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Influence of form IA RubisCO and environmental dissolved inorganic carbon on the $\delta^{13}C$ of the clam-chemoautotroph symbiosis *Solemya velum*

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Summary

Many nutritive symbioses between chemoautotrophic bacteria and invertebrates, such as *Solemya velum*, have $\delta^{13}C$ values of ~–30 to –35‰, considerably more depleted than phytoplankton. Most of the chemoautotrophic symbionts fix carbon with a form IA ribulose 1,5-bisphosphate carboxylase (RubisCO). We hypothesized that this form of RubisCO discriminates against $^{13}CO_2$ to a greater extent than other forms. *Solemya velum* symbiont RubisCO was cloned and expressed in *Escherichia coli*, purified and characterized. Enzyme from this recombinant system fixed carbon most rapidly at pH 7.5 and 20–25°C. Surprisingly, this RubisCO had an ε-value (proportional to the degree to which the enzyme discriminates against $^{13}CO_2$) of 24.4‰, similar to form IB RubisCOs, and higher than form II RubisCOs. Samples of interstitial water from *S. velum*'s habitat were collected to determine whether the dissolved inorganic carbon (DIC) could contribute to the negative $\delta^{13}C$ values. *Solemya velum* habitat DIC was present at high concentrations (up to ~5 mM) and isotopically depleted, with $\delta^{13}C$ values as low as ~ –6‰. Thus environmental DIC, coupled with a high degree of isotopic fractionation by symbiont RubisCO likely contribute to the isotopically depleted $\delta^{13}C$ values of *S. velum* biomass, highlighting the necessity of considering factors at all levels (from environmental to enzymatic) in interpreting stable isotope ratios.

Introduction

The stable carbon isotope compositions ($\delta^{13}C$ values) of chemoautotrophic symbioses fall into two clusters that are distinct from those typically observed in phytoplankton. Bivalves with chemoautotrophic symbionts range from ~– 30 to –34‰, and hydrothermal vent vestimentiferan tubeworms cluster at –8.8 to –16‰, whereas phytoplankton range from –18 to –28‰ (Conway *et al*., 1989; Fisher, 1990; Goericke *et al*., 1994; Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996). These values are expressions of the stable carbon isotope composition of a sample, with $\delta^{13}C = [(R_{sample}/R_{std}) - 1]*1000$, where $R_{sample} = {}^{13}C/{}^{12}C$ of a sample, and $R_{std} = {}^{13}C/{}^{12}C$ of Pee Dee Belemnite (Michener and Schell, 1994). Given that phytoplankton and chemoautotrophic endosymbionts all use RubisCO (ribulose 1,5-bisphosphate-carboxylase/oxygenase) and the Calvin-Benson cycle to fix CO_2 (Cavanaugh, 1983; Fisher, 1990; Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996), it is surprising that their $\delta^{13}C$ values are so different from each other.

However, the RubisCO enzymes are now known to vary in their structure and kinetic characteristics. Based on amino acid sequences, four forms of RubisCO, or RubisCO-like proteins, are currently known, with form I further subdivided into four clusters, A–D (Tabita, 1999). Form I RubisCOs consist of eight large and eight small subunits. Between forms A–D, the large subunits share at maximum 80% amino acid sequence identity (Tabita, 1995). Form IA is present in many chemoautotrophic bacteria, including symbionts from most chemoautotrophic bivalves, and is also found in some marine picophytoplankton (Shimada *et al*., 1995; Watson and Tabita, 1997; Schwedock *et al*., 2004). Form IB, which has been most extensively studied, is present in most cyanobacteria and green plastids. Form IC is found in some chemoautotrophs and anaerobic phototrophic bacteria, whereas form ID occurs in diatoms and other cells with non-green plastids (Delwiche and Palmer, 1996). Catalytic units of form II RubisCO consist of dimers of a single subunit evolutionarily related to the large subunits of form I, though they only share about 30% amino acid sequence identity (Tabita, 1995). Form II RubisCOs are present in some dinoflagellates and many free-living photo- and chemoautotrophic bacteria, as well as in the symbionts of vestimentiferan tubeworms (Robinson and Cavanaugh, 1995; Morse *et al*., 1995; Whitney *et al*., 1995; Cavanaugh and Robinson, 1996). Form III RubisCOs are

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present in Archaea (Watson *et al*., 1999), and form IVs (now referred to as RubisCO-like proteins), while clearly evolutionarily related to other RubisCOs, are not catalytically active as carboxylases and are present in a variety of microorganisms, including Archaea and Gram-positive bacteria (Hanson and Tabita, 2001).

Given these differences in primary structure, it is not surprising that RubisCO enzymes demonstrate considerable heterogeneity in the degree to which they fractionate carbon during fixation. Although all RubisCOs deal with the same substrate, they exhibit different fractionations because the activiation–energy barrier for ^{13}C is higher than that for ^{12}C (Melander and Saunders, 1980; Cook, 1998). This arises because: (i) ^{13}C is always bound more tightly than ^{12}C, both in the substrate and in the transition state, and (ii) bonds to the reactive carbon are inevitably weaker in the transition state than in the substrate. The net result is that the binding-energy difference between ^{12}C and ^{13}C is smaller in the transition state than in the substrate. Therefore, the activation energy barrier for ^{13}C is higher than that for ^{12}C. Differences in isotopic