Q HR 10/10 anion-exchange column. The column was equilibrated with 2 mM phosphate buffer, pH 8.0, and washed with 100 mM phosphate buffer before the enzyme was eluted with a linear gradient containing 100 to 300 mM phosphate buffer. This gradient was modified for extracts containing both forms of RubisCO so that form I and form II could be separated (the first elution was done with a 100 to 180 mM gradient, the wash was done with 180 mM phosphate buffer, and then another elution was done with a 180 to 300 mM gradient). Fractions (1.0 ml) were collected, and those fractions corresponding to peaks of absorption at 280 nm were assayed for carboxylase activity. Active fractions were pooled and concentrated with Centriprep30 concentrators. The purity of each preparation was monitored by both nondenaturing polyacrylamide gel electrophoresis (PAGE) (performed at 4°C) and sodium dodecyl sulfate (SDS)-PAGE. The acrylamide concentration was 6 or 7.5% for nondenaturing gels and 15% for SDS-PAGE gels. Proteins were visualized with Coomassie blue (0.05%) in 50% methanol and 10% acetic acid.

Kinetic measurements. The CO_2/O_2 ratio specificity factor (τ) was determined by the standard method (19, 47) as previously described (41). All calculations were made with an O_2 concentration of 1.23 mM for 100% O_2-flushed reactions. Concentrations of CO_2 were determined from the concentration of HCO_3^-, with 6.12 being used as the pK′ of CO_2/HCO_3^- at equilibrium. K_{RuBP}, K_{CO_2}, V_{CO_2}, and K_{O_2} were determined as previously described (41).

Fallover assay. The enzymes were incubated with 20 mM $NaH^{14}CO_3$ and 10 mM $MgCl_2$ in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.0) for 5 min at 30°C. RuBP was added to a final concentration of 4 mM. Aliquots containing enzyme were taken at various times (0 to 40 min) after the addition of RuBP, and the reaction was terminated with 100 μl of propionic acid.

ER activation experiment. Nonactivated enzymes were prepared after gel filtration of the proteins through an Econo-Pac 10DG column (Bio-Rad) equilibrated with 80 mM HEPES buffer, pH 8.0. RuBP, at 0.5 mM, was then added to the nonactivated form of RubisCO and incubated in 80 mM HEPES buffer, pH 8.0, for 30 min at 25°C. The RuBP-bound unactivated enzymes (ER) were then activated in 20 mM $NaHCO_3$, 10 mM $MgCl_2$, and 4 mM RuBP. Samples containing enzyme were taken at various times (30 s to 30 min) after activation and assayed for carboxylase activity for 1 min as described previously (12).

Sensitivity to effector molecules. Enzymes were preincubated in 0.8 mM RuBP or 20 mM $NaH^{14}CO_3$ and 10 mM $MgCl_2$ in 80 mM HEPES buffer (pH 8.0) for 30 min at 30°C. After the 30-min incubation, the missing substrate was added and the reaction was terminated at different times (30 s to 10 min) upon the addition of 100 μl of propionic acid. The effect of 6-phosphogluconate (PGN) at various concentrations (0 to 1.5 mM) was determined after it was incubated with 2 μg of enzyme in 20 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, and 80 mM HEPES buffer (pH 8.0) for 5 min. A standard carboxylase assay was performed, and the specific activity was determined (12).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the GenBank-EMBL data bank under accession numbers L42940 (*cbbL cbbS*) and L37437 (*cbbM*).

A

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CCC CAG CCC TGA ACA CGA TAA CCG TTC ACG CAG GAG TCA GTC ATG GAT CAA TCC GCA CGT      60
                                                        Met Asp Gln Ser Ala Arg
                                                    cbbM ---->

TAC GCA GAC CTC TCG CTC AAG GAA GAG GAC CTG ATC AAG GGT GGC CGC CAC ATC CTG GTC     120
Tyr Ala Asp Leu Ser Leu Lys Glu Glu Asp Leu Ile Lys Gly Gly Arg His Ile Leu Val

GCC TAC AAG ATG AAA CCG AAG TCC GGC TAC GGC TAC CTC GAG GCC GCC GCG CAC TTC GCC     180
Ala Tyr Lys Met Lys Pro Lys Ser Gly Tyr Gly Tyr Leu Glu Ala Ala Ala His Phe Ala

GCC GAG TCG TCG ACC GGT ACC AAC GTC GAA GTC TCG ACG ACC GAC GAC TTC ACC AAG GGC     240
Ala Glu Ser Ser Thr Gly Thr Asn Val Glu Val Ser Thr Thr Asp Asp Phe Thr Lys Gly

GTC GAC GCG CTC GTC TAC TAC ATC GAC GAA GCC AGC GAA GAC ATG CGG GTC GCC TAT CCG     300
Val Asp Ala Leu Val Tyr Tyr Ile Asp Glu Ala Ser Glu Asp Met Arg Val Ala Tyr Pro

CTC GAG CTG TTC GAC CGT AAC GTC ACC GAC GGC CGT TTC ATG CTG GTC TCG TTC CTG ACG     360
Leu Glu Leu Phe Asp Arg Asn Val Thr Asp Gly Arg Phe Met Leu Val Ser Phe Leu Thr

CTC GCG ATC GGC AAC AAC CAG GGC ATG GGT GAC ATC GAA CAC GCC AAG ATG ATC GAC TTC     420
Leu Ala Ile Gly Asn Asn Gln Gly Met Gly Asp Ile Glu His Ala Lys Met Ile Asp Phe

TAC GTG CCC GAG CGC TGC ATC CAG ATG TTC GAC GGC CCC GCG ACC GAC ATC TCC AAC CTG     480
Tyr Val Pro Glu Arg Cys Ile Gln Met Phe Asp Gly Pro Ala Thr Asp Ile Ser Asn Leu

TGG CGC ATC CTC GGC CGC CCG GTG GTC AAC GGC GGT TAC ATC GCC GGC ACC ATC ATC AAG     540
Trp Arg Ile Leu Gly Arg Pro Val Val Asn Gly Gly Tyr Ile Ala Gly Thr Ile Ile Lys

CCC AAG CTC GGT CTG CGT CCC GAG CCG TTC GCC AAG GCC GCC TAC CAG TTC TGG CTC GGT     600
Pro Lys Leu Gly Leu Arg Pro Glu Pro Phe Ala Lys Ala Ala Tyr Gln Phe Trp Leu Gly

GGC GAC TTC ATC AAG AAC GAC GAA CCC CAG GGC AAC CAG GTC TTC TGC CCG CTG AAG AAG     660
Gly Asp Phe Ile Lys Asn Asp Glu Pro Gln Gly Asn Gln Val Phe Cys Pro Leu Lys Lys

GTG CTG CCG CTC GTG TAC GAC GCG ATG AAG CGC GCG CAG GAC GAT ACC GGT CAG GCC AAG     720
Val Leu Pro Leu Val Tyr Asp Ala Met Lys Arg Ala Gln Asp Asp Thr Gly Gln Ala Lys

