Substitutions at Methionine 295 of *Archaeoglobus fulgidus* Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Affect Oxygen Binding and CO$_2$/O$_2$ Specificity*

Nathaniel E. Kree and F. Robert Tabita

From the Department of Microbiology and the Plant Molecular Biology/Biotechnology Program, Ohio State University, Columbus, Ohio 43210-1292

*Archaeoglobus fulgidus* RbcL2, a form III ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), exhibits unique properties not found in other well studied form I and II Rubiscos, such as optimal activity from 83 to 93 °C and an extremely high $k_{\text{cat}}$ value (23 s$^{-1}$). More interestingly, this protein is unusual in that exposure or assay in the presence of oxygen and high levels of CO$_2$ resulted in substantial loss (85–90%) of activity compared with assays performed under strictly anaerobic conditions. Kinetic studies indicated that *A. fulgidus* RbcL2 possesses an unusually high affinity for oxygen ($K_i = 5 \mu M$); O$_2$ is a competitive inhibitor with respect to CO$_2$, yet the high affinity for O$_2$ presumably accounts for the inability of high levels of CO$_2$ to prevent inhibition. Comparative bioinformatic analyses of available archaeal Rubisco sequences were conducted to provide clues as to why the RbcL2 protein might possess such a high affinity for oxygen. These analyses suggested the potential importance of several unique residues, as did additional analyses within the context of available form I–III Rubisco structures. One residue unique to archaeal proteins (Met-295) was of particular interest because of its proximity to known active-site residues. Recombinant M295D *A. fulgidus* Rubisco was less sensitive to oxygen compared with the wild-type enzyme. This residue, along with other potential changes in conserved residues of form III Rubiscos, may provide an understanding as to how Rubisco may have evolved to function in the presence of air.

Several eukaryotic and prokaryotic organisms are able to obtain all needed carbon by directly assimilating and reducing CO$_2$. The major mechanism by which CO$_2$ is assimilated in nature is via the Calvin-Benson-Basham (CBB) reductive pentose phosphate pathway. There are two unique enzymatic reactions in this pathway that allow CO$_2$ to serve as the sole carbon source for growth. The first of these is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme that catalyzes the actual CO$_2$ fixation reaction. The other unique enzyme is phosphoribulokinase, which catalyzes the synthesis of the substrate or CO$_2$ acceptor compound for Rubisco, ribulose 1,5-bisphosphate (RuBP). The mechanism of Rubisco catalysis is well defined (1). Briefly, Rubisco initially catalyzes formation of an enediolate intermediate between the second and third carbons of RuBP; this allows for a nucleophilic attack by the gaseous substrate CO$_2$, resulting in the formation of a six-carbon intermediate that is subsequently lysed into two molecules of 3-phosphoglycerate (3-PGA). Alternatively, molecular oxygen may also serve as a gaseous substrate through a similar nucleophilic attack at the same enediol enzyme complex, forming instead a five-carbon peroxide intermediate that is also subsequently cleaved to form two different products, one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). The 3-PGA product formed by Rubisco is further utilized in the CBB pathway to regenerate RuBP to supply other carbon intermediates for growth. Unfortunately, the 2-PG product formed as a consequence of O$_2$ fixation to the enediolate intermediate is further oxidatively metabolized, leading to the eventual loss of carbon from the organism, making the oxygenase function of Rubisco inimical to maximizing net CO$_2$ fixation. The relative rates of the competing carboxylase and oxygenase reactions ($v_c/v_o$) catalyzed by Rubisco, performed at particular CO$_2$ and O$_2$ concentrations, provide a means to quantify the enzyme’s inherent ability to distinguish between the two substrates such that $v_c/v_o = \Omega[C\text{O}_2]/[O\text{2}]$, where $\Omega$ is the specificity factor. In addition, $\Omega = V_{max}/K_m$ reflect the catalytic efficiencies for the carboxylase ($V_{o}/K_o$) and oxygenase ($V_{c}/K_c$) reactions, respectively.

Rubisco is arguably the most abundant protein on earth (2, 3). This protein plays an important role in the photosynthetic tissue of higher forms of eukaryotic life such as terrestrial plants, but may also be found in lower phototrophic eukaryotes such as green, red, and brown algae and in prokaryotic cyanobacteria and phototrophic eubacteria. Moreover, large numbers of chemosynthetic prokaryotes, which grow in the absence of light and use dark chemical reactions to provide the energy to support growth, depend on Rubisco and the CBB CO$_2$ fixation pathway (2). Rubisco molecules from these sources have been previously categorized into two groups (forms I and II) based on their sequence homology and structural similarities (2). The form I Rubisco structure is a complex holoenzyme composed of eight large (L; catalytic) subunits and eight small (S) subunits in an (L$_2$)$_4$(S$_2$)$_4$ configuration. The form I enzymes...
are found in virtually all eukaryotic photosynthetic organisms and also in all cyanobacteria and most eubacteria that use the CBB pathway to assimilate CO₂. The form II Rubiscos have a more simple structure, composed solely of large subunits in an \((L_2)_n\) configuration, which share \(\sim 25\% - 30\%\) amino acid identity with form I large subunits. Form II proteins are found in various purple photosynthetic bacteria, chemotrophs, and eukaryotic marine dinoflagellates (2). The important kinetic constants of diverse forms of Rubisco, particularly \(K_c\) and \(V_c\), differ considerably even among closely related and structurally similar proteins (4, 5); however, there is little molecular understanding as to the basis for this variation. Although all Rubisco proteins conserve key residues for known mechanistically important functions (1, 2), it is apparent that different non-conserved residues and regions of the protein must influence the specificity of the substrates carbon dioxide and oxygen. The focus of engineering a more efficient Rubisco would involve changing the specificity factor of the enzyme by either increasing the carboxylase or decreasing the oxygenase activity or somehow altering the relative affinity for either CO₂ or O₂ (6). Clearly, before such molecular engineering feats should be considered, it will be most important to understand the factors that mitigate and influence the different kinetic properties of the enzyme.

