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Kinetic isotope effect and biochemical characterization of form IA RubisCO from the marine cyanobacterium *Prochlorococcus marinus* MIT9313

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Abstract

In an effort to better understand the factors influencing carbon fixation by *Prochlorococcus*, and to elucidate the effects of these cyanobacteria on the ocean carbon cycle, the kinetic parameters and isotopic discrimination of form IA ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) from *Prochlorococcus marinus* MIT 9313 were determined. The RubisCO genes (*cbbL* and *cbbS*) were cloned and expressed in *Escherichia coli*. Enzyme was purified via ammonium sulfate precipitation, and the optimum pH and temperature, as well as Michaelis–Menton constants, were determined radiometrically. The degree to which this RubisCO discriminates against $^{13}CO_2$ during fixation was determined by the high-precision substrate depletion method. Purified enzyme had a pH optimum of 7.5, was most active between 20°C and 30°C, had a moderate V_{max} (0.41 μmol min^{-1} mg protein^{-1}), and had the highest K_{CO2} value (0.75 mmol L^{-1}) for a form I RubisCO characterized to date. The ε value, $\varepsilon = 1{,}000[(k^{12}/k^{13}) - 1]$, for the enzyme was determined to be 24.0‰ (95% C.I. = 22.2–25.6‰), within the range observed for other form I RubisCOs. This ε value is a critical baseline for interpreting the $\delta^{13}C$ values of marine environmental samples, particularly those collected from the open ocean, where *P. marinus* is responsible for a substantial fraction of carbon fixation.

Marine picophytoplankton are a substantial portion of all microbial plankton despite their minute size (cell diameters ranging from 0.2 to 2 μm). These organisms are present worldwide at concentrations ranging from 10^3 to 10^5 mL^{-1} throughout the euphotic zone (Partensky et al. 1999; Dandonneau et al. 2006). They are estimated to be ~40% of global phytoplankton biomass and, along with other picophytoplankton, are responsible for approximately 40% of global marine carbon fixation (Maranon et al. 2001). One group that dominates picophytoplankton in the oligotrophic open ocean is *Prochlorococcus* (Dandonneau et al. 2006), which, on the basis of rRNA gene sequences, falls within the phylum Cyanobacteria and forms a tight group nested within the marine *Synechococcus* cluster (Urbach et al. 1998).

Prochlorococcus spp. occur throughout the euphotic zone because of the presence of divinyl chlorophylls (*a* and *b*) that absorb blue light effectively and enable some strains to photosynthesize near the bottom of the euphotic zone (Goericke and Welschmeyer 1993; Moore and Chisholm 1999). *Prochlorococcus marinus* MIT 9313 is a low-light–adapted strain that was originally isolated from the Gulf Stream (Moore and Chisholm 1999). Subsequently, it has been determined to be one of the dominant low-light–adapted ecotypes of *P. marinus* present at ~100 m depth in the oligotrophic Atlantic Ocean between 35°N and 35°S (Johnson et al. 2006).

P. marinus MIT 9313, like other *Prochlorococcus* and marine *Synechococcus* strains (Watson and Tabita 1996), has a ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) more similar to those present in Proteobacteria than in other Cyanobacteria (Rocap et al. 2003). RubisCO, the CO_2-fixing enzyme of the Calvin–Benson cycle, is broadly divided into six large groups of enzymes, called form IA-D, form II, and form III RubisCO, which vary greatly in structure and kinetic parameters although they catalyze the same reactions. Form I enzymes consist of eight large and eight small subunits, whereas form II and form III consist of dimers or decamers of a single subunit, which are evolutionarily related to the form I large subunits (Tabita 1999). A fourth group, known as 'RubisCO-like proteins', are evolutionarily related to RubisCO large subunits but are not catalytically active as carboxylases and might play a role in sulfur metabolism (Hanson and Tabita 2001).