CTG TTC TCG ATG AAC ATC ACC GCT GAC GAC CAC TAC GAG ATG TGC GCC CGC GCC GAC TAC     780
Leu Phe Ser Met Asn Ile Thr Ala Asp Asp His Tyr Glu Met Cys Ala Arg Ala Asp Tyr

GCG CTC GAA GTC TTC GGC CCC GAC GCC GAC AAG CTG GCG TTC CTG GTC GAC GGC TAC GTC     840
Ala Leu Glu Val Phe Gly Pro Asp Ala Asp Lys Leu Ala Phe Leu Val Asp Gly Tyr Val

GGC GGC CCC GGC ATG GTG ACC ACG GCC CGT CGG CAG TAC CCC GGC CAG TAC CTG CAC TAC     900
Gly Gly Pro Gly Met Val Thr Thr Ala Arg Arg Gln Tyr Pro Gly Gln Tyr Leu His Tyr

CAC CGT GCC GGC CAC GGT GCC GTG ACC TCG CCT TCG GCC AAG CGT GGC TAC ACC GCT ATC     960
His Arg Ala Gly His Gly Ala Val Thr Ser Pro Ser Ala Lys Arg Gly Tyr Thr Ala Ile

GTG CTC GCC AAG ATG AGC CGC GTG CAG CGC GCC AGC GGC GAC TGG ACC GGC ACC ATG GGC    1020
Val Leu Ala Lys Met Ser Arg Val Gln Arg Ala Ser Gly Asp Trp Thr Gly Thr Met Gly

TTC GGC CCG ATG GAC GGC GAG TCT AGC GAG CGC ACC ATC GCC TAT ATG CTG ACC GAG GAC    1080
Phe Gly Pro Met Asp Gly Glu Ser Ser Glu Arg Thr Ile Ala Tyr Met Leu Thr Glu Asp

CAG CCC CAG GGG CCC TTC TAC CGT CTC TCC TGC GCG CGC GAT ACG GCA TGT AGC GCG ATG    1140
Gln Pro Gln Gly Pro Phe Tyr Arg Leu Ser Cys Ala Arg Asp Thr Ala Cys Ser Ala Met

TGT AGC CGC GGC ATG ATC GGA CTG CGC ATG CCC GGC TCC TTC GAG AAC CTC GGA AAT CCC    1200
Cys Ser Arg Gly Met Ile Gly Leu Arg Met Pro Gly Ser Phe Glu Asn Leu Gly Asn Pro

ACT GTT ATC TAC ACG GCG GGC GCC GCC GCC TTC GGC CAT ATC GAC GGC CCG GTC GAC GCC    1260
Thr Val Ile Tyr Thr Ala Gly Ala Ala Ala Phe Gly His Ile Asp Gly Pro Val Asp Ala

GCA CGG TCG TCA CGT CAT GCC TGG CAT GCA TGG AGA GAC GGC GTT CGG GTA CTG GAC TAC    1320
Ala Arg Ser Ser Arg His Ala Trp His Ala Trp Arg Asp Gly Val Arg Val Leu Asp Tyr

GCC CGC GAG CAC AAG GTA CTC GCG CGC GAC TTC AAG TCC TCC GCC GGC GAC GCC GAC GAG    1380
Ala Arg Glu His Lys Val Leu Ala Arg Asp Phe Lys Ser Ser Ala Gly Asp Ala Asp Glu

ATC TAT CCG CGC TGG CGC AAG TCC ATG GGC GTC TAG                                    1416
Ile Tyr Pro Arg Trp Arg Lys Ser Met Gly Val  *
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B

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1 MDQSARYADLSLKEEDLIKGGRHILVAYKMKPKSGYGYLEAAAHFAAESS      50
2 MDQSNRYARLDLQEADLIAGGRHVLCAYVMKPKAGYGYLETAAHFAAESS      50
3 MDQSSRYVNLALKEEDLIAGGEHVLCAYIMKPKAGYGYVATAAHFAAESS      50
4 LDQSSRYADLSLTEEDLVKNGKHVLVAYIMN-QGGYDYLATAAHVAAESS      49
5 MDQSNRYADLTLTEEKLVADGNHLLVAYRLKPAAGYGFLEVAAHVAAESS      50
  .***.**. *.* *..*. .* *.* ** ..  .**.....*** *****

1 TGTNVEVSTTDDFTKGVDALVYYIDEAS-----EDMRVAYPLELFDRNVT      95
2 TGTNVEVSTTDDFTRGVDALVYEIDPEK-----EIMKIAYPVELFDRNII      95
3 TGTNVEVCTTDDFTRGVDALVYEVDEAR-----ELTKIAYPVALFHRNIT      95
4 TGTNVNVCTTDDFTKTVDALVYYIDPEN-----EEMKIAYPVPLFDRNIT      94
5 TGTNVEVSTTVDFTRGVDALVYEIDEAAFGDKGGLMKIAYPVDLFDPNLI      100
  *****.*.** ***..****** .* .       .  ..***. **..*..

1 DGRFMLVSFLTLAIGNNQGMGDIEHAKMIDFYVPERCIQMFDGPATDISN      145
2 DGRAMLCSFLTLTIGNNQGMGDVEYAKMHDFYVPPCYLRLFDGPSMNIAD      145
3 DGKAMIASFLTLTMGNNQGMGDVEYAKMHDFYVPEAYRALFDGPSVNISA      145
4 DGRAMMCSVLTLSIGNNQGNGDVEYGKIYDIYFPPSYLRFFDGPACSILD      144
5 DGHYNVSHMWSLILGTNHGMGDHDGLRMLDFSAPEKMVTRFDGPATDISD      150
  **.  .    .* .*.*.* **  .  .. *.  *       ****. .* .

1 LWRILGRPVVNGGYIAGTIIKPKLGLRPEPFAKAAYQFWLGGDFIKNDEP      195
2 MWRVLGRDVRNGGMVVGTIIKPKLGLRPKPFADACHEFWLGADFIKNDEP      195
3 LWKVLGRPEVDGGLVVGTIIKPKLGLRPKPFAEACHAFWLGGDFIKNDEP      195
4 MWRILGRDMTDGGLVVGTIIKPKLGLQPKPFGEACYAFGQGGDFIKNDEP      194
5 LWKVLGRPEVDGGYIAGTIIKPKLGLRPEPFAKACYDFWLGGDFIKNDEP      200
  .*..***   .** ..**********.*.**..* ..*  *.********

1 QGNQVFCPLKKVLPLVYDAMKRAQDDTGQAKLFSMNITADDHYEMCARAD      245
2 QGNQTFAPLKETIRLVADAMKRAQDETGEAKLFSANITADDHYEMVARGE      245
3 QGNQPFAPLRDTIALVADAMRRAQDETGEAKLFSANITADDPFEIIARGE      245
4 QGNQVFCQMNECIPEVVTAMKACIKETGSEKLFSANITADDPAEMIARGK      244
5 QANQNFCPMEVVIPKVAEAMDRAQQATGQAKLFSANVTADFHEEMIKRGE      250
  *.** * ..   .  * .**     ..** .**** *.*** . *.  *..

