Unusual Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase of Anoxic Archaea

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The predominant pool of organic matter on earth is derived from the biological reduction and assimilation of carbon dioxide gas, catalyzed primarily by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). By virtue of its capacity to use molecular oxygen as an alternative and competing gaseous substrate, the catalytic efficiency of RubisCO and the enzyme's ability to assimilate CO₂ may be severely limited, with consequent environmental and agricultural effects. Recent genomic sequencing projects, however, have identified putative RubisCO genes from anoxic Archaea. In the present study, these potential RubisCO sequences, from Methanococcus jannaschii and Archaeoglobus fulgidus, were analyzed in order to ascertain whether such sequences might encode functional proteins. We also report the isolation and properties of recombinant RubisCO using sequences obtained from the obligately anaerobic hyperthermophilic methanogen M. jannaschii. This is the first description of an archaeal RubisCO sequence; this study also represents the initial characterization of a RubisCO molecule that has evolved in the absence of molecular oxygen. The enzyme was shown to be a homodimer whose deduced sequence, along with other recently obtained archaeal RubisCO sequences, differs substantially from those of known RubisCO molecules. The recombinant M. jannaschii enzyme has a somewhat low, but reasonable kₐₐₜ, however, unlike previously isolated RubisCO molecules, this enzyme is very oxygen sensitive yet it is stable to hyperthermal temperatures and catalyzes the formation of the expected carboxylation product. Despite inhibition by oxygen, this unusual RubisCO still catalyzes a weak yet demonstrable oxygenase activity, with perhaps the lowest capacity for CO₂/O₂ discrimination ever encountered for any RubisCO.

Carbon dioxide (CO₂) is a greenhouse gas whose relative concentration is thought to be increasing in the earth's atmosphere (23). CO₂ is also the sole carbon source for the predominant life-forms on this planet, and its efficient incorporation into organic matter is directly related to the productivity of important ecosystems, including agriculturally significant plants (34). For most organisms, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO) catalyzes the primary step of CO₂ fixation (6, 8, 25); despite the fact that it is the most abundant protein found on earth (3), RubisCO's catalytic efficiency is severely limited by the capacity to catalyze a competing O₂ fixation reaction. This leads to inefficient CO₂ fixation and low productivity. At this time, the molecular basis for CO₂/O₂ discrimination is not completely understood; however, the relative capacity of this enzyme to favor either carboxylation or oxygenation is not immutable but varies for different sources of RubisCO (9, 20). Recently, the complete genomes of the hyperthermophilic archaeons Methanococcus jannaschii and Archaeoglobus fulgidus were sequenced (2, 10). These organisms are representative of prokaryotic organisms (Archaea) that are considered to be distinct from eubacteria; they also possess many characteristics common to eukaryotes and are thus thought to be representative of a third kingdom of life (31). Since both methanogenic and sulfate-reducing archaea are anoxic organisms that fix CO₂ by acetyl coenzyme A and reductive tricarboxylic acid-like pathways (5, 22, 24, 32), it was most surprising to find that both genes contain sequences that potentially encode the large subunit of RubisCO.

Indeed, A. fulgidus possesses two putative RubisCO genes. RubisCO is the key enzyme of the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway (6, 8, 25), a route that is quite distinct from the known CO₂ assimilatory pathways of these organisms. Despite the fact that the CBB pathway and RubisCO are undoubtedly responsible for the bulk of organic carbon on earth, the CBB route has never been unequivocally established to be significant in obligately anoxic prokaryotes. Since RubisCO also catalyzes a physiologically important oxygenase reaction, such that CO₂ and O₂ compete for the enzyme-bound enediolate of RuBP (6, 8, 25), it is perhaps understandable that obligately anoxic prokaryotes employ other CO₂ fixation reaction schemes. However, the unexpected potential opportunity to examine RubisCO from organisms that evolved in the complete absence of oxygen presumably provides an unprecedented opportunity to discern how the active site of diverse RubisCO enzymes may have evolved to function at different CO₂ and O₂ tensions. This study was directed at examining the potential of archaeal RubisCO sequences to encode for functional enzymes. The results of this study indicate that recombinant M. jannaschii RubisCO catalyzes a bonafide RubisCO reaction, albeit with many unusual properties, including a rather unique interaction with oxygen.