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Form I RubisCOs show considerable divergence among the four groups, IA–ID, on the basis of amino acid sequences (Tabita 1999). Forms IA and IB share ~80% identity, as do forms IC and ID, whereas the subgroup including IA and IB share only ~60% identity with the subgroup including IC and ID. In general, the catalytic properties of form I RubisCO make it more efficient than form II under low CO_2, high O_2 conditions. Form I enzymes generally have higher affinities for CO_2 and demonstrate a higher specificity for CO_2 relative to O_2 (Tabita 1999).

Many species of marine *Synechococcus* and *Prochlorococcus*, as well as the majority of alpha, beta, and gamma proteobacterial chemoautotrophs characterized to date, encode and express form IA RubisCO (Watson and Tabita 1996; Rocap et al. 2003). Form IB is found in most cyanobacteria and green chloroplasts. Within the form I group, these are the best characterized. Form IC is found in a limited number of proteobacteria, and form ID is found in most nongreen chloroplasts. Form II is found scattered throughout the Proteobacteria and in some dinoflagellates (Tabita 1999), whereas form III is present in Archaea (Finn and Tabita 2003). In accordance with the broad distribution of form I RubisCOs among phytoplankton, when RubisCO genes are amplified from plankton collections, forms IA, IB, IC, and ID are present (Pichard et al. 1997).

Catalytic differences among these forms of RubisCO might contribute to the considerable heterogeneity in $\delta^{13}C$ values observed in phytoplankton. Marine phytoplankton samples have $\delta^{13}C$ values ranging from −16‰ to −28‰ (Goericke et al. 1994). The scatter in these $\delta^{13}C$ values has been attributed to diffusive limitation (including the influences of cell size, cell shape, and growth rate), active transport, and C_4 pathways for carbon fixation (see Hayes 2001 for review). However, some of this scatter in plankton $\delta^{13}C$ values could also occur because of carbon fixation by different RubisCO enzymes that vary in their degree of isotopic fractionation (Cavanaugh and Robinson 1996). The degree to which a given RubisCO discriminates against ^{13}C is measured as a ratio of the reaction rate constants for each isotope ($k^{12} : k^{13}$). For convenience, it is helpful to convert this ratio to an epsilon value—$\varepsilon = 1{,}000[(k^{12}/k^{13}) - 1] = \varepsilon_f$, the discrimination due specifically to carboxylation), which is directly proportional to the discrimination against ^{13}C that occurs between the substrate pool (CO_2) and the product (phosphoglycerate; Hayes 2001). Early studies with combustion methods showed variation in ε values from RubisCOs isolated from different organisms (Estep et al. 1978). Subsequent work with high precision methods has indicated that form I enzymes discriminate against ^{13}C to a greater extent than form II. Form IA RubisCO from the gamma proteobacterial chemolithoautotrophic endosymbionts of the protobranch bivalve *Solemya velum* has an ε value of 24.5‰ (Scott et al. 2004*b*), whereas form IB RubisCOs from spinach and from the freshwater cyanobacterium *Synechococcus* PCC6301 have ε values of 29‰ and 22‰, respectively (Roeske and O'Leary 1984; Guy et al. 1993; McNevin et al. 2006). Fractionation by form II RubisCOs from the photosynthetic alpha proteobacterium *Rhodospirillum rubrum* and from the symbionts of the hydrothermal vent tubeworm *Riftia pachyptila* is less than that demonstrated by the form I RubisCOs. For enzyme from *R. rubrum*, three separate investigations with high-precision methods are in agreement and have determined a fractionation factor of between 17‰ and 22‰ for the enzyme, depending on the concentration of Mg^{+2} (Roeske and O'Leary 1985; Guy et al. 1993; McNevin et al. 2006). By use of the same methods, the form II enzyme from *R. pachyptila* symbionts was found to have an ε value of 19.5‰ (Robinson et al. 2003). This smaller ε value in form II RubisCOs is reflected by the more isotopically enriched stable isotope compositions measured in most hydrothermal vent organisms expressing form II (Cavanaugh and Robinson 1996).