1 YALEVFGPDAD--KLAFLVDGYVGGPGMVTTARRQYPGQYLHYHRAGHGA      293
2 YILETFGENAD--HVAFLVDGYVTGPAAITTARRQFPRQFLHYHRAGHGA      293
3 YVLETFGENAS--HVALLVDGYVAGAAAITTARRRFPDNFLHYHRAGHGA      293
4 YILGQFGPMAE--NCAFLVDGYVAGGTAVTVARRNFPKQFFHYHRAGHGA      292
5 YVLGEFAKYGNEKHVAFLVDGFVTGPAGVTTSRRAFPDTYLHFHRAGHGA      300
  * *. *.  ..  . *.****.*.* . .*..** .*  ..*.*******

1 VTSPSAKRGYTAIVLAKMSRVQGASGDHTGTMGFGKLEGEGSERTIAYML      343
2 VTSPQSMRGYTAFVLSKMARLQGASGIHTGTMGYGKMEGEAADKIMAYML      343
3 VTSPQSKRGYTAFVHCKMARLQGASGIHTGTMGFGKMEGESSDRAIAYML      343
4 VTSPQTQRGYTAFVHTKISRVIGASGIHVGTMSFGKMVGDASDKGIAYML      342
5 VTSYKSPMGMDPLCYMKLARLMGASGIHTGTMVYGKMEGHNDERVLAYML      350
  ***   . .* ...    *..*. **** *.*** .**. *..... .****

1 TEDQPQGPFFRQS-WARDTACSAMASGGMHGLRMPGSFENLGEPDVVLTA      392
2 TDEAAEGPFYRQTGWGS-KATTPIISGGMNALRLPGFFDNLGHSNVIQTS      392
3 TQDEAQGPFYRQS-WGGMKACTPIISGGMNALRMPGFFENLGNANVILTA      392
4 QQDAAGGPYYHQK-WEGVVQTTPIISGGMNALRLPAFFENLGHSYVILTA      391
5 ERDECQGPYFYQK-WYGMKPTTPIISGGMDALRLPGFFENLGHGNVINTC      399
    ..  **.. *. *     . ... ****..**.*. *.***.  *. *

1 GGGTFGHIDGPVDAARANRH---AWEAWRDGV--------RVLDYAREHK      431
2 GGGAFGHLDGGTAGAKSLRQ---SHEAWMAGV--------DLVTYAREHR      431
3 GGGAFGHIDGPVAGARSLRQ---AWQAWRDGV--------PVLDYAREHK      431
4 GGGTFGHKDGPKQGATSCRQDEEAWKLWKAGTYGDVSLSDGVIEYAKTHE      441
5 GGGSFGHIDSPAAGGISLGQAYACWKT-----------GAEPIE---APR      435
  ***.*** *.  ... .   .              .              ..   ..

1 VLARDFKSWAGDADEIYPRWRKSMGV-----------------      457
2 ELARAFESFPADADKFYPGWRDRLHR-------AA---------      459
3 ELARAFESFPGDADQIYPGWRKALGVEDTRSALPA---------      466
4 EIKGAFLTFQKDSDQIYPGWKEKLGYTGESSVQAASFDWQKKAA      485
5 EFARAFESFPGDADKIFPGWREKLGVHK----------------      463
   .  .* ... *.*...* *.. .
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FIG. 2. (A) Nucleotide sequence and deduced amino acid sequence for the *T. denitrificans cbbM* gene. Predicted amino acid residues are shown below the respective codons. Ribosome binding sites are underlined. The arrows indicate the start site and direction of translation. (B) Alignment of deduced protein sequences from the *cbbM* genes from (1) *T. denitrificans*, (2) *R. sphaeroides*, (3) *R. rubrum*, (4) *Gonyaulax* sp., and (5) *H. marinus*. Perfectly conserved (asterisks) and well-conserved (dots) residues are indicated.

RESULTS

Sequencing of the RubisCO genes. The sequences of both *cbbL cbbS* (Fig. 2A) and *cbbM* (Fig. 3A) were determined. The *cbbL* gene is preceded by a ribosome binding site (AGGAGA) starting at position −11. The gene starts with ATG and extends 1.42 kb to a TAA stop codon at position 1561. The *cbbS* gene is separated from *cbbL* by a 114-bp spacer region which also contains a ribosome binding site (AGGA) starting at the −10 position. The start codon of *cbbS* is ATG as well, and *cbbS* is 354 bp long. A TAA stop codon at position 2028 marks the end of the gene, which is separated from an inverted repeat by 24 bp. The *cbbM* gene also starts with ATG and is preceded by a ribosome binding site (AGGA) at position −11. The gene extends 1.37 kb to a TAG stop codon at position 1389. The deduced amino acid sequence of the *T. denitrificans* form II enzyme was determined to be homologous to its counterparts from photosynthetic (*R. sphaeroides* [57] and *R. rubrum* [35]) and nonphotosynthetic (*Hydrogenovibrio* sp. [60]) prokaryotes as well as the recently determined eukaryotic dinoflagellate (*Gonyaulax* sp. [33]) enzyme (Fig. 2B). From this limited number of form II-containing α and β eubacteria and the one group of eukaryotic organisms which contain form II RubisCO enzymes (33, 59), it would appear that the form II enzyme is conserved throughout evolution. However, form I RubisCO may be divided into four subclasses according to sequence relatedness (51); the form I *T. denitrificans* large subunit most nearly resembles the proteins from *Chromatium vinosum* (22), a hydrothermal vent symbiont (48), and *Thiobacillus ferrooxidans* (23), with which 93, 89, and 86% identities, respectively, were observed (Fig. 3B). These proteins have been classified in class IA (51). An identity of only 57% was obtained with the *R. sphaeroides* form I enzyme (11) in class IC (51).

Expression of *T. denitrificans* RubisCO genes. A RubisCO deletion mutant of *R. sphaeroides* (strain 16) has been used as a host for the expression of RubisCO genes from several sources. Expression was achieved by complementing strain 16 with plasmids containing genes that encode both forms of RubisCO. Such fragments presumably contain a promoter that is recognized by the *R. sphaeroides* RNA polymerase for subsequent transcription and translation. Alternatively, a promoter-vector molecule specifically constructed for foreign RubisCO gene expression may be employed to complement the mutant to CO_2-dependent growth (7). Three plasmid (pLARF5) constructs, each containing genes encoding the *T.*