Recently completed fully sequenced genomes from Archaea have indicated the presence of Rubisco genes in several organisms, although there is no evidence that the CBB reductive pentose phosphate pathway provides a major means by which these organisms assimilate CO₂ (7–12). Rather, Rubisco and a pentose phosphate pathway in methanogenic Archaea (13). Our laboratory has determined that the archaeal Rubisco genes from Archaeoglobus fulgidus, Methanocaldococcus jannaschii, and Methanosarcina acetivorans encode bona fide Rubiscos, capable of catalyzing substantial activity, both in the native organisms as well as within Escherichia coli (11, 12). Moreover, the archaeal genes can be expressed in a phototrophic eubacterial host in which the native Rubisco genes have been inactivated such that the archaeal genes may complement the organism to allow CO₂-dependent growth (12). On the basis of sequence homologies, archaeal Rubiscos represent a special class of enzyme, termed form III (2, 14) to distinguish these enzymes from previously characterized form I and II Rubiscos. Even with these considerable differences in primary sequence, the form III enzymes retain many features characteristic of all forms of Rubisco, mainly the ability to carry out carboxylation as a consequence of conservation of the key residues implicated in catalysis (11), as discussed above.

The \textit{A. fulgidus} \textit{rbcL2} gene encodes a protein (RbcL2) of 441 amino acids with a monomer molecular mass of 48.5 kDa. This form III Rubisco exists as a homodimer, as do the enzymes from \textit{M. jannaschii} and \textit{M. acetivorans} (11, 12). In this respect, the quaternary structure of the three form III archaeal enzymes resembles the bacterial form II Rubisco from \textit{Rhodospirillum rubrum} (15). However, \textit{A. fulgidus} RbcL2 exhibits unique properties not found in other forms of Rubisco such as optimal activity at temperatures exceeding 80 °C. In addition, this protein is highly sensitive yet reversibly inhibited upon exposure to air levels of oxygen, necessitating that the enzyme be prepared under strictly anaerobic conditions to obtain optimal activity. This enzyme is derived from a very strict anaerobe, and like other Rubiscos, there are no motifs that suggest oxygen involvement in stability. In this study, we have focused on this unusual reversible inhibition by low concentrations of oxygen and examined the possible involvement with a unique residue (Met-295) that appears to be located at an influential site within the structure of the protein. The unique oxygen sensitivity of the form III archaeal Rubiscos may provide clues as to how the active site of this enzyme has evolved to become more stable in the presence of oxygen in more evolutionarily advanced form I and II Rubisco proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, and Growth Conditions—**All cloning steps were performed in \textit{E. coli} JM109 (16) prior to transformation into \textit{E. coli} BL21 (Stratagene, La Jolla, CA) for overexpression of the \textit{rbcL2} gene. \textit{E. coli} cultures were grown in LB medium containing 1% Tryptone, 0.5% yeast extract, and 1% (w/v) \(\text{NaCl}\). \textit{A. fulgidus} \textit{rbcL2} (AF\_1638, NCBI accession number NC\_000917) was cloned directly from genomic DNA. Primers designed with an NdeI restriction site (5’-GGATTCCATATGGCGGAGTTTGAGATTTACAGA-3’) at the \(\text{N}\) terminus and a BamHI restriction site (5’-GCGGATCTTA-GATTGGCGTAAACCCTG-3’) at the C terminus were used to amplify the \textit{rbcL2} gene from \textit{A. fulgidus} genomic DNA with Pfu polymerase. The gene was ligated into PCR-Script\textsuperscript{®} (Stratagene) and sequenced for PCR-incorporated mutations. The gene was subcloned into pET11a using the NdeI and BamHI sites in that vector.

**Site-directed Mutagenesis—**Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) (17). The Met-295 \textit{ATG} sequence within the \textit{A. fulgidus} \textit{rbcL2} gene was replaced with \textit{GGG}, \textit{TTC}, and \textit{GAC} to obtain alanine, phenylalanine, and aspartate, respectively, at this position. Automated sequencing was performed to confirm the sequences of mutant genes using a Model 3730 DNA analyzer system (Applied Biosystems, Foster City, CA) at the Ohio State University Plant-Microbe Genomics Facility. The mutant genes were inserted into fresh pET11a plasmid after digestion with NdeI and BamHI.

**Overexpression of the \textit{rbcL2} Gene and Purification of Recombinant \textit{RbcL2} Protein—**\textit{E. coli} BL21 cells were transformed with a pET11a vector containing the \textit{A. fulgidus} \textit{rbcL2} gene, and overnight tube cultures were used to inoculate 2.8-liter broad-bottom flasks containing 2 liters of medium. The cultures were incubated at 37 °C and shaken at 120 rpm to minimize aeration until the culture reached \(A_{600} = 0.4\). The temperature of the cultures was then raised to 42 °C for 30 min to heat-shock the cultures, resulting in an increase in the amount of soluble recombinant protein (12). The cultures were allowed to cool to room temperature before inducing with 0.1 mM isopropyl \(\beta\)-thiogalactopyranoside and allowed to shake at 120 rpm for 16 h at room temperature. Cells were harvested and washed with anaerobic wash buffer (100 mM Bicine-NaOH (pH 8.3), 10 mM MgCl₂, and 1 mM EDTA) and placed in an anaerobic chamber. Cells were centrifuged again in anaerobic screw cap centrifuge tubes with rubber seals. Cell pellets were recovered in
the chamber and stored at −70 °C before further protein purification.

All subsequent preparation and manipulation of the cell material were performed in an anaerobic chamber. Prior to column chromatography, cells were resuspended in wash buffer supplemented with 10 mM phenylmethylsulfonyl fluoride and 50 μg/ml DNase I and then disrupted using a pressurized French pressure cell (at 110,000 kilopascals) flowing directly into a sealed anaerobic serum vial sparged with argon gas. The lysed cells were then centrifuged at 16,000 × g for 20 min at 4 °C in screw cap centrifuge tubes with rubber seals. The supernatant was decanted into a serum vial, placed in an 80 °C water bath for 20 min, and plunged into an ice bath for 1 h. The heat-stable extract was transferred to a fresh screw cap centrifuge tube with a rubber seal and centrifuged at 30,000 × g for 30 min at 4 °C. The supernatant was syringe-filtered using 0.22-μm filters before loading onto columns.

Column chromatography was performed under anaerobic conditions using a BioLogic HR workstation (Bio-Rad). Heat-stable extract was loaded onto a Q-Sepharose strong anion-exchange column equilibrated with wash buffer supplemented with 50 mM NaHCO₃ and 10 mM β-mercaptoethanol (Buffer A). Samples were eluted in a 0–2 M NaCl gradient in Buffer A; recombinant A. fulgidus RbcL2 eluted at ~0.4 M NaCl. Fractions were monitored for activity using a modified protocol of the standard Rubisco assay under anaerobic conditions (18). Fractions with high activity were pooled, concentrated with a Millipore concentrator (molecular weight cutoff of 30,000), and loaded onto a 110-ml Superose 12 gel filtration column. Fractions with high activity were again pooled and further purified based on hydrophobic interaction using a phenyl-Sepharose column. Samples were eluted with buffers of decreasing salt content starting with 2 M (NH₄)₂SO₄. Recombinant A. fulgidus RbcL2 was found to elute at ~0.4 M (NH₄)₂SO₄. Purified protein was stored in 20% glycerol at −70 °C in anaerobically sealed serum vials.