MATERIALS AND METHODS

Expression vector construction. An M. jannaschii RubisCO expression plasmid was constructed beginning with plasmid pAMJEV50, which was obtained from The Institute for Genomic Research, Rockville, Md. It contains the putative RubisCO large subunit gene, MJ1235, plus another hypothetical open reading frame, MJ1234. After being treated with AseI, plasmid pAMJEV50 yielded a 1.4-kb fragment containing the M. jannaschii RubisCO gene plus a truncated fragment of MJ1233. The 5' overhangs of the AseI fragment were filled and subcloned into plasmid pK18 by using the Smal site of the multicloning region.

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After sequencing to establish orientation, the resulting plasmid, pK18Gw, was digested with BamHI and KpnI and ligated into the (His)6-tagged vector pPROEXHTa (Life Technologies, Inc., Gaithersburg, Md.) to produce expression vector pGwHa.

Synthesis and purification of recombinant Rubisco. The recombinant M. jannaschii Rubisco was purified from crude extracts of Escherichia coli DH5α (pGwHa) grown anaerobically at 35°C in Terrific (glycerol)-fumarate growth medium, by centrifuging (per liter of distilled water) 12 g of typtone, 24 g of yeast extract, 4 g of fumaric acid, and 0.5% (vol/vol) glycerol, buffered at pH 7.2 with 90 mM potassium phosphate buffer containing 100 μg of ampicillin/ml. Seed cultures of 100 ml were grown aerobically in shake flasks overnight and inoculated into a fermenter vessel containing 10 liters of medium which was sparged with either nitrogen or argon gas at a rate of 2 l/min. When the culture reached an optical density at 590 nm (OD590) of between 0.2 and 0.3, isopropyl β-D-galactopyranoside was added to a concentration of 0.1 mM. After the culture reached an OD590 of between 0.4 and 0.6, the cells were harvested anaerobically by using a continuous flow centrifuge system flushed with nitrogen gas. The cell pellet was placed in a 250-ml centrifuge bottle in an anaerobic chamber and then stored at −70°C. At the desired time, the frozen cells were thawed and centrifuged anaerobically at 16,000 × g at 4°C for 10 min to remove leftover broth. The cell pellet was washed twice anaerobically with 250 ml of lysis buffer (50 mM Tris-HCl [pH 8.5] containing 10 mM β-mercaptoethanol and 0.8 M KC1). The washed pellet, in 50 ml of lysis buffer containing 1 mM phenylmethysulfonyl fluoride, was disrupted by using a pressurized French pressure cell (at 102,000 kPa) with the crude extract extruded into a 160-ml sealed serum bottle flushed with argon gas. The crude extract was placed in a 250-ml centrifuge bottle in the anaerobic hood, which was then sealed and centrifuged at 16,000 × g at 4°C for 10 min. The supernatant was decanted into a fresh 160-ml serum bottle in the anaerobic hood; the bottle was pressurized with argon to 68 kPa and then placed into an 85°C water bath for 15 min with gentle shaking. The heat-treated crude extract was transferred to a 50-ml anaerobic centrifuge tubes and centrifuged at 30,000 × g for 30 min at 4°C. This supernatant was added to an argon-pressurized (68 kPa) 160-ml serum bottle and stored at 4°C or used (at room temperature) for the subsequent column chromatography step. Nickel chelate chromatography, following protocols provided with the ProEX HT Prokaryotic Expression System kit (Life Technologies, Inc.), was performed in the anaerobic hood. Fractions were collected in 2-ml sealed serum vials, and samples were assayed for activity by standard procedures. Recombinant Rhodospirillum rubrum Rubisco was purified by a previously established procedure (27) except that a Green-A agarose dye affinity column was substituted for the DEAE-cellulose column step (19). Polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) was performed by standard procedures (20), at 10 and 8% acrylamide, respectively. In some cases, non-denaturing gradient gels (from 4 to 20% acrylamide) were used.