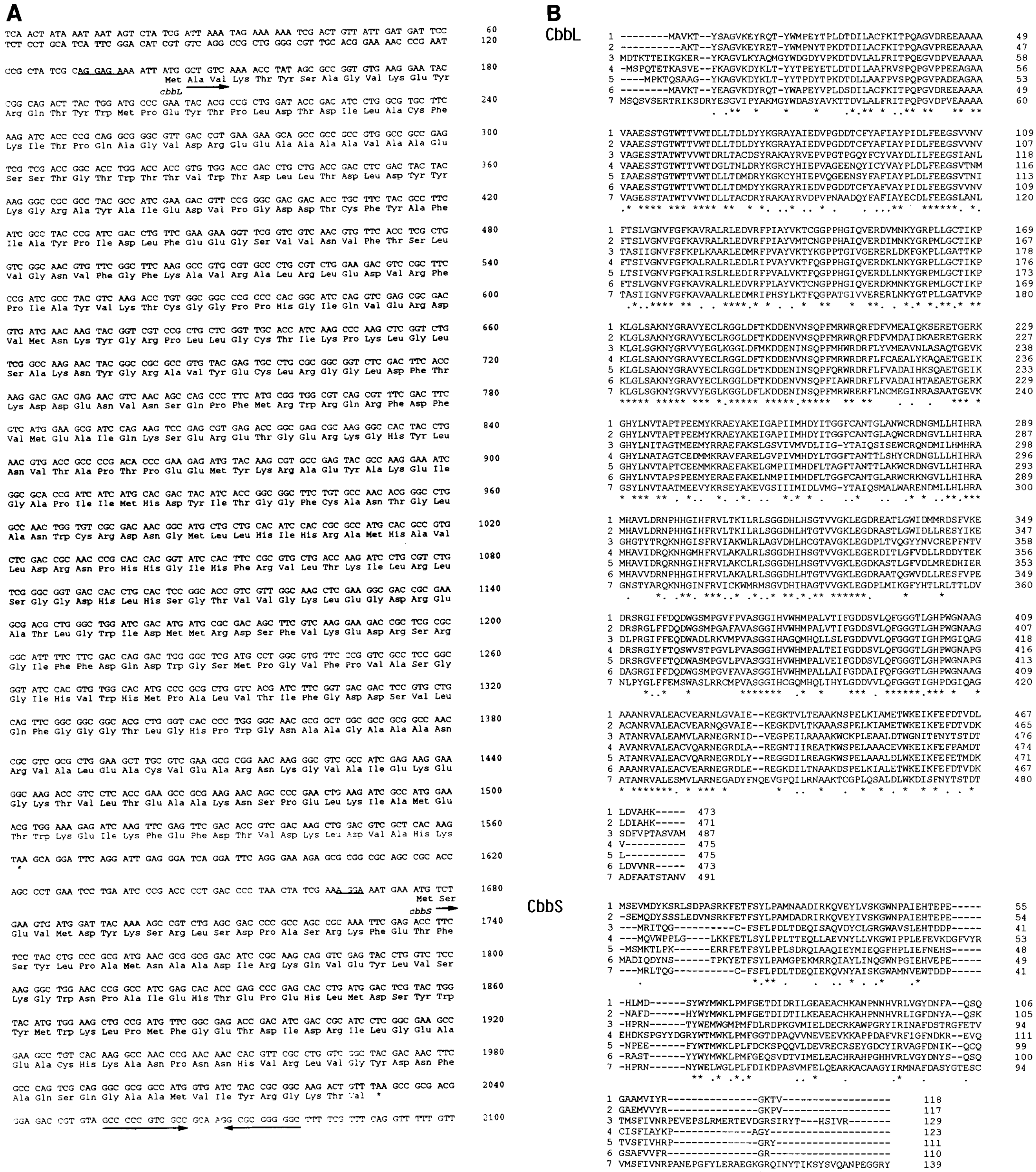

FIG. 3. (A) Nucleotide sequence and deduced amino acid sequence for the *T. denitrificans cbbL cbbS* genes. Predicted amino acid residues are shown below the respective codons. Ribosome binding sites are underlined. The short arrows indicate the start sites and directions of translation, and the long arrows indicate hairpin inverted repeats. (B) Alignment of deduced protein sequences from the *cbbL cbbS* genes from (1) *T. denitrificans*, (2) *C. vinosum*, (3) *R. sphaeroides*, (4) spinach, (5) *Synechococcus* sp. strain 6301, (6) *T. ferrooxidans*, and (7) *Cylindrotheca* sp. (strain N1). Perfectly conserved (asterisks) and well-conserved (dots) residues are indicated. The alignment was obtained with Clustal W (1.5) multiple sequence alignment software.

TABLE 1. Photoheterotrophic and photoautotrophic growth and RubisCO activity of *R. sphaeroides* 16 complemented with plasmids pTDF1, pTDF2, and pTDF12

Plasmid[a]	Growth conditions[b]	Doubling time (h)	Sp act (U/mg of protein)
pTDF1	Heterotrophic	7.3	0.02
	Autotrophic	27.1	0.59
pTDF2	Heterotrophic	8.4	0.30
	Autotrophic	22.2	0.19
pTDF12	Heterotrophic	6.3	0.22
	Autotrophic	32.2	0.66

[a] Plasmid pTDF1 contains the *T. denitrificans cbbL* and *cbbS* genes, plasmid pTDF2 contains the *T. denitrificans cbbM* gene, and plasmid pTDF12 contains the *T. denitrificans cbbL cbbS* and *cbbM* genes.

[b] Heterotrophic cultures were grown in 0.4% malate minimal medium, and autotrophic cultures were grown in minimal medium bubbled with 1.5% CO_2 in H_2.

denitrificans form I RubisCO (pTDF1), form II RubisCO (pTDF2), or both enzymes (pTDF12), were transformed into *E. coli* S17-1 (trimethoprim resistant and tetracycline sensitive). Transformants of S17-1 containing each pLARF5 construct (the constructs confer tetracycline resistance [21]) were then used in matings with *R. sphaeroides* 16. Cells having the proper phenotype and containing plasmids with the genes specifying either or both forms of *T. denitrificans* RubisCO were grown photoheterotrophically (0.4% malate minimal medium) and photoautotrophically (in carbon-free minimal media bubbled with 1.5% CO_2–98.5% H_2) in the presence of trimethoprim and kanamycin (Table 1). The complemented cells grew to high density and exhibited growth rates similar to those previously observed for *R. sphaeroides* 16 cells that expressed foreign RubisCO genes (7, 8). In addition, RubisCO activity levels (Table 1) indicated that transcription of the *cbb*

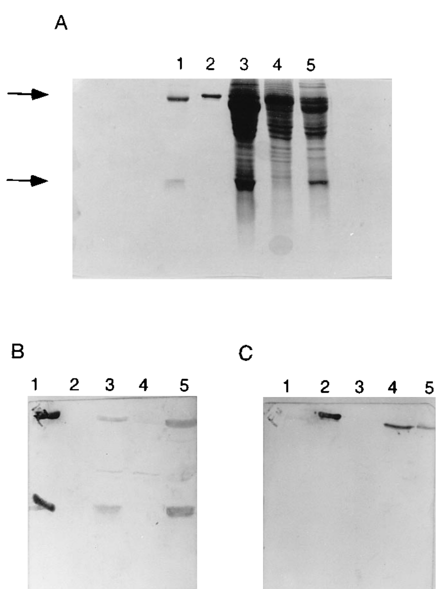

FIG. 4. Expression of *T. denitrificans cbbL cbbS* and *cbbM* genes in *R. sphaeroides* 16 by SDS-PAGE analysis (A) and Western immunoblot analysis using antibodies against form I RubisCO from *Synechococcus* sp. 6301 (B) and antibodies against form II RubisCO from *R. sphaeroides* (C). Lane 1, form I RubisCO from *Synechococcus* sp. 6301; lane 2, form II RubisCO from *R. sphaeroides*; lane 3, *R. sphaeroides* 16(pTDF1) heat-treated extract; lane 4, *R. sphaeroides* 16(pTDF2) heat-treated extract; lane 5, *R. sphaeroides* 16(pTDF12) heat-treated extract. The arrows indicate the large and small subunits of RubisCO.