Radiometric Rubisco Assays—A. fulgidus RbcL2 was assayed for activity under a strictly anaerobic atmosphere unless noted otherwise. The previously described assay was used and modified to optimize carboxylase activity (18). Buffers and substrates were bubbled with argon gas in sealed glass serum vials prior to use. In an anaerobic chamber, enzyme was prepared in glass serum vials in wash buffer supplemented with 0.3 M NaCl. Vials were sealed in the chamber and then placed in a Reacti-Therm III™ heating/stirring module (Pierce) set at 83 °C after the addition of 50 mM NaHCO₃ in Bicine-NaOH buffer containing 0.22-μm filters before loading onto columns.

Kinetic Measurements—Purified enzymes were used for all kinetic measurements of $k_{cat}$, $K_{CO_2}$, $K_{O_2}$, $K_{RuBP}$ ($K_m$ for RuBP), and Ω unless noted otherwise. The $K_{CO_2}$ was determined under strictly anaerobic conditions using sealed vials as described previously (20) with few modifications. Dilutions of NaH¹⁴CO₃ were prepared in 100 mM Bicine-NaOH buffer with 10 mM MgCl₂, The pH of the buffer was usually ~8.3, and the exact pH was recorded for each assay. Assays were performed at 83 °C, initiated by the addition of anaerobic RuBP, and terminated after 30 s by the addition of aerobic propionic acid. Vials were unsealed and dried overnight in a vacuum oven at 65 °C. Products were resuspended in 1 N HCl and counted in scintillation mixture. Results were plotted using SigmaPlot 2002 (Version 8.0), deriving the $K_{CO_2}$ and $K_{O_2}$ by fitting values to a hyperbolic curve and double-reciprocal plot. The concentration of CO₂ was derived using the pH and the Henderson-Hasselbach relationship. The solubility of CO₂ at 83 °C was calculated from published values (21) to obtain an equation that was extrapolated to 83 °C. Various concentrations of oxygen were introduced into the vials by removing a certain percent of the anaerobic headspace and replacing it with the same amount of oxygen from a sealed serum vial sparged with pure oxygen. The solubility of oxygen was determined using available solubility charts (Unisense A/S, Aarhus, Denmark). The salinity of the buffer used for each assay was calculated. The amount of soluble oxygen (micromolar) based on the salinity and temperature under the assay conditions was provided by the charts and used in determining the amount of oxygen present in each vial.

The $K_{RuBP}$ was measured as described for the $K_{CO_2}$ determined under strictly anaerobic conditions in sealed serum vials at 83 °C. Various concentrations of RuBP were prepared and sparged with argon gas. Assays were initiated by the addition of RuBP to the assay vials containing activated enzyme; the reaction was stopped after 30 s by the addition of aerobic propionic acid. Samples were dried overnight, resuspended, and counted in scintillation mixture. Results were plotted using SigmaPlot 2002 (Version 8.0).

Specificity was measured under conditions of saturating O₂ with 100 mM NaHCO₃ in 100 mM Bicine-NaOH buffer (pH 8.3) and 10 mM MgCl₂. The concentration of CO₂ was calculated from the Henderson-Hasselbach relationship as described above for the $K_{CO_2}$. The solubility of oxygen was determined using available solubility charts as described above. The solubility of CO₂ at 83 °C was calculated from published values (21) to obtain an equation that was extrapolated to 83 °C. Reactions were initiated by the addition of [1-³H]RuBP and incubated at 83 °C for 2 h. Reaction products were separated with a Mono Q resin using a Dionex DX500 chromatography system and detected with an in-line scintillation counter (β-RAM, IN/US Systems, Inc., Tampa, FL) as described (22).

Modeling of A. fulgidus RbcL2 was performed using DeepView Swiss-PdbViewer Version 3.7 (23). The template used to model the dimer form of the enzyme was the Thermococcus kodakaraensis strain KOD1 crystal structure (Protein Data Bank code 1GEH) (24); this is the closest related Rubisco large subunit and is 72% identical at the amino acid level to A. fulgidus RbcL2.

RESULTS

General Properties of A. fulgidus RbcL2—It is clear that Rubisco from the Archaea A. fulgidus (RbcL2) may be considered a member of a rather discrete class of proteins that are
FIGURE 2. Coomassie Blue-stained SDS-polyacrylamide gel of samples from Rubisco purification. The A. fulgidus RbcL2 gene was expressed in E. coli, and samples were obtained from uninduced cells (lane 1), soluble extract of French press-disrupted E. coli cells after induction (lane 2), supernatant obtained after centrifugation of the heat-treated extract for 20 min at 90 °C (lane 3), Q-Sepharose anion-exchange chromatography (lane 4), Superose 12 gel filtration (lane 5), and phencyl-Sepharose hydrophobic chromatography (lane 6).

Met-295 of Form III Archaeal Rubisco

A. fulgidus RbcL2 enzyme is a homodimeric protein (12), with each monomer composed of 441 amino acids with a molecular mass of 48.5 kDa. RbcL2 shows 41% amino acid identity to the large subunit of the Synechococcus spp. strain PCC 6301 Rubisco, a representative form I enzyme, and shows 33% amino acid identity to the R. rubrum enzyme, a typical form II Rubisco. The form I Synechococcus PCC 6301 and form II R. rubrum large subunits exhibit 33% identity to each other. Moreover, among other well studied form III Rubiscos, A. fulgidus RbcL2 shows close sequence (72%) identity to T. kodakaraensis strain KOD1 RbcL, but only 44% identity to M. jannaschii RbcL. The crystal structures of the form I Synechococcus PCC 6301 (Protein Data Bank code 1RBL), form II R. rubrum (code 5RUB), and form III T. kodakaraensis (code 1GEH) Rubiscos have been solved (27–29). Despite overall low sequence homology between representative enzymes from different groups, there is conservation of almost all critical active-site residues that are within 3.3 Å of the bound substrate analog 2-carboxyarabinitol 1,5-bisphosphate within the active site of the Synechococcus PCC 6301 enzyme (28), with the lone exception being position 170 (Fig. 1). It is also clear that what might be termed the Rubisco motif (GXDFXXKXE) is conserved in all Rubiscos, with only the phenylalanine residue within this region replaced with leucine or tyrosine in the form II enzymes from different groups, there is conservation of almost all critical active-site residues that are within 3.3 Å of the bound substrate analog 2-carboxyarabinitol 1,5-bisphosphate within the active site of the Synechococcus PCC 6301 enzyme (28), with the
easy distinguished from the well described form I and II Rubiscos (2, 6, 14, 25, 26). However, the form III A. fulgidus enzyme possesses characteristic Rubisco motifs found in both form I and II Rubisco large subunits (1, 25, 26). More specifically, the