The $\delta^{13}C$ values of picophytoplankton such as *Prochlorococcus* and *Synechococcus* have not been measured to date. However, when plankton are size-fractionated, those cells with diameters <3 μm, which would include these cyanobacteria, have the most negative values, ranging from −24‰ to −25.5‰ (Rau et al. 1990). Given the frequent occurrence of form IA RubisCO in these organisms, we hypothesized that the more negative $\delta^{13}C$ values of picophytoplankton are due to a higher degree of isotopic fractionation by their form IA RubisCOs.

Given their role in producing a substantial portion of primary productivity in both coastal areas and the open ocean, stable carbon isotope compositions of *Prochlorococcus* and other picophytoplankton must strongly influence the $\delta^{13}C$ of biogenic material exported from the photic zone, or cycling through the microbial loop. Understanding the factors that influence the $\delta^{13}C$ of picophytoplankton are therefore key to understanding the $\delta^{13}C$ of ocean biomass. To elucidate the environmental factors that could influence carbon fixation by these organisms *in situ*, to provide a baseline on which cyanobacterial picophytoplankton $\delta^{13}C$ values can be interpreted, and to better constrain the assumptions used to interpret oceanic $\delta^{13}C$ values, the kinetic properties and ε value of RubisCO from *P. marinus* MIT 9313 were determined.

Materials and methods

Expression of cyanobacterial RubisCO enzymes in E. coli—To obtain the large quantities of RubisCO necessary for ε determination, RubisCO genes (*cbbL* and *cbbS*) from *P. marinus* MIT 9313 were cloned and expressed in *E. coli.* Primers based on published genome sequence data (Rocap et al. 2003) were designed to amplify the region of the cyanobacterial chromosomes including the *cbbL* and *cbbS* genes (forward primer: 5′-CACCATGAGCAAGAAGTATGACGCA-3′; reverse primer: 5′-ACAACGGCCTTCAAATC-3′). Amplicons were cloned into the pET101/D-TOPO vector (Invitrogen). As in (Schwedock et al. 2004), the amplicon-containing plasmid was used to transform *E. coli* LL308 carrying pGroESL, which encodes the molecular chaperonins GroES and GroEL. To express both RubisCO and chaperonins, cells were cultivated at 37°C in Luria broth until exponential phase growth was reached. Gene expression was induced by

bringing the cultures to 1 mmol L^{-1} IPTG, incubating, and agitating at room temperature at 130 rpm for 48 h. Cells were harvested via centrifugation and sonicated (Schwedock et al. 2004). RubisCO was purified from cell extracts by adding $(NH_4)_2SO_4$ to 20% saturation, which precipitated the RubisCO. Pelleted enzyme was resuspended in RubisCO storage buffer (50 mmol L^{-1} bicine, pH 7.5, 30 mmol L^{-1} $MgCl_2$, 66 mmol L^{-1} $NaHCO_3$, 5 mmol L^{-1} dithiothreitol [DTT], 20% glycerol). This RubisCO solution was frozen with liquid nitrogen and stored at −80°C. RubisCO purity was monitored during expression and purification with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and activity was measured via radiometric carbon fixation assays (Schwedock et al. 2004).

RubisCO characterization—The responses of enzyme activity to pH and temperature were determined radiometrically by an isotopic disequilibrium technique (Scott et al. 2004*b*). Preliminary experiments indicated that the K_{CO2} values for these enzymes were quite large, requiring incubations in the presence of very high concentrations of CO_2. Because the CO_2 concentrations necessary to meet and exceed the K_{CO2} values were high enough to result in significant volatilization of CO_2 during an extended assay, the more rapid isotopic disequilibrium technique was used to determine K_{CO2} and V_{max} values for these enzymes as well.