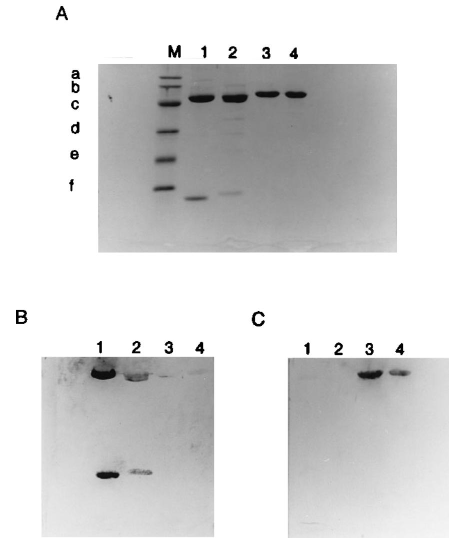

FIG. 5. SDS-PAGE analysis (A) and Western immunoblot analysis of highly purified preparations of *T. denitrificans* form I and form II RubisCO with antibodies against form I RubisCO from *Synechococcus* sp. 6301 (B) and antibodies against form II RubisCO from *R. sphaeroides* (C). Lane 1, form I RubisCO from *Synechococcus* sp. 6301; lane 2, form I RubisCO from *T. denitrificans*; lane 3, form II RubisCO from *R. sphaeroides*; lane 4, form II RubisCO from *T. denitrificans*. M refers to commercial molecular weight standards (a, 97,400; b, 66,220; c, 45,000; d, 31,000; e, 21,500; f, 14,400).

gene was regulated in much the same way as transcription of endogenous *cbb* genes is regulated by wild-type *R. sphaeroides* under these growth conditions (7). Only the strain expressing *cbbM* and exhibiting less than a twofold difference in RubisCO activity levels under the two growth conditions differed from the norm. Since photoautotrophic cultures synthesized both enzymes at high levels, subsequent purification of RubisCO was entirely feasible. SDS-PAGE and Western immunoblot analysis of heat-treated crude extracts obtained from cells grown photoautotrophically confirmed that the *T. denitrificans* RubisCO enzymes were synthesized to high levels (Fig. 4). In addition, it was found that the *T. denitrificans* form I enzyme reacted better to antiserum against RubisCO from *Synechococcus* sp. strain PCC6301 (Fig. 4) than against antiserum to form I RubisCO from *R. sphaeroides* (results not shown). The *T. denitrificans cbbL cbbS* and *cbbM* genes were subsequently subcloned into plasmid pK18 (a kanamycin-resistant derivative of pUC) in order to express the genes in *E. coli*. For unknown reasons, this attempt was unsuccessful, as extracts from induced cells showed no activity and SDS-PAGE indicated that the enzymes were not being synthesized. Given the fact that the levels of expression in *R. sphaeroides* were high, combined with previous experience in purifying RubisCO from this organism, expression in *E. coli* was not pursued further.

Purification of the RubisCO enzymes. Form I and form II RubisCO from *T. denitrificans* were purified from *R. sphaeroides* extracts containing one or both enzymes. SDS-PAGE, nondenaturing PAGE, and Western immunoblot analysis showed that the enzymes were purified to near homogeneity (Fig. 5). The native molecular weights of the form I and form II enzymes were determined to be about 500,000 and about 360,000, respectively, with nondenaturing gels and several standards at polyacrylamide concentrations of 6 and 7.5% being used (data not shown).

Sensitivity to known effector molecules. The phosphorylated intermediates, xylulose 1,5-bisphosphate and 3-keto-D-arabini-

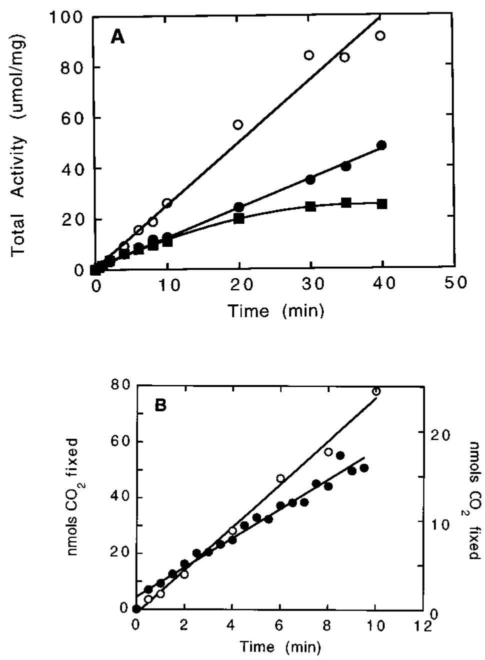

FIG. 6. (A) Activity time courses for the form I (●) and form II (○) RubisCO enzymes from *T. denitrificans* and the spinach RubisCO (■). Enzymes were preincubated for 5 min at 30°C with 20 mM $NaHCO_3$–10 mM $MgCl_2$ prior to the addition of RuBP to a final concentration of 0.4 mM. The reaction was terminated at various times after the addition of RuBP. (B) Activity time courses for the form I (●) and form II (○) enzymes from *T. denitrificans*; the enzymes were preincubated in the same conditions described above, and the reaction was terminated at various times after the addition of RuBP over a shorter interval (10 min). The scale on the left applies to form II, and the scale on the right applies to the form I enzyme.

tol 1,5-bisphosphate, which are formed as a result of the isomerization or epimerization of the enediol of RuBP, inhibit most form I RubisCO enzymes. In instances in which form I RubisCO is not inhibited by these compounds, a distinct lack of an effect on the time course of the reaction is observed. This is the case for the cyanobacterial enzyme (1, 27); the form II RubisCO enzymes from *R. rubrum* and *R. sphaeroides* are also presumably not affected, since these enzymes show no fallover (13). To examine if the *T. denitrificans* RubisCO enzymes exhibited fallover, the activity of both enzymes was monitored over a 40-min period. It is apparent from these results that the *T. denitrificans* form II enzyme did not exhibit fallover over this time interval (Fig. 6A), which is a response similar to those of the *R. sphaeroides* and *R. rubrum* form II enzymes (13). Interestingly, the behavior of the form I *T. denitrificans* enzyme also did not indicate fallover (Fig. 6A). Even by careful examination during the early phase of the time course experiment, it was clear that the reaction was linear (Fig. 6B). Moreover, a direct comparison with the classic fallover response exhibited by spinach RubisCO indicated that the form I enzyme of *T. denitrificans* is quite different from the spinach enzyme in this respect (Fig. 6A).