FIGURE 1. Partial amino acid sequence alignments of form III archaeal (A. fulgidus, M. jannaschii, and T. kodakaraensis) and representative form I (Synechococcus sp. strain PCC 6301) and form II (R. rubrum) Rubiscos. Multiple sequence alignments were performed using ClustalW (32). The NCBI accession numbers for each deduced large subunit gene sequence are as follows: A. fulgidus rbcL2, O93627; M. jannaschii rbcL, Q58632; R. rubrum rbcL, O93627; Synechococcus (Synechococcus) PCC 6301 rbcL, P00880; and R. rubrum cbbM, P04718. Residue identities are marked with asterisks; conserved substitutions are marked with colons; and semiconserved substitutions are marked with periods (32). Known active-site residues determined to be within 3.3 Å of the bound substrate transition state analog 2-carboxyarabinitol 1,5-bisphosphate in the Synechococcus PCC 6301 enzyme are shown in boldface and labeled C for catalytic and R for RuBP binding properties. Where these residues are identical in all three sequences, they are highlighted in black. The characteristic Rubisco motif sequence (GXDFXXKXE) is conserved in all Rubiscos, with only the phenylalanine residue within this region replaced with leucine or tyrosine in the form II enzymes from different groups, there is conservation of almost all critical active-site residues that are within 3.3 Å of the bound substrate analog 2-carboxyarabinitol 1,5-bisphosphate within the active site of the Synechococcus PCC 6301 enzyme (28), with the lone exception being position 170 (Fig. 1). It is also clear that what might be termed the Rubisco motif (GXDFXXKXE) is conserved in all Rubiscos, with only the phenylalanine residue within this region replaced with leucine or tyrosine in the form III enzymes from M. jannaschii and A. fulgidus/T. kodakaraensis, respectively. It is the lysine residue within this motif that becomes carbamylated during “activation” of the enzyme, with the negatively charged aspartate and glutamate residues functioning to bind divalent cations to stabilize the carbamylated lysine (1). At this time, it is not known whether Phe-170 or residues substituted in this position are required for catalysis (1, 30). Although the deduced sequence of A. fulgidus RbcL2 is more homologous to form I large subunits, no small subunit has been detected in the genome of this organism, and residues previously shown to make contact with small subunits of the form I large subunits (11) are poorly conserved within the large subunit polypeptide of A. fulgidus RbcL2. Some of these small subunit contact residues are, however, conserved in the form II R. rubrum enzyme, which also does not have small subunits (31).

The A. fulgidus rbcL2 gene was overexpressed in E. coli BL21, and the resultant recombinant protein was purified to virtual homogeneity under strictly anaerobic conditions (Fig. 2). By nondenaturing polyacrylamide gel electrophoresis and gel f-
tration chromatography, purified \textit{A. fulgidus} RbcL2 was found to exist as a dimer of large subunits, similar to the previously determined holoenzyme structure of form III \textit{M. jannaschii} RbcL (11, 12). Like the \textit{M. jannaschii} (12) and \textit{T. kodakaraensis} (8) archaeal Rubiscos, the \textit{A. fulgidus} enzyme was highly active at temperatures up to 93 °C, with a temperature optimum for activity of 83 °C. Interestingly, activity was detected as low as 23 °C, quite different from the \textit{M. jannaschii} enzyme (12). Optimal activity for \textit{A. fulgidus} RbcL2 was also achieved in the presence of 0.3 m NaCl. Under strictly anaerobic conditions at 83 °C, the expected product, 3-[\textsuperscript{14}C]PGA, was produced in stoichiometric amounts by the \textit{A. fulgidus} RbcL2-catalyzed reaction using a \textsuperscript{14}CO\textsubscript{2} incorporation assay or a nonradioactive coupled PGA enzyme assay (11). These results demonstrate that this enzyme is a \textit{bona fide} Rubisco. Furthermore, \textit{A. fulgidus} RbcL2 was found to possess an unusually high \(k_{\text{cat}}\) of 23 s\textsuperscript{-1} at 83 °C (specific activity of 28.9 µmol/min/mg) compared with the characteristically low \(k_{\text{cat}}\) values of 3–5 s\textsuperscript{-1} reported for other forms of Rubisco assayed at their optimal temperatures (26, 32).

\textbf{Interactions with Molecular Oxygen}—Aside from its extreme thermostability and high intrinsic activity, \textit{A. fulgidus} RbcL2, unlike its \textit{T. kodakaraensis} homolog (8), was found to be sensitive to molecular oxygen. Thus, substantial activity loss was obtained in preparations exposed to oxygen and/or assayed in the presence of oxygen even in the presence of overwhelming excesses of bicarbonate. Our experiments, as well as previous studies (11, 12), indicated that the activity lost after exposure of \textit{A. fulgidus} RbcL2 to molecular oxygen could be recovered. When the enzyme was exposed to molecular oxygen for 30 min and assayed in the presence of air, ~10–15\% of the overall carboxylase activity was obtained compared with enzyme kept fully anaerobic and assayed under strictly anaerobic conditions. When the oxygen-exposed enzyme was subsequently injected into an anaerobic vial and assayed under strictly anaerobic conditions, 65\% of the carboxylase activity was obtained compared with enzyme preparations maintained and assayed under anaerobic conditions (Fig. 3A). Several repetitions of these experiments with different enzyme preparations indicated that the levels of recovered activity ranged from 65\% to nearly full recovery. The fact that the level of recovered activity varied suggested that perhaps differing amounts of oxygen might have been carried over from the oxygen incubation vials to the anaerobic assay tubes. Thus, experiments were designed to scrub out all vestiges of oxygen from enzyme preparations that had been exposed to oxygen and then transferred to and diluted in the anaerobic assay vials. Scrubbing was accomplished by incorporating commercially available protocatechuate 3,4-dioxygenase and its substrate, protocatechuic acid, into the Rubisco assay mixture to “fix” any oxygen that remained in solution. The protocatechuate 3,4-dioxygenase scrubbing system was highly effective, resulting in the recovery of all available carboxylase activity (Fig. 4). O\textsubscript{2}-mediated inhibition was thus fully reversible; partial restoration (65\%) of activity obtained in vials lacking the oxygen-scrubbing system was clearly attributable to oxygen carried over from the initial incubation.