RubisCO ε values—Isotopic fractionation by cyanobacterial RubisCO was determined by the substrate depletion technique, as in Scott et al. (2004*b*). Ribulose 1,5-bisphosphate and gravimetrically verified $MgCl_2$ solutions were prepared (Scott et al. 2004*b*). Approximately 0.5 mg of enzyme was dialyzed overnight in dialysis buffer (50 mmol L^{-1} bicine, 25 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} DTT, 10 mmol L^{-1} $NaHCO_3$, pH 7.5) at 4°C. Dialyzed RubisCO was diluted to a volume of 25 mL with N_2-purged reaction buffer (dialysis buffer plus 40 μg mL^{-1} bovine carbonic anhydrase), sealed in a glass gas-tight syringe with a stir bar, and activated for 10 min at 25°C. To begin the reaction, ribulose 1,5-bisphosphate was injected into the syringe to a final concentration of 5 mmol L^{-1}. Portions of 2–4 mL were removed from the continuously stirred gas-tight syringe over the next ~8 h. For each portion, the dissolved inorganic carbon was quantified with a gas chromatograph (Dobrinski et al. 2005) and purified via cryodistillation for injection into a mass spectrometer (Scott et al. 2004*b*). As in Scott et al. (2004*a*), ε values were determined from the slope of the line with $x = \ln[DIC]$ and $y = \ln(R)$, where [DIC] is the concentration of dissolved inorganic carbon and R is the isotope ratio of the DIC. Data from replicate runs were combined to generate mean ε values with the use of Pitman estimators (Scott et al. 2004*a*).

Results

Expression of cyanobacterial RubisCO in E. coli—RubisCO cloned from *P. marinus* MIT 9313 was successfully expressed in *E. coli*, and purified to ~90% with ammonium sulfate (Fig. 1). Purified RubisCO was catalytically active and demonstrated carboxylase activity only in the presence of ribulose 1,5-bisphosphate.

RubisCO biochemical characterization—*P. marinus* MIT 9313 RubisCO was most active between pH 7 and 8.5, with an optimum pH of 7.5 (Fig. 2A). When incubated at pH 7.5, this enzyme was active at a broad range of temperatures, with an optimum between 20°C and 30°C (Fig. 2B). The K_{CO2} at pH 7.5 and 20°C was very high (mean ± SD, 0.75 ± 0.16 mmol L^{-1}; $n = 2$: 0.86, 0.64) and it has a moderate V_{max} (0.41 ± 0.05 μmol min^{-1} mg $protein^{-1}$; $n = 2$: 0.44, 0.38; Fig. 3).

RubisCO ε values—ε values from three independent experiments fell within 1.2‰ of each other, with a mean value of 24.0‰ and a 95% confidence interval of 22.2–25.6‰ (Fig. 4).

Discussion

This is the first description of the catalytic properties of a cyanobacterial form IA RubisCO enzyme, providing insight into the physiology of *P. marinus* MIT 9313 carbon fixation, as well as a critical baseline for interpreting environmental and laboratory stable carbon isotope values of *P. marinus*–generated organic carbon. This is particularly

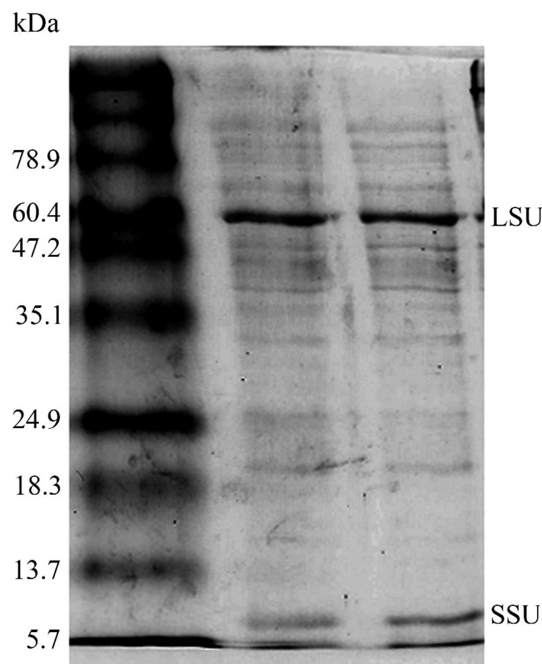

Fig. 1. *Prochlorococcus marinus* MIT 9313 RubisCO. Heterologously expressed RubisCO was pelleted from cell-free extracts by bringing $(NH_4)_2SO_4$ to 20% saturation. For SDS-PAGE, the pellets were redissolved in SDS-PAGE sample buffer, and 2.5-μg portions were loaded into each lane. Results from two independent purifications are shown here. The gel was stained with Coomassie Blue; molecular mass markers were run in the far left lane. LSU and SSU = large and small subunit, respectively.