RuBP is also a potent inhibitor of in vitro RubisCO activation, because it can form an ER complex that is incapable of being carbamylated (24). As in the case of fallover, cyanobacterial (27) and form II (13) RubisCO enzymes are notable exceptions. To determine the effect RuBP has on the *T. denitrificans* enzymes, each enzyme in its unactivated form was incubated in 0.5 mM RuBP for 30 min. After the subsequent simultaneous addition of Mg^{2+} and HCO_3^-, the activity of the enzymes was monitored over time (Fig. 7). The *T. denitrificans* form II RubisCO showed a rapid recovery from RuBP preincubation, quickly reaching levels of activity exhibited by the fully activated enzyme complex. The form I enzyme, on the other hand, did not recover much of its activity, reaching only 15% of the activity levels exhibited by the fully activated form of the enzyme. These results indicate that RuBP is a strong inhibitor of the form I enzyme, presumably by binding to this enzyme and forming the ER complex. Interestingly, this behavior differentiates the *T. denitrificans* form I enzyme from cyanobacterial RubisCO, which rapidly recovers from RuBP treatment under similar conditions (29).

The results of the experiments presented in Fig. 6 and 7 indicated that the form II *T. denitrificans* enzyme responded much like the well-described *R. rubrum* and *R. sphaeroides* enzymes. The form I *T. denitrificans* enzyme did not show any tendency to fallover, and yet the form I RubisCO could not recover from RuBP preincubation. Previous studies had indicated that there was a linear rate of CO_2 fixation for the form II *R. sphaeroides* enzyme but not the form I enzyme, even in the presence of RuBP (13, 52). Thus, the two *T. denitrificans* unactivated enzymes were preincubated with either RuBP or HCO_3^- and Mg^{2+} for 30 min, prior to an assay of activity at various times after the addition of the missing substrates (Fig. 8). The form II enzyme showed only a slight effect after preincubation with RuBP, compared with the activity levels achieved by preincubation with HCO_3^- and Mg^{2+}. By contrast, the form I enzyme was severely inhibited by preincubation with RuBP, which prevented it from reaching the levels of activity for enzyme preincubated with HCO_3^- and Mg^{2+}. The results of this experiment confirmed and extended the studies of fallover (Fig. 6) and ER activation (Fig. 7).

Another known effector molecule is PGN. All sources of form I RubisCO show various degrees of inhibition by PGN. Form II enzymes from *R. rubrum* and *R. sphaeroides*, on the other hand, seem to be either not affected or less affected (12, 13, 43, 53). To investigate the effects of this phosphorylated compound, the *T. denitrificans* enzymes were preincubated with various concentrations of PGN prior to the initiation of the reaction. The *T. denitrificans* enzymes followed the trends established previously (data not shown) in that the form I enzyme was inhibited by low concentrations of PGN (50% inhibition at 150 μM) while the form II enzyme showed only slight inhibition at up to 1.5 mM PGN.

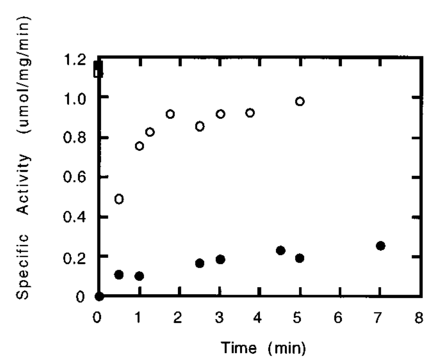

FIG. 7. Time-dependent activation of the form I and form II RubisCO-RuBP (ER) complex. The form I (●) and form II (○) RubisCO enzymes from *T. denitrificans* were preincubated in 0.5 mM RuBP. After incubation for 30 min at 25°C, the enzymes were added to a reaction mixture containing 20 mM $NaHCO_3$, 10 mM $MgCl_2$, and 4 mM RuBP. The carboxylase activity (micromoles of CO_2 fixed per milligram per minute) was measured by a 1-min assay at various times after activation. The activity of the fully activated enzymes was also determined for both form I (■) and form II (□) *T. denitrificans* RubisCO.

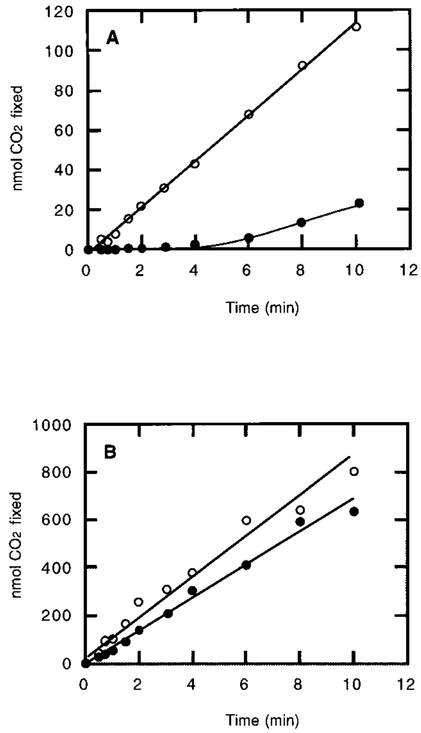

FIG. 8. Preincubation of form I RubisCO (A) and form II RubisCO (B) from *T. denitrificans* with 0.8 mM RuBP (●) or 20 mM $NaHCO_3$–10 mM $MgCl_2$ (○). Enzymes were preincubated for 30 min at 30°C prior to the addition of the missing substrates at time 0. The carboxylase activity (nanomoles of CO_2 fixed) was measured at various times after the reaction was initialized.

Kinetic analysis of the *T. denitrificans* RubisCO enzymes. The specificity factor, or τ value, may be determined at any concentration of CO_2 and O_2 by simultaneously measuring the carboxylase and oxygenase activities. As described in Materials and Methods, this determination entails the separation of the products of the carboxylase and the oxygenase reactions with specifically labeled [1-H^3]RuBP and $^{14}CO_2$. The specificity factor (τ) is thus a measure of the ability of RubisCO to discriminate between CO_2 and O_2 at any given CO_2/O_2 ratio and provides a means of comparing the efficiencies of different RubisCO enzymes. For the form I enzyme, the specificity factor was found to be 46 (Table 2), which is somewhat higher than the value reported for two cyanobacterial enzymes (26, 41). For various form I RubisCO enzymes for which specificity factors have now been determined, this is a relatively low value. Like those of other form II enzymes, the τ value for *T. denitrificans* form II RubisCO is extremely low. Additional kinetic analyses revealed that the K_{CO_2} for the form I enzyme was lower than that for the form II enzyme (Table 2) and approached the K_{CO_2} for cyanobacterial RubisCO (41). As expected, the form II enzyme exhibited a high K_{CO_2}, which partially explains the low τ value. The K_{O_2} was also examined, and not surprisingly, the form I enzyme exhibited a much higher K_{O_2} than the form II enzyme. Finally, the K_{RuBP} was determined for both RubisCO enzymes. The value obtained for the form I enzyme was almost four times as large as the value obtained for the form II enzyme (Table 2).