\textbf{Kinetics of Oxygen-mediated Inhibition}—Inhibition by low amounts of oxygen, especially in the presence of a large excess of bicarbonate (\(\text{CO}_3\)) in the otherwise anaerobic

\textbf{FIGURE 3.} Recovery of carboxylase activity of \textit{A. fulgidus} RbcL2 upon oxygen exposure. The wild-type (A) and M295D (B) enzymes were assayed initially under strictly anaerobic conditions (Anaerobic). The enzyme was then exposed to molecular oxygen for 30 min and assayed in the presence of air (O\textsubscript{2} Exposed). An aliquot was taken from the vial containing oxygen-exposed enzyme, injected into an anaerobic vial, and assayed (Recovered). No O\textsubscript{2}-scavenging system was present in the “recovered” vials. Assay conditions were the same in all cases using the Rubisco assay as described under “Experimental Procedures,” with the exception of the “oxygen-exposed” samples, in which the assay vials were not sealed with rubber septas and were crimped with aluminum caps.

\textbf{FIGURE 4.} Reversibility of oxygen inhibition of \textit{A. fulgidus} RbcL2. Assays were performed in the absence (○) or presence (□) of molecular oxygen, followed by removal of molecular oxygen and replacement with an anaerobic atmosphere at 20 min and the addition of an O\textsubscript{2}-scavenging system containing protocatechuate 3,4-dioxygenase (20) at 30 min (indicated by the arrow). In all cases, the enzyme was dialyzed in a \textit{CO}_3- and O\textsubscript{2}-free buffer (wash buffer supplemented with 0.4 m NaCl).
must be quite efficient because simply diluting oxygen-exposed enzyme into an anaerobic assay with high levels of bicarbonate was not sufficient to fully re activate the enzyme. Furthermore, full activity was restored only after the addition of the O2-scavenging system. Thus, experiments were initiated to measure the affinity of the A. fulgidus enzyme for its substrates CO2, O2, and RuBP. The usual anaerobic methods were employed as described under “Experimental Procedures,” and the $K_{\text{CO2}}$, $K_{\text{O2}}$, and $K_{\text{RuBP}}$ were determined at 83 °C. The $K_{\text{CO2}}$ was determined to be $51 \pm 8 \mu M$ (Fig. 5). To calculate the $K_{\text{O2}}$, various fixed concentrations of pure oxygen were injected into vials that were assayed with varying amounts of CO2 as described for the $K_{\text{CO2}}$ determination. Double-reciprocal plots (Fig. 5, upper panel) clearly show that O2 was a competitive inhibitor with respect to CO2. In addition, replotting of the data allowed the $K_{\text{O2}}$ or $K_i$ for O2 to be determined ($5 \pm 1 \mu M$) (Fig. 5, lower panel). The $K_{\text{RuBP}}$ was determined to be $20 \pm 5 \mu M$ (Table 1). The $K_{\text{CO2}}$ and $K_{\text{RuBP}}$ values fall within the range of values reported for other form I and II Rubiscos (2). The most notable difference was the extremely low $K_{\text{O2}}$ for A. fulgidus RbcL2. Form I and II Rubiscos typically have $K_{\text{O2}}$ values ranging from 500 to 1000 $\mu M$; thus, it is apparent that the unusual sensitivity of A. fulgidus RbcL2 activity to oxygen may be attributed to the extremely high affinity of this form III archaeal enzyme for oxygen.

Bioinformatic Analysis of Different Forms of Rubisco—The low residual carboxylase activity seen with the A. fulgidus enzyme in the presence of low concentrations of oxygen was also observed for other form III archaeal enzymes from M. jannaschii and M. acetivorans (11, 12). Moreover, as a result of various genome sequencing projects, there are now several available archaeal Rubisco sequences in the data base. These archaeal sequences, along with many additional form I and II Rubisco sequences available, prompted a bioinformatic analysis of all available form I–III sequences to determine whether there might be unique residues within structurally significant regions of the enzyme that might perhaps influence the unusual kinetic properties of the archaeal Rubisco.
Eight known form III Rubiscos from Archaea, *A. fulgidus* RbcL2, *T. kodakaraensis* (NCBI accession number AB018555), *M. jannaschii* (AAB99239), *M. acetivorans* (AAM07894), Methanosarcina mazei (AAM30945), Pyrococcus abyssi (CAB50122), Pyrococcus furiosus (AAL81280), and Pyrococcus horikoshii (BAA30036), were aligned using ClustalW (33). Of the 441 amino acids in *A. fulgidus* RbcL2, there are 107 amino acids that are identical among the eight archaeal Rubiscos. To determine the uniqueness of these 107 amino acids, all eight form III Rubisco sequences were compared with a large representative group of form I and II Rubiscos. Of the 107 amino acids, 55 of these differed from the form I and II proteins. These 55 amino acids were then examined with respect to their positions within the crystal structures of representative form I (*Synechococcus* PCC 6301) and form II (*R. rubrum*) Rubiscos as well as to a homology model of the structure of dimeric *A. fulgidus* RbcL2.

Site-directed Mutagenesis and Properties of Altered Enzymes—Met-295 of the *A. fulgidus* rbcL2 gene was altered according to established site-directed mutagenesis protocols, with the recombinant M295F mutant *A. fulgidus* protein synthesized and purified to mimic the form I enzymes at this position and the M295A protein produced to mimic the form II enzymes. Finally, a recombinant M295D enzyme was synthesized and purified to introduce a charged amino acid that is not present at this position in any of the three forms of Rubisco. All three mutant proteins (M295A, M295D, and M295F) were initially analyzed in extracts prepared from small-scale cultures (25 ml) along with the wild-type enzyme, produced from cells grown under exactly the same conditions. Large-scale growths were also performed to obtain greater amounts of protein when required (see “Experimental Procedures”). For these initial assays, anaerobically prepared heat-treated supernatant fractions were used as the source of enzyme because significant amounts of heat-labile *E. coli* proteins could be removed (Fig. 2). Each sample was assayed under strictly anaerobic conditions.