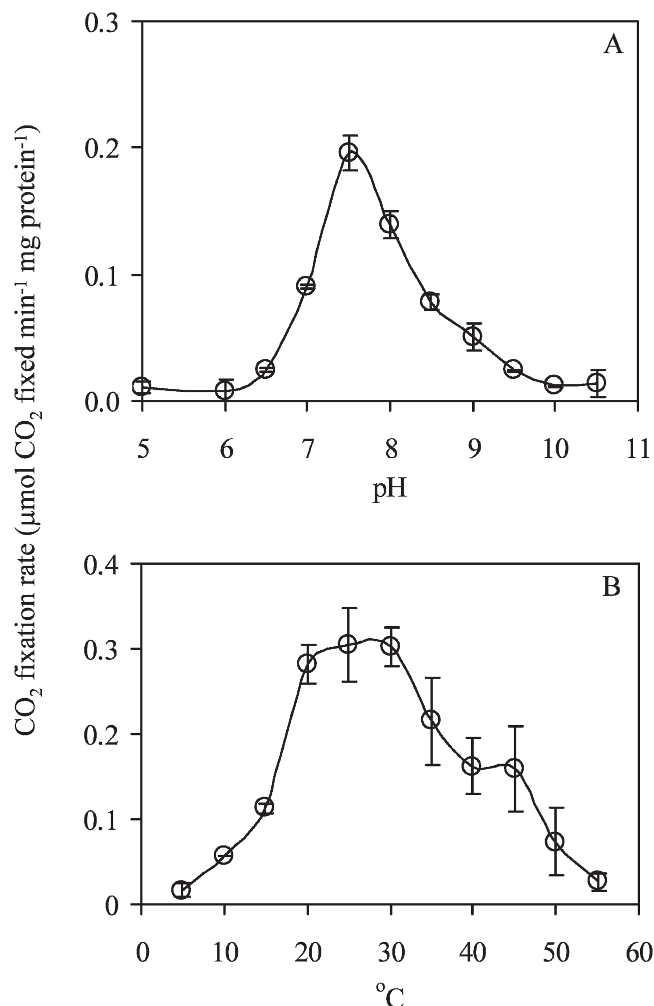

Fig. 2. (A) pH and (B) temperature optima for *Prochlorococcus marinus* MIT 9313 RubisCO. Error bars are the standard deviations of the carbon fixation rates.

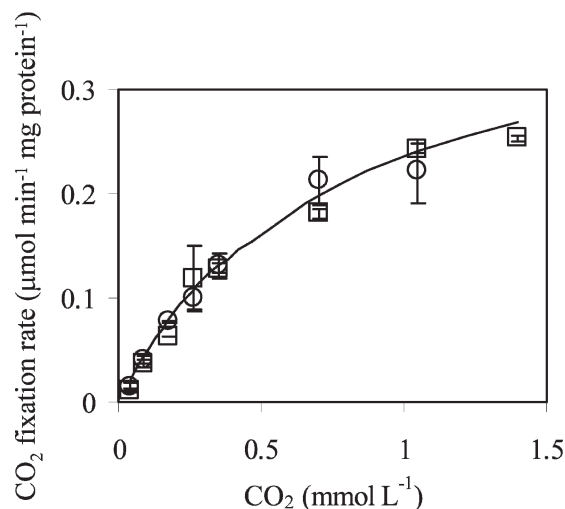

Fig. 3. *Prochlorococcus marinus* MIT 9313 RubisCO Michaelis–Menton kinetics. Results from two independent experiments are plotted with different symbols. The rectangular hyperbola depicted with the data has K_{CO2} = 0.75 mmol L^{-1} and V_{max} = 0.41 μmol min^{-1} (mg protein)$^{-1}$.