DISCUSSION

T. denitrificans is a chemolithotrophic microorganism and a member of the β proteobacteria group and uses thiosulfate or other sulfur compounds as an energy source and nitrate as an electron acceptor when it is cultured under anaerobic conditions. This organism is one of the many obligate autotrophs that fixes CO_2 via the Calvin cycle. McFadden and Denend (32) purified and partially characterized a RubisCO enzyme from this organism and determined that it had an apparent molecular weight of 350,000. More recently, the genes for two distinct RubisCO enzymes were identified in this organism and cloned (6). The presence of *cbbM*, which encodes form II RubisCO, presumably confirms the observations of McFadden and Denend, since form II RubisCO with an M_r of 350,000 is often isolated. Additional RubisCO genes (*cbbL* and *cbbS*) encoding form I RubisCO are also present in this organism. In *T. denitrificans*, the genes for both forms of RubisCO are separated by only 17 kb and are transcribed in opposite directions (Fig. 1), leading to speculation that the region between the two sets of genes may have regulatory significance (6). Other than what is known of the form II RubisCO from *R. rubrum* and *R. sphaeroides*, little is known of the structure-function relationships of form II RubisCO from nonphotosynthetic organisms. Indeed, organisms such as *T. denitrificans* often are found in environments where the oxygen and carbon dioxide concentrations may vary considerably. Since a large amount of evidence has recently accumulated to indicate that RubisCO may have evolved to accommodate rather specific environmental growth conditions (42), examination of such enzymes has the potential to reveal novel structural determinants that influence the specificity of this enzyme towards its gaseous substrates. Thus, the present study was initiated to characterize the enzymes from *T. denitrificans* further and compare them with other well-known RubisCO enzymes for which the kinetic properties have been well established, particularly the specificity factor. Although much work has focused on known determinants (15, 16), it was of interest to examine RubisCO from an anaerobic denitrifier and known acid-tolerant organism, *T. denitrificans*, given the diversity of organisms known to fix CO_2 via the Calvin cycle.

For unknown reasons, it has been difficult to express *Thiobacillus* RubisCO genes in *E. coli* (6, 49), although low levels of active enzyme were reportedly obtained with the *cbbM* gene of *T. intermedius* (49). One alternative would be to purify the enzyme from the native organism, i.e., *T. denitrificans*. Unfor-

TABLE 2. Kinetic properties of RubisCO enzymes from *T. denitrificans*

Enzyme	τ ($V_{CO_2} \cdot K_{O_2}/V_{O_2} \cdot K_{CO_2}$)	V_{CO_2} (μmol/min/mg)[a]	V_{O_2} (μmol/min/mg)[b]	V_{CO_2}/V_{O_2}	k_{cat}[c] s^{-1}	K_{RuBP} (μM)	K_{CO_2} (μM)	K_{O_2} (μM)	K_{O_2}/K_{CO_2}
Form I	46 ± 1.6	1.8 ± 1.1	0.5	3.6	1.9	43 ± 0.9	138 ± 5.1	1,637 ± 650	12
Form II	14 ± 0.8	3.3 ± 0.9	0.6	5.5	3.5	13.3 ± 1.9	256 ± 61	619 ± 237	2.4

[a] V_{CO_2} valves were obtained with Lineweaver-Burk plots from multiple assays.
[b] V_{O_2} valves were obtained from the equation $\tau = V_{CO_2} \cdot K_{O_2}/V_{O_2} \cdot K_{CO_2}$.
[c] k_{cat} valves were calculated from V_{CO_2}, with the assumption that the *T. denitrificans* form I enzyme has a molecular weight of 500,000 and the form II RubisCO has a molecular weight of 360,000.

tunately, the difficulty in obtaining the needed massive quantities of *T. denitrificans* is a notorious shortcoming for enzymological studies with such organisms. However, it was found that high levels of active enzyme could be obtained with an *R. sphaeroides* expression system in which *R. sphaeroides* 16, a RubisCO deletion strain (7), was found to be complemented to autotrophic growth with foreign RubisCO genes and either a promoter specific for the foreign RubisCO genes or, alternatively, a heterologous promoter constructed on a specific RubisCO expression plasmid (7). With regard to the *T. denitrificans* genes, it was found that broad-host-range plasmids containing *cbbL cbbS*, *cbbM*, or both sets of genes and their cognate promoters could complement strain 16 to autotrophic growth. It is interesting that under both photoautotrophic and photoheterotrophic conditions, the *R. sphaeroides* 16(pTDF2) cultures exhibit a longer lag period before exponential growth ensues.

Western immunoblot analysis of crude extracts revealed that the form I RubisCO reacted better with antiserum against *Synechococcus* sp. strain PCC 6301 RubisCO than with antiserum directed at *R. sphaeroides* form I RubisCO, suggesting greater homology to the cyanobacterial enzyme. This suggestion was borne out by a direct comparison of their deduced amino acid sequences. The form II *T. denitrificans* enzyme reacted strongly only to antiserum against *R. sphaeroides* form II RubisCO, suggesting that these enzymes may be homologous. Indeed, analysis of the deduced amino acid sequence of the form II RubisCO of *T. denitrificans* showed 66% identity to the sequence of the *R. sphaeroides* form II protein and 69% identity to the sequence of the *R. rubrum* enzyme.

The native molecular weight of each *T. denitrificans* RubisCO enzyme was approximated by nondenaturing (6 and 7.5%) PAGE. By this analysis, the molecular weight of the form I enzyme was calculated to be ~500,000, on the low end of the general range of molecular weights exhibited for form I RubisCO from various sources (50). We determined that the *cbbM* gene of *T. denitrificans* encoded a form II RubisCO with a molecular weight of about 360,000, which is very close to the 350,000 value reported previously (32). In nondenaturing gels, the *T. denitrificans* form II enzyme migrated more slowly than *R. sphaeroides* form II RubisCO, perhaps indicating that the former is somewhat larger; thus, the *T. denitrificans* enzyme is more compatible with an L_6 or L_8 quaternary structure, since the *cbbM* gene encodes a polypeptide with an M_r of 50,328. In the final analysis, more detailed studies will be required to determine the precise quaternary structure of the two *T. denitrificans* enzymes; however, all form I RubisCO proteins are L_8S_8 molecules, while form II RubisCO is most properly described as L_x, with the exception of the *R. rubrum* and *H. marinus* enzymes, which are definitely L_2 proteins.