Eight known form III Rubiscos from Archaea, *A. fulgidus* RbcL2, *T. kodakaraensis* (NCBI accession number AB018555), *M. jannaschii* (AAB99239), *M. acetivorans* (AAM07894), Methanosarcina mazei (AAM30945), Pyrococcus abyssi (CAB50122), Pyrococcus furiosus (AAL81280), and Pyrococcus horikoshii (BAA30036), were aligned using ClustalW (33). Of the 441 amino acids in *A. fulgidus* RbcL2, there are 107 amino acids that are identical among the eight archaeal Rubiscos. To determine the uniqueness of these 107 amino acids, all eight form III Rubisco sequences were compared with a large representative group of form I and II Rubiscos. Of the 107 amino acids, 55 of these differed from the form I and II proteins. These 55 amino acids were then examined with respect to their positions within the crystal structures of representative form I (*Synechococcus* PCC 6301) and form II (*R. rubrum*) Rubiscos as well to monitor activity; an aliquot of the enzyme preparation was then exposed to molecular oxygen and re-assayed. The wild-type, M295A, and M295F enzymes each had 11–17% activity compared with anaerobic controls. Surprisingly, the M295D enzyme showed the least sensitivity to oxygen exposure and retained 35% of its activity after the same oxygen exposure regimen.

These results prompted additional studies of the M295D enzyme; accordingly, large-scale growths allowed significant amounts of purified recombinant protein to be prepared, much like the recombinant wild-type enzyme (Fig. 2). Purified wild-type and M295D proteins were assayed both anaerobically and aerobically after 30 min of exposure to oxygen. Although the purified wild-type enzyme again showed 85–90% loss of activity upon oxygen exposure and assay in the presence of air, the
M295D enzyme lost only 60–65% of its activity (Fig. 3B), similar to results obtained with the partially purified M295D protein. Clearly, the M295D enzyme was altered in such a way that the normal response to molecular oxygen was changed; this enzyme appeared much less susceptible to the deleterious effects of oxygen. As with the wild-type enzyme (Fig. 5), the kinetic constants for each of the substrates (K_{CO2}, K_{O2}, and K_{RbPB}) were determined for the M295D enzyme at 83 °C (Table 1). Clearly, there was little change in the K_{CO2} and K_{RbPB} for the M295D enzyme. However, in agreement with the recovery experiment, there was an ~5-fold increase in the K_{O2} (from 5 ± 1 μM for the wild-type enzyme to 24 ± 7 μM determined for the M295D protein). Although double-reciprocal plots at varying CO2 concentrations and several fixed O2 levels for the M295D enzyme (Fig. 6, upper panel) showed more scatter than for the wild-type enzyme (Fig. 5), replots of the data gave a linear response (Fig. 6, lower panel) such that accurate and reproducible kinetic constants could be determined. Again, the M295D enzyme showed the expected competitive inhibition by O2 with respect to CO2. Furthermore, the M295D enzyme retained significantly more activity than did the wild-type enzyme when both enzymes were incubated with concentrations of oxygen ranging from 1 to 100% in the gas phase (data not shown). Like the wild-type enzyme (Fig. 4), the M295D enzyme recovered fully after the oxygen-exposed enzyme was incubated in anaerobic vials containing the oxygen-scrubbing system to remove carryover oxygen from the incubation vials.

The low residual activity that was retained by the wild-type A. fulgidus enzyme upon exposure to oxygen suggested that it would be feasible to determine the CO2/O2 specificity of the enzyme, i.e. the specificity factor (Ω). Thus, experiments were performed with both the wild-type and M295D enzymes using established methods to separate and quantitate the specific carboxylase and oxygenase reaction products (3-[3H]PGA and 2-[3H]PG, respectively) obtained from a reaction mixture containing [1-3H]RuBP and both gaseous substrates CO2 and O2 (22). The results of this experiment indicated that the wild-type A. fulgidus RbcL2 enzyme catalyzed, albeit weakly and over a long time period, oxygen-dependent formation of 2-[3H]PG (Fig. 7A), which was not formed in the absence of oxygen (Fig. 7C). Clearly, comparisons of the chromatographic profiles in the presence and absence of oxygen indicated that the level of 3-[3H]PGA produced was greatly reduced under an oxygen atmosphere, in agreement with the 14CO2 incorporation assays showing inhibition of carboxylase activity in the presence of oxygen. In addition, it was apparent that the M295D enzyme produced significantly more 3-[3H]PGA than did the wild-type enzyme (Fig. 7B). Presumably, the increase in the K_{O2} or K_i for O2 for the M295D enzyme impinges on the fact that the carboxylation reaction is less inhibited in the presence of oxygen compared with the wild-type enzyme. The levels of 3-[3H]PGA and 2-[3H]PG produced at the concentrations of O2 and CO2 utilized in this reaction allowed a calculation to be made of the relative CO2/O2 substrate specificity factor (Ω) value for this archaeal enzyme and the M295D mutant protein (Table 1). The consequences of enhanced carboxylase activity in the presence of oxygen for the M295D enzyme and the increase in the K_{O2},
Met-295 of Form III Archaeal Rubisco

 caused an ~3-fold increase in the Ω value compared with the wild-type protein.

To assess whether the role of the methionine-to-aspartic acid substitution at position 295 in the enzyme might be unique, substitutions were considered at other positions in close proximity to the active site. Similar to how Met-295 is positioned with regard to the active site, Ser-363, ~10 Å from Met-295 according to the modeled structure, is situated in what appears to be a hydrophobic pocket that surrounds one side of the active site. In addition, the model structure shows an ionic interaction of the side chain of Ser-363 with the highly conserved and catalytically important residues Gly-313 and Thr-314 of A. fulgidus RbcL2. Gly-313 and Thr-314, found in all forms of Rubisco, show no ionic interactions with the amino acid residue equivalent to Ser-363 of RbcL2 in form I and II enzymes. This unique interaction and positioning of Ser-363 in a key hydrophobic pocket of RbcL2, similar to Met-295, thus suggested that Ser-363 of RbcL2 might be a likely candidate for further investigation by site-directed mutagenesis. Sequence alignments of form I–III enzymes show that alanine is uniquely conserved at this position in the form I enzymes and that isoleucine is uniquely conserved at this same position in the form II enzymes. In the form III enzymes, serine is present at this position for A. fulgidus, T. kodakaraensis, and M. jannaschii, whereas the remaining methanogens and all of the pyrococci have alanine present at this position. Because of the residues found at this position in the above enzymes, Ser-363 was changed to alanine, isoleucine, or valine in A. fulgidus RbcL2. Initial analysis of heat-stable enzyme and 86% activity for the M295D/S363V enzyme (Fig. 8). However, as a consequence of changing these two residues near the active site, the absolute activity levels (specific activities or $k_{cat}$) of these two double mutants were much lower than those of the wild-type and single mutant enzymes.