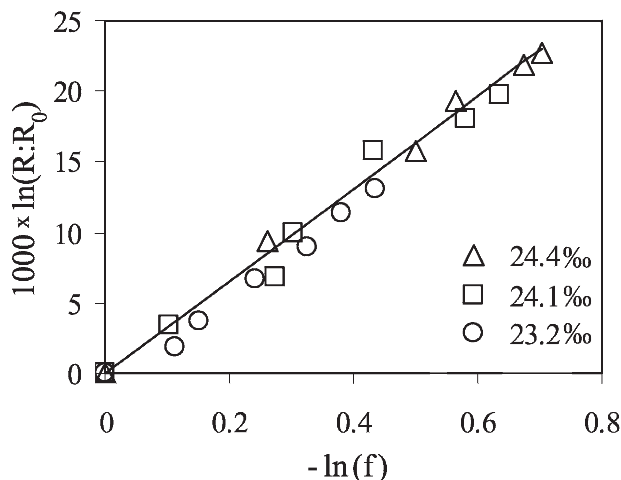

Fig. 4. *Prochlorococcus marinus* MIT 9313 RubisCO ε value determination. Three independent experiments were conducted and are depicted with different symbols. R = $^{13}C/^{12}C$ of the dissolved inorganic carbon (DIC), f = [DIC]/[DIC_0], and R_0 and [DIC_0] are the R and dissolved inorganic carbon concentration for the first time point. ε values were calculated for each experiment from the slope of the line ln R versus ln[DIC] (see Materials and Methods). The data are plotted here as ln($R:R_0$) versus ln(f) for simplicity because R_0 and [DIC_0] varied between experiments. The line has a slope corresponding to a mean ε value of 24.0‰.

relevant for the oligotrophic open ocean, where *P. marinus* primary productivity accounts for as much as 30% of the total (Goericke and Welschmeyer 1993).

Some aspects of *P. marinus* MIT 9313 RubisCO are as expected on the basis of the organism's habitat, but others were quite surprising. The pH optimum for the enzyme is consistent with the range of pH values typical for neutrophilic bacterial cytoplasm (7–8; e.g., Kashket 1985; Dobrinski et al. 2005), and the temperature optimum of 20–30°C is consistent with ocean temperatures (>17°C) where *P. marinus* MIT 9313 is abundant (Johnson et al. 2006).

However, the high K_{CO2} value (750 μmol L^{-1}) was unexpected, in that other form IA enzymes have K_{CO2} values ranging from 30 to 140 μmol L^{-1} (Tabita 1999; Schwedock et al. 2004). This low affinity suggests that *P. marinus* MIT 9313 has a carbon-concentrating mechanism (CCM) because surface ocean CO_2 concentrations are typically 10–20 μmol L^{-1} (Zeebe and Wolf-Gladrow 2003). CCMs enable many cyanobacteria to grow when the ambient CO_2 concentration is lower than the K_{CO2} of their RubisCO enzymes by enabling them to utilize extracellular bicarbonate, which is more plentiful than CO_2 under alkaline conditions (e.g., seawater) (Zeebe and Wolf-Gladrow 2003; Badger et al. 2006). In many cyanobacteria, bicarbonate is actively transported into the cytoplasm (Badger et al. 2006 and references cited therein). Carbon dioxide is generated when cytoplasmic bicarbonate diffuses into carboxysomes, which are intracellular inclusions packed with RubisCO and a trace of carbonic anhydrase (Badger et al. 2006 for review). Most carbon dioxide that escapes utilization by carboxysomal RubisCO and diffuses

into the cytoplasm is prevented from diffusing through the cellular envelope by conversion to bicarbonate by vectoral thylakoid-associated carbonic anhydrase, which is believed to couple carbon dioxide hydration to electron transport (references cited in Badger et al. 2006). Cyanobacteria appear to fall into two groups on the basis of the CCM genes that are present: α-cyanobacteria (*P. marinus* MIT 9313 and other *Prochlorococcus* and marine *Synechococcus* species), and β-cyanobacteria (a more diverse group of freshwater and marine species). Many β-cyanobacteria have two or three bicarbonate transporters encoded in their genome (BCT1, SbtA, and BicA), and two vectoral carbonic anhydrase complexes, as well as carboxysomal genes and form IB RubisCO (Badger et al. 2006).