Analysis and quantitation of reaction products. Radiometric 14CO2 fixation and coupled 3-phosphoglyceric acid (3-PGA) assays were performed as previously described (26). The transition state analog 2-carboxyarabinitol 1,5-bisphosphate (CABP) and [1-3H]RuBP were synthesized by established procedures (12, 17). To quantitate the level of Rubisco reaction products, [1-3H]RuBP (80 μM) was incubated with the M. jannaschii enzyme (66 μg/ml) or the R. rubrum enzyme (2 μg/ml) in 0.5 ml of 80 mM HEPES-NaOH buffer, pH 7.2, containing 10 mM β-mercaptoethanol. The reactions were quenched with NaBH₄, and the mixtures were deproteinized and applied and eluted from a MonoQ anion exchange column (HR5/5, 5 by 50 mm; Pharmacia Biotech, Inc., Piscataway, N.J.), as previously described (7). Radioactivity was continuously monitored with a β-RAM detector (IN/US, Tampa, Fla.).

RESULTS AND DISCUSSION

Analysis of potential archaeal Rubisco sequences. As a first attempt to determine if the archaeal sequences encode active Rubisco, we carefully analyzed the M. jannaschii and A. fulgidus deduced amino acid sequences. Phylogenetic analysis (Fig. 1) of representatives of all known classes of Rubisco shows that the putative M. jannaschii and A. fulgidus proteins fall into a group that is quite distinct from previous groupings of known form I and form II enzymes (30). Indeed, the distinctness of the M. jannaschii and A. fulgidus deduced sequences are such that one might question whether they could possibly encode functional proteins. The putative proteins possess characteristic motifs of both form I and form II Rubisco large subunits, the two structurally distinct types of catalytic polypeptide here-tofore described (6, 8, 25) (Fig. 2). The M. jannaschii protein shows 33% amino acid identity to a typical form II subunit (that from the nonsulfur purple bacterium R. rubrum) and exhibits 41% identity to the closest representative form I Rubisco large subunit (that from the cyanobacterium Synechococcus sp.

strain PCC 6301); the R. rubrum and Synechococcus sequences are themselves 33% identical. The crystal structures of both these bacterial Rubisco have been solved (14, 15). Interestingly, the A. fulgidus 1 and A. fulgidus 2 deduced sequences show only 41 and 45% identity, respectively, to the putative M. jannaschii Rubisco. However, comparisons of the deduced amino acid sequences of the M. jannaschii and A. fulgidus Rubisco to the Synechococcus form I and R. rubrum form II enzymes (Fig. 2) indicated that almost all the critical active-site residues were present. For example, of 14 active-site residues (Fig. 3) determined to be within 3.5 Å of the bound transition state analog CABP (15) in the Synechococcus enzyme, 13 residues are identical in the M. jannaschii and A. fulgidus sequences. The only variant residues are at position 193 of the alignment (Fig. 2). It should be noted that the phenylalanine in the consensus pattern Gly-x-D-x-F-x-K-x-X-D-E found in all functional Rubisco so far identified, is replaced by a leucine in the M. jannaschii sequence and by isoleucine or tyrosine in the A. fulgidus proteins. While the lysine becomes carbamylated during “activation” of the enzyme and the second aspartate is a magnesium ligand (1), to our knowledge the phenylalanine of this motif has not been shown to be directly required for catalysis. Although the deduced sequences of the putative archaeal Rubisco proteins are more similar to that of the form I enzyme, no small subunit sequence was detected in either genome. Consequently, we have found that the putative archaeal proteins contain either
FIG. 2. Deduced amino acid sequence alignment of archaean (M. jannaschii and A. fulgidus) and representative form I (Synechococcus sp. strain PCC 6301) and form II (R. rubrum) RubisCO molecules. Multiple sequence alignments were performed by using ClustalW (29). The accession numbers for each deduced large subunit sequence are as follows: Synechococcus PCC 6301, P00880; M. jannaschii, Q58632; A. fulgidus rbcL1, O28685; A. fulgidus rbcL2, O28635; R. rubrum, (P04718). Residue identities are marked with an asterisk, conserved substitutions are marked with a colon, and semiconserved substitutions are marked with a period (29). Known active-site residues determined to be within 3.3 Å of the bound transition state analog CABP in the Synechococcus PCC 6301 enzyme are labeled A. Where these residues are identical in all three sequences they are in boldface type. Residues known to make contact with small subunits in the Synechococcus enzyme are labeled S. The characteristic RubisCO motif sequence, GXDFXKXDE, is shown in boldface and underlined. The alignments were adjusted manually to take into account known structural considerations.
poorly conserved residues or, in fact, do not possess residues that have been previously shown to make contact with small subunits of the form I enzyme (Fig. 2). Where these residues are conserved, most of the conservations are also found in the R. rubrum enzyme, which also does not have small subunits (28).