**DISCUSSION**

A previous study demonstrated, via $^{14}$CO$_2$ radiometric assays and Western immunoblotting, that crude cell extracts of A. fulgidus do indeed contain functional Rubisco (12). Because A. fulgidus is a thermophilic strict anaerobe isolated from the bottom of the ocean near hydrothermal vents, it is not surprising that Rubisco from this organism adapts to function under similar extreme conditions in vitro. Indeed, this form III homodimer catalyzes a reaction with a $k_{cat}$ that is 4–5-fold higher than other forms of Rubisco. Of considerable interest (and unlike other well studied Rubiscos) is the substantial loss of carboxylase activity of the A. fulgidus enzyme even when CO$_2$ levels are in great excess. Of molecular oxygen even when CO$_2$ levels are in great excess. Of molecular oxygen even when CO$_2$ levels are in great excess.

FIGURE 8. Wild-type and mutant A. fulgidus RbcL2 activities after exposure to oxygen. Purified recombinant wild-type, M295D, S363I, S363V, M295D/S363I, and M295D/S363V enzymes were assayed for activity under strictly anaerobic conditions. These preparations were then exposed to pure oxygen as described under “Experimental Procedures” and assayed in the presence of air. The percent activity retained is the amount of carboxylase activity obtained under anaerobic conditions compared with the amount of carboxylase activity obtained after oxygen exposure and assay in the presence of air. Absolute specific activities (nanomoles of CO$_2$/fixed per min/mg) obtained under both conditions are listed below each sample.

| Activity Remaining (%) | Wild-type | M295D | S363I | S363V | M295D/S363I | M295D/S363V |
|------------------------|-----------|-------|-------|-------|-------------|-------------|
| Anaerobic$^{a,b}$      | 10396 ± 637 | 12732 ± 376 | 15854 ± 692 | 15468 ± 919 | 615 ± 23 | 2715 ± 170 |
| O$_2$-Exposed$^{a,b}$  | 1255 ± 18  | 6019 ± 664 | 7699 ± 855 | 6617 ± 170 | 505 ± 89 | 2361 ± 124 |

$^a$Average of at least three independent assays.

$^b$Specific activities given in mmol CO$_2$/min/mg.

Wild-type and mutant A. fulgidus RbcL2 activities after exposure to oxygen.
related Rubisco from _T. kodakaraensis_ (29), suggested that it might be feasible to design experiments to elucidate the molecular basis for the unusual properties exhibited by the _A. fulgidus_ enzyme. Of particular interest is the extremely high _k_\text{cat} and oxygen sensitivity of this enzyme. The response to molecular oxygen was clearly shown to be a classic competition with CO\textsubscript{2} for the enediolate intermediate of the enzyme, as observed for all Rubisco proteins. However, what distinguished the _A. fulgidus_ enzyme from other sources of Rubisco was the extremely high affinity this enzyme showed for molecular oxygen, with _K_\text{f} values (~5 \mu M) that were nearly 3 orders of magnitude lower than those of typical form I or II enzymes. Clearly, this high affinity for molecular oxygen underscores why inhibition of carboxylase activity was initially obtained even in reaction mixtures that contained levels of CO\textsubscript{2} that normally overcomes the inhibitory effects of oxygen on form I and II Rubiscos.

Obviously, Rubisco from organisms like _A. fulgidus_ is not ever expected to encounter molecular oxygen. With the interesting in vitro response of this enzyme to oxygen noted in this study, we proceeded to further analyze this protein with expectations that such studies might eventually provide clues as to how the active site of Rubisco evolved in more evolutionary advanced organisms. In our analysis of the linear sequences of the _A. fulgidus_ (and other archaeal) Rubiscos compared with those of other well studied form I and II enzymes, Met-295 was singled out for further attention. After this residue was altered by site-directed mutagenesis, and recombinant M295A, M295D, and M295F proteins were prepared, it was apparent that the M295D enzyme showed substantially less sensitivity to molecular oxygen than did the wild-type protein. A homology model of the homodimeric structure of _A. fulgidus_ RbcL2 was constructed (Fig. 9) based on the solved structure of the highly homologous (72% amino acid identity) _T. kodakaraensis_ enzyme (29). Like the large subunits of all Rubiscos, known residues necessary for catalysis (1) are conserved and are positioned within the _A. fulgidus_ structure in the same locale as in other Rubisco structures. As for Met-295, it was found to be situated in a hydrophobic pocket created by residues along the active site and in close proximity to a highly conserved residue, Arg-279 (Figs. 1 and 10), found in all other forms of Rubisco and shown to be necessary for catalysis and binding of the five-carbon substrate RuBP. The model structure predicts no ionic interactions between Arg-279 and Met-295 in the wild-type form of the enzyme (A). In the M295D mutant, the model predicts an ionic interaction between the hydroxyl group of Asp-295 and the amino group of Arg-279 (broken purple line).
aspartate residue with one of the side chain nitrogen atoms of Arg-279 (Fig. 10B). All other amino acid mutations made at position 295 suggested either unfavorable conformations or no ionic interactions with Arg-279. In addition, many rotamers were available for the aspartic acid substitution at the methionine position; the rotamers with the lowest score, thus the most favorable conformation, all had hydrogen bond interactions with Arg-279. Although this occurrence is seen within the model structure, perhaps stabilization of Arg-279 is necessary for the carboxylation activity to function in the presence of oxygen, or perhaps there is some significance to the presence of hydrophobic pockets surrounding the active site. Thus, the amino acid side chain situated in these pockets might play a role in the enzyme’s overall carboxylation activity in the presence of oxygen, as demonstrated for Met-295. This could perhaps be the reason why hydrogen bonding is observed at a different position in other solved crystal structures, but not in the model of A. fulgidus RbcL2. Further investigation into this localized structural change led us to another amino acid, Ser-363, which we predicted might have a similar effect on oxygen sensitivity. A possible alteration of the hydrogen bond interactions with the highly conserved Arg-279, independent of the suggested hydrogen bonding between M295D and Arg-279, might be influenced by mutations to Ser-363. Again, the model structure indicates that this amino acid is on β-strand 7, situated in a hydrophobic pocket adjacent to the active site. Alanine is strictly conserved at this same position in the form I enzymes, and isoleucine in the form II enzymes. Form III enzymes have either serine or alanine. For A. fulgidus RbcL2, the S363I and S363V mutant enzymes showed increased retention of activity when assayed in the presence of oxygen, whereas the S363A mutant enzyme mimicked the wild-type enzyme and lost activity when assayed in the presence of oxygen. These results were quite reminiscent of what was found for enzymes containing substitutions at Met-295. Additionally, the recombinant M295D/S363I and M295D/S363V double mutants retained very high levels of activity when assayed in the presence of oxygen, suggesting an additive effect of mutations at two influential residues in hydrophobic pockets situated close to the active site. The mutations at Ser-363 and the double mutations of M295D and the Ser-363 mutants will prompt further investigations of these interactions and how they influence the activity of the enzyme and, more importantly, the interaction with molecular oxygen. In addition, the potential to biologically select (20) double mutants that retain high absolute activity is something that might be quite feasible and most revealing.