Once the *T. denitrificans* RubisCO enzymes were purified, it was possible to examine their kinetic properties in more detail. This was the major objective of the current study, since very little is known of the properties of RubisCO from bacteria capable of growing in unusual environments where key kinetic properties might be altered to allow the organism to thrive. Initially, the sensitivity of the enzymes to known effector molecules was examined. Fallover is one manifestation of the inhibition of RubisCO activity and is caused by products that invariably form during catalysis. It was interesting that the *T. denitrificans* form II enzyme also did not exhibit fallover, much like the two photosynthetic bacterial form II enzymes, providing another similarity and, most importantly, indicating that the structural basis for this property is probably conserved among diverse form II enzymes. These results also suggest that form II RubisCO enzymes somehow avoid inhibition by the products of the isomerase and epimerase activities of RubisCO (3-keto-D-arabinito 1,5-bisphosphate and xylulose 1,5-bisphosphate, respectively), both of which have been shown to be responsible for fallover (5, 62). Perhaps form II enzymes are generally more efficient in protecting the enediol intermediate from misprotonation, which is an excellent possibility, since an *R. rubrum* mutant enzyme which does exhibit fallover was recently isolated (28). Form I enzymes, on the other hand, have consistently been found to be sensitive to these five-carbon compounds; consequently, they exhibit fallover. The only exceptions are the cyanobacterial enzymes (1, 27) and perhaps an algal RubisCO (61). *T. denitrificans* form I RubisCO is an interesting case, since it does not exhibit fallover. This is one more similarity to the cyanobacterial RubisCO; yet unlike the cyanobacterial enzyme, RuBP is a strong inhibitor of the *T. denitrificans* form I RubisCO. With the exception of the cyanobacterial (27) and form II (13, 55) enzymes, formation of an ER complex by unactivated enzyme and RuBP has been observed for most RubisCO enzymes. As expected, the form II enzyme from *T. denitrificans* was not inhibited by RuBP, which is consistent with the behavior of the other two form II enzymes that have been characterized. Both *T. denitrificans* enzymes behaved in the expected manner towards the effector PGN.

The specificity factor (τ), which is a measure of the ability of RubisCO to discriminate between CO_2 and O_2 at a given CO_2/O_2 concentration ratio, was originally used by Jordan and Ogren (18) to compare RubisCO enzymes from diverse sources. The *T. denitrificans* form I enzyme was found to have a τ value slightly higher than the reported values for cyanobacterial RubisCO (Table 2), which is low for form I RubisCO. Interestingly, carboxysomes are present in cyanobacteria and most *Thiobacillus* species. These are polyhedral bodies normally enclosed by a membrane but mainly consisting of protein, most of which is RubisCO. One of the proposed functions of this prokaryotic organelle is to act as a CO_2-concentrating mechanism to favor the carboxylase activity of RubisCO while protecting the enzyme from the inhibitory effects of O_2 (4). Although *T. denitrificans* does not appear to contain carboxysomes, the reason for the low τ value exhibited by the *T. denitrificans* form I RubisCO may well be due to the fact that this enzyme may have evolved in an environment containing a very high CO_2/O_2 concentration ratio; therefore, the requirement for an enzyme highly efficient in discriminating between CO_2 and O_2 would be superfluous. Since *T. denitrificans* can grow under anaerobic CO_2-fixing conditions, the need for carboxysomes is moot in any case. Like other form II enzymes, the τ value for the *T. denitrificans* form II RubisCO is extremely low, although slightly higher than the value for the *R. sphaeroides* form II enzyme (Table 2). Other kinetic parameters correlated well with the τ values obtained for each of the *T. denitrificans* RubisCO enzymes. The K_{CO_2} was high for the form II enzyme, contributing in large part to its low τ value. The form I enzyme exhibited a lower K_{CO_2} than the form II enzyme and approached the values obtained for cyanobacterial RubisCO. Even so, the K_{CO_2} for these form I enzymes is high relative to the values obtained for eukaryotic and most prokaryotic RubisCO enzymes. Interestingly, the K_{CO_2} for form I RubisCO enzymes from other thiobacilli are even five- to sixfold higher (39, 46). If these latter determinations are accurate, the fact that these enzymes were all isolated from organisms that contain carboxysomes may be significant. Curiously, the form I RubisCO from *T. ferrooxidans* has a K_{CO_2} value of 28 μM (17), suggesting that *T. ferrooxidans*, which grows at pHs lower than 2.0, was pressured to devise an adaptive mechanism to compensate for low CO_2 concentrations in its normal environment. Certainly, now that many of the RubisCO genes have been or are being sequenced from such organisms, the ability to relate specific residue alterations to specific kinetic properties will provide a large impetus to ongoing structure-function studies in a number of laboratories, including our own. The K_{RuBP} value for the form II *T. denitrificans* enzyme was lower than that for the *R. sphaeroides* form II enzyme and approaches the reported value of 11 μM for the *R. rubrum* enzyme (25). The form I enzyme, on the other hand, has a somewhat higher K_{RuBP} value than cyanobacterial RubisCO, but the form I K_{RuBP} is also lower than the value reported for the K_{RuBP} of the *T. ferrooxidans* enzyme and approaches the K_{RuBP} of the *R. sphaeroides* form I enzyme (9). These data, taken together with the specificity factor results, again suggest that the form II *T. denitrificans* enzyme is very similar to other RubisCO enzymes of this type and that the form I *T. denitrificans* RubisCO, in general, has kinetic properties very similar to those of cyanobacterial RubisCO.

The similarities of the general properties of the *T. denitrificans* form II RubisCO and those of the previously characterized form II enzymes of nonsulfur photosynthetic bacteria undoubtedly reflect their homology; however, the form I *T. denitrificans* large subunits are even more homologous to other form I proteins, and yet the kinetic and other properties vary significantly. Whatever the molecular and structural basis of the properties, the current study indicates that form II enzymes from phylogenetically different organisms are well conserved throughout microbial evolution, suggesting that this type of RubisCO has evolved for a particular purpose. Indeed, homologous form II RubisCO enzymes are also present in eukaryotic photosynthetic dinoflagellates (33, 59), although such proteins have yet to be isolated as catalytically active proteins. The function of form II RubisCO in an organism that also synthesizes form I is still not totally resolved; however, it does appear that the form II enzyme is more important under conditions of carbon excess in both *R. sphaeroides* (20) and *T. denitrificans* (6) and that form I RubisCO is presumably required for growth under limiting concentrations of CO_2 in the presence of oxygen (30).

A new classification of RubisCO enzymes based on their sequence relatedness has been proposed (51). Interestingly, even closely related RubisCO enzymes show vastly different τ values, suggesting that judicious comparisons of known key regions will help point the way to the structural determinants that influence specificity. The *T. denitrificans* form II RubisCO obviously belongs to the type II RubisCO group, making this the most uniform group. It is interesting that the form I RubisCO from *T. denitrificans* resembles the class IA enzymes but that many of its enzymatic properties closely resemble those of cyanobacterial RubisCO (class IB) (51). The most interesting exception is the strong ability of RuBP to prevent activation of the *T. denitrificans* enzyme. Given the close immunological relatedness of the cyanobacterial and *T. denitrificans* enzymes and the fact that the cyanobacterial genes easily hybridize to *T. denitrificans cbbL* and *cbbS* sequences, it is not surprising that the complete sequences reflect significant similarities; however, there are also notable differences which presumably also have some influence on function. Since cyanobacterial RubisCO is not inhibited by RuBP and does not exhibit fallover, future experiments will be directed at defining determinants important for this behavior; the known sequence plus subsequent enzymological studies of the closely related *T. denitrificans* form I enzyme (this study), combined with recent cyanobacterial RubisCO X-ray structural models (36), may help establish the molecular basis for the relative inability of RuBP to bind to cyanobacterial RubisCO.

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