Initially, we focused on the M. jannaschii sequence, simply because it was available before those of A. fulgidus. It is apparent that the M. jannaschii sequence is notably different from those of the form I and II enzymes in the following regions: a large gap in the alignment from position 463 to 474 and a 14-amino-acid truncation at the C terminus relative to the R. rubrum enzyme. Similar gaps are found in the A. fulgidus deduced sequences. The C-terminal truncation of these putative proteins is of interest since removal of amino acids downstream of Pro-478 of the R. rubrum RubisCO yielded an enzyme that lost >99% of its ability to bind CABP and also tended to form an octameric structure (18). In general, the sequences surrounding the 14 active-site residues are more similar between the Synechococcus and M. jannaschii sequences. However, the region downstream of the catalytically important loop-6 region (residues 364 to 373 of the alignment) is of some interest since this sequence is similar to that of the equivalent region from R. rubrum but diverges greatly from those of the Synechococcus protein and other form I Rubisco.

Overall, the near absolute conservation of critical residues suggests the strong possibility that selective pressures have maintained the functionality of the M. jannaschii protein. Finally, three-dimensional coordinates were obtained for the putative M. jannaschii sequence and the A. fulgidus rbcL2 sequences by homology modeling with the ExPasy server (16). A comparison with the known Synechococcus structure clearly demonstrated that the M. jannaschii sequence and the A. fulgidus rbcL2 sequences can be modeled in such a way that all active-site residues found within 3.3 Å of the bound transition state analog CABP (15) are found at the same coordinates in the predicted archaeal Rubisco structures (Fig. 3). Indeed, the active-site residues and many other major features of these structures are virtually superimposable. Major features and differences between the enzymes are highlighted in Fig. 3 (see also the legend to Fig. 2). The confidence level for this model is very high for >90% of the residues.

Properties of recombinant M. jannaschii Rubisco. The available “in silico” evidence indicated that the M. jannaschii and A. fulgidus sequences might encode functional Rubisco.

To provide experimental proof for this suggestion, the M. jannaschii Rubisco sequence was cloned from total M. jannaschii DNA or subcloned from a plasmid previously shown to contain the gene of interest (2). The putative M. jannaschii Rubisco

FIG. 3. Tertiary structure prediction of archaeal Rubisco molecules. The predicted tertiary structure of the M. jannaschii sequence (A) and the A. fulgidus rbcL2 sequence (B) is compared to that of the known structure of the Synechococcus large subunit (C). The Synechococcus small subunit is also shown to the lower left of the structure (in amber). Label sizes and shading reflect the distance from the viewer with the smaller and darker, respectively, being further from the viewer. The main features are highlighted as follows: yellow, active-site residues within 3.3 Å of the bound transition state analog CABP (15) are found at the same coordinates in the predicted archael Rubisco structures (Fig. 3). Indeed, the active-site residues and many other major features of these structures are virtually superimposable. Major features and differences between the enzymes are highlighted in Fig. 3 (see also the legend to Fig. 2). The confidence level for this model is very high for >90% of the residues.
sequence was then subcloned into vector pProExHTa and then expressed in *E. coli* to produce a (His)_6-tagged recombinant protein. Similarly, expression vectors containing the *A. fulgidus* RubisCO sequences have also been prepared (33). The *M. jannaschii* recombinant fusion protein was recovered as a homogeneous preparation from metal-chelate columns (Fig. 4A) and migrated in SDS-polyacrylamide gels as a protein with a molecular weight of about 55,000, consistent with its deduced molecular mass of 51,726 Da. The amino-terminal amino acid sequence of recombinant protein prepared from constructs that do not contain a (His)_6-tag sequence (i.e., prepared by using a pK vector) was shown to agree with the amino terminus of the deduced sequence. On a nondenaturing 8% acrylamide gel (not shown), the (His)_6-tagged protein migrated at a position which would suggest that it is a homodimer with a calculated molecular weight of approximately 105,000 (Fig. 4B). The recombinant *M. jannaschii* protein, whether prepared from a (His)_6-tagged or a non-(His)_6-tagged vector, possessed specific activities which ranged from 1 to 2 nmol of CO_2 fixed/min/mg of protein in crude extracts. When purified, the fusion protein had a k_cat that ranged from 0.5 to 1.6 s\(^{-1}\) for several different preparations. This value is somewhat lower than what is usually obtained for RubisCO from eubacteria and eukaryotes (k_cat of 3 to 5 s\(^{-1}\)) but may reflect our current inability to measure the *M. jannaschii* enzyme under optimum conditions specific for this protein. Specific proteolytic cleavage of the (His)_6-tagged sequence from the amino terminus had no effect on the enzymatic activity.