Acknowledgments—We thank the staff of the Plant-Microbe Genomics Facility for automated DNA sequencing and Cedric Bobst and Sriram Satagopan for discussions and technical advice.

REFERENCES

1. Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., and Lorimer, G. H. (1998) Chem. Rev. 98, 549–562
2. Tabita, F. R. (1999) Photosynth. Res. 60, 1–28
3. Ellis, R. J. (1979) Trends Biochem. Sci. 4, 241–244
4. Jordan, D. B., and Ogren, W. L. (1981) Nature 291, 513–515
5. Horken, K. M., and Tabita, F. R. (1999) Arch. Biochem. Biophys. 361, 183–194
6. Spreitzer, R. J. (1999) Photosynth. Res. 60, 29–42
7. Deppenmeier, U., Johann, A., Hartsch, T., Merkl, R., Schmitz, R. A., Martinez-Arias, R., Henne, A., Wiezer, A., Baumer, S., Jacob, C., Bruggemann, H., Lientard, T., Christmann, A., Bomecke, M., Steckel, S., Bhattacharyya, A., Lykidis, A., Overbeek, R., Klenk, H. P., Gunsalus, R. P., Fritz, H. J., and Gottschalk, G. (2002) J. Mol. Microbiol. Biotechnol. 4, 453–461
8. Ezaki, S., Maeda, N., Kishimoto, T., Atomi, H., and Imanaka, T. (1999) J. Biol. Chem. 274, 5078–5082
9. Galagan, J. E., Nusbaum, C., Roy, A., Endrizzi, M. G., MacDonald, P., FitzHugh, W., Calvo, S., Engels, R., Smirnov, S., Atwood, D., Brown, A., Allen, N., Naylor, J., Stange-Thomann, N., DeArellano, K., Johnson, R., Linton, L., McEwan, P., McKernan, K., Talamas, J., Tirrell, A., Ye, W., Zinner, A., Barber, R. D., Cann, I., Graham, D. E., Grahame, D. A., Guss, A. M., Hedderich, R., Ingram-Smith, C., Kuettern, H. C., Krzycki, J. A., Leigh, J. A., Li, W., Liu, J., Mukhopadhyay, B., Reeve, J. N., Smith, K., Springer, T. A., Umayam, L. A., White, O., White, R. H., Conway de Macario, E., Ferry, J. G., Jarrell, K. F., Jing, H., Macario, A. J., Paulsen, I., Pritchett, M., Sowers, K. R., Swanson, R. V., Zinder, S. H., Lander, E., Metcalf, W. W., and Birren, B. (2002) Genome Res. 12, 532–542
10. Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D., Richardson, D. L., Kerlavage, A. R., Graham, D. E., Kirkpides, N. C., Fleischmann, R. D., Quackenbush, J., Lee, N. H., Sutton, G. G., Gill, S., Kirkness, E. F., Dougherty, B. A., McKenney, K., Adams, M. D., Loftus, B., Peterson, S., Reich, C. I., McNeil, L. K., Badger, J. H., Gloced, A., Zhou, L., Overbeek, R., Gocayne, J. D., Weidman, J. F., McDonald, L., Utterback, T., Cotton, M. D., Spriggs, T., Artiach, P., Kaine, B. P., Sykes, S. M., Sadow, P. W., D’Andrea, K. P., Bowman, C., Fujii, C., Garland, S. A., Mason, T. M., Olsen, G. J., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1997) Nature 390, 364–370
11. Watson, G. M. F., Yu, J., and Tabita, F. R. (1999) J. Bacteriol. 181, 1569–1575
12. Finn, M., and Tabita, F. R. (2003) J. Bacteriol. 185, 3049–3059
13. Finn, M., and Tabita, F. R. (2004) J. Bacteriol. 186, 6360–6366
14. Watson, G. M. F., and Tabita, F. R. (1997) FEMS Microbiol. Lett. 146, 13–22
15. Tabita, F. R., and McFadden, B. A. (1974) J. Biol. Chem. 249, 3459–3464
16. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
17. Papworth, C., Bauer, J. C., Braman, J., and Wright, D. A. (1996) Strategies 9, 3–4
18. Tabita, F. R., Caruso, P., and Whitman, W. (1978) Anal. Biochem. 84, 462–472
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Smith, S., and Tabita, F. R. (2003) J. Mol. Biol. 331, 557–569
21. Dean, J. A. (ed) (1985) Lange’s Handbook of Chemistry, 13th Ed., McGraw-Hill Book Co., New York
22. Harpel, M. R., Lee, E. H., and Hartman, F. C. (1993) Anal. Chem. 209, 367–374
23. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
24. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) Nucleic Acids Res. 28, 235–242
25. Gutteridge, S., and Gatenby, A. A. (1995) Plant Cell 7, 809–819
26. Hartman, F. C., and Harpel, M. R. (1994) Annu. Rev. Biochem. 63, 197–234
27. Lundqvist, T., and Schneider, G. (1991) J. Biol. Chem. 266, 12604–12611
28. Newman, J., and Gutteridge, S. (1993) J. Biol. Chem. 268, 25876–25886
29. Maeda, N., Kitano, K., Fukui, T., Ezaki, S., and Imanaka, T. (1999) J. Mol. Biol. 293, 57–66
30. Andersson, I., Knight, S., Schneider, G., Lundqvist, Y., Lundqvist, T., Branden, C.-I., and Lorimer, G. H. (1989) Nature 337, 229–234
31. Tabita, F. R., and McFadden, B. A. (1974) J. Biol. Chem. 249, 3459–3466
32. Hartman, F. C., and Harpel, M. R. (1993) Adv. Enzymol. Relat. Areas Mol. Biol. 6, 71–75
33. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1989) Nucleic Acids Res. 22, 4673–4680
34. Zhang, K. Y. J., Cascio, D., and Eisenberg, D. (1994) Protein Sci. 3, 64–69