In α-cyanobacteria, some bicarbonate transporters and vectoral carbonic anhydrases are absent, and they have RubisCO (form IA) and carboxysome genes that are more similar to those present in Proteobacteria (Badger et al. 2006). Specifically, *P. marinus* MIT 9313 has only one of the three known cyanobacterial bicarbonate transporters (BicA) encoded in its genome, which has been demonstrated to be a relatively low-affinity bicarbonate transporter in other cyanobacteria (Badger et al. 2006). Furthermore, genes encoding the components of both membrane-linked vectoral carbonic anhydrase systems are missing in *P. marinus* MIT 9313 (Badger et al. 2006). *P. marinus* MIT 9313 might be able to survive with a scaled-back CCM. However, given its extremely low-affinity RubisCO, it seems likely that it uses an as yet undescribed high-affinity bicarbonate transporter.

P. marinus MIT 9313 RubisCO has an ε value, 24.0‰, within the range determined for the values measured for three other form I enzymes. This value is quite similar to that of the *S. velum* chemolithoautotrophic symbiont form IA RubisCO (24.5‰; Scott et al. 2004*b*), and falls between the two values observed for form IB RubisCO ($\varepsilon = 22‰$ for *Synechococcus* PCC6301; $\varepsilon = 29–30‰$ for *Spinacia oleracea*; Roeske and O'Leary 1984; Guy et al. 1993). It is possible that ε values between 22‰ and 25‰ are more 'typical' for form I enzymes than the spinach $\varepsilon = 29‰$, highlighting the danger of applying the value from spinach to bacteria-dominated systems. However, it is important to remember that two form IA enzymes and two form IB enzymes are a sparse sampling of the full phylogenetic breadth of form I RubisCOs. Further studies with more form I RubisCOs are necessary to demonstrate what is 'typical' for these enzymes.

The *P. marinus* RubisCO ε value will be particularly useful for interpreting the $\delta^{13}C$ values of *Prochlorococcus* cells collected from the ocean. Plankton-derived organic carbon typically has $\delta^{13}C$ values between −16‰ and −28‰ (Goericke et al. 1994); picophytoplankton-sized cells have somewhat isotopically depleted values (−24‰ to −25.5‰; Rau et al. 1990). At this point, $\delta^{13}C$ values have yet to be reported for *Prochlorococcus* cells from environmental samples. A value of about −33‰ is predicted for *Prochlorococcus* biomass solely on the basis of the RubisCO ε value (24.0‰) and the $\delta^{13}C$ value of CO_2 from waters between 18°C and 25°C (~9‰; Zeebe and Wolf-Gladrow 2003; Johnson et al. 2006). It is likely that the actual $\delta^{13}C$ values of *Prochlorococcus* cells are more positive than this because of the diffusive limitation of CO_2 or the operation of a CCM (references cited in Hayes 2001). Alternatively, more positive $\delta^{13}C$ values could suggest that these organisms obtain a portion of their cellular carbon from environmental organic carbon pools. Indeed, *P. marinus* MIT9313 can utilize organic nitrogen sources such as urea and amino acids (Moore et al. 2002; Zubkov et al. 2003) and has predicted amino acid transporters (Rocap et al. 2003). The measurement of $\delta^{13}C$ values from environmental *Prochlorococcus* samples, in combination with this *Prochlorococcus* RubisCO ε value, will help clarify these and other remaining mysteries for this minute, yet dominant, global primary producer. Furthermore, ε values from these cyanobacteria and other primary producers are essential for the use $\delta^{13}C$ values in realistic modeling of carbon fixation, gas exchange, and contributions to global carbon fixation.

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