This unusual RubisCO was further characterized. In keeping with its novel source, the *M. jannaschii* enzyme was shown to be very stable to high temperatures with no loss of activity at 85°C for up to 60 min; in addition, maximum activity was obtained in the presence of 0.6 M KCl (results not shown). The *M. jannaschii* enzyme produced the expected product, 3-PGA, in the absence of O_2, in a reaction which was coincident with the incorporation of ^14^CO_2 into acid-stable product (not shown), yielding a stoichiometry of 1.96 mol of PGA produced per mol of RuBP carboxylated. Carboxylation and 3-PGA formation were specifically inhibited by the RubisCO transition-state analog CABP (Fig. 5A). The production of [^3H]3-PGA from a CO_2 fixation reaction mixture containing [1-^3H]RuBP was also established (Fig. 5B), further indicating that the *M. jannaschii* enzyme catalyzes a bona fide RubisCO activity.

Exposure of the enzyme to air under normal laboratory
conditions resulted in considerable loss of enzymatic activity over time (Fig. 6A), precluding routine kinetic measurements in the presence of oxygen. However, inhibition by O₂ was found to be reversible, as removal of this gas followed by the addition of an O₂ scavenging system resulted in the recovery of full enzymatic activity (Fig. 6B). Yet, O₂ binding must be quite efficient since simply diluting air-treated enzyme into an anaerobic assay was not sufficient to reactivate the enzyme (Fig. 6A). The observed reversibility of O₂ inhibition, and the known mechanism of RubisCO catalysis (8), suggests that CO₂ and O₂ may be competing for enzyme-bound enediolate, although it is conceivable that O₂ may interact at a second site on the anoxic *M. jannaschii* enzyme. Certainly, further kinetic studies are very much in order. Most significantly, the low residual activity retained upon exposure of the enzyme to air levels of oxygen suggested that it might be possible to determine if this enzyme catalyzes oxygenase activity, i.e., the oxygenolysis of RuBP.

This, advantage was taken of known methods to separate and quantify the specific carboxylase and oxygenase reaction products, which could be determined unambiguously even at low levels of activity by isolating [³H]2-phosphoglycolate (2-PG) and [³H]3-PGA from a reaction mixture containing [¹-³H]RuBP under an air atmosphere. The results of such an experiment indicated that the *M. jannaschii* enzyme catalyzed, albeit weakly and over a long time period, oxygen-dependent formation of 2-PG (Fig. 7). In addition, it would appear that O₂ interacts with the enzyme in such a way that carboxylase activity is diminished, since the level of 3-PGA produced is greatly reduced in these experiments in marked contrast to that of the *R. rubrum* enzyme. Indeed, the levels of 3-PGA and 2-PG produced (Fig. 7), at the concentrations of O₂ and CO₂ employed in this reaction, allow a calculation to be made of the relative CO₂/O₂ substrate specificity (τ) of the archaeal enzyme (7). For three separate experiments, with assays performed in the presence of air or molecular oxygen, the *M. jannaschii* enzyme yielded a CO₂/O₂ specificity factor of about 0.5, the lowest value ever reported for RubisCO from any source. The calculated specificity value of the *M. jannaschii* enzyme, and the long time required to produce the reaction products at room temperature in the presence of oxygen, resembles the low specificity and low activity obtained for a mutant *R. rubrum* enzyme (13). It should be noted that assays were performed at room temperature, far from the optimum temperature for activity (65°C) of the *M. jannaschii* enzyme. This was done to minimize the degradation of RuBP, which was exacerbated at this temperature in the high-salt environment of the specificity conditions.

**FIG. 6.** Effects of molecular oxygen. (A) RubisCO activity after incubation of the *M. jannaschii* enzyme at room temperature for the indicated times in the presence (■) or absence (□) of air (in an argon atmosphere) and assayed anaerobically at 65°C in the presence (■, □) or absence (□, □) of 10 mM β-mercaptoethanol by a standard 10-min ¹⁴CO₂ incorporation assay. (B) Reversibility of oxygen inhibition for assays run in the absence (□) or presence (■) of air, followed by removal of air and replacement with a nitrogen atmosphere at 18 min and the addition of an O₂ scavenging system containing protocatechuate dioxygenase (7) at 29 min. In all cases, the enzyme was dialyzed in a CO₂-free, O₂-free buffer of 80 mM HEPES (pH 7.2) containing 1 mM EDTA and 10 mM MgCl₂.

**FIG. 7.** Anion exchange chromatographic separation of products from a reaction containing [¹-³H]RuBP and the recombinant *M. jannaschii* enzyme incubated in the absence (a) or presence of air (b) at room temperature. In profiles a and b, the reaction was allowed to continue for 5 days. A similar reaction containing the *R. rubrum* RubisCO was performed in the presence of air for 60 min at room temperature (c). Assays were run at room temperature to limit the time-dependent degradation of RuBP that occurs under these conditions.
assay. Further rigorous CO₂/O₂ specificity and kinetic studies of this archaenal enzyme are obviously in order and should provide answers as to why this enzyme has such low substrate specificity and low activity in the presence of oxygen.

In conclusion, analysis of sequences which encode putative RibulCO proteins in *M. jannaschii* and *A. fulgidus* indicate, especially for *M. jannaschii*, the potential for the formation of a catalytically active enzyme. The preparation of homogeneous *M. jannaschii* recombinant protein with the capacity to catalyze a demonstrable RibulCO reaction, at a *k*₄₀ that is somewhat lower, yet approximates, that obtained for previously studied proteins, indicates that this recombinant enzyme is indeed functional. These analyses, however, do not predict what physiological role RibulCO might have in *M. jannaschii* or *A. fulgidus*. Genomic sequences obtained from these organisms do not show the presence of a recognizable gene(s) for phosphoribulokinase (21), the enzyme needed to complete the CBB pathway and also to generate the CO₂ acceptor RuBP. In part, this might be because only few phosphoribulokinase sequences are available in the current database; those that are available from eubacteria and eukaryotes show as little as 13% identity (25). Thus, phosphoribulokinase sequences that might yield a potential match to one or more open reading frames of unknown function in the genomes of *M. jannaschii* and *A. fulgidus* (2, 10) may be unrecognizable. In any case, the *M. jannaschii* enzyme purified here represents a form of RibulCO that has hitherto not been encountered and may be limited to anoxic extremophile representatives of the archaea. Indeed, determining how this enzyme functions at high temperatures and copes with O₂, a substrate that the *M. jannaschii* or *A. fulgidus* enzymes should never encounter, has considerable fundamental interest and may lead to an understanding of how “conventional” RibulCO molecules discriminate between CO₂ and O₂. In this regard, the apparent unusually high sensitivity of the *M. jannaschii* enzyme to O₂, a molecule that serves as both substrate and inhibitor, is unique to this source of RibulCO. As the signature property of RibulCO, CO₂/O₂ specificity plays an important role in global productivity and CO₂ sequestration and is a property that seems to have evolved to different extents for different sources of RibulCO (9, 20). Further studies of the archaenal enzyme are likely to be particularly cogent and should provide answers as to how the active site of this important enzyme has adapted to function at various levels of CO₂ and O₂ in different organisms in diverse environments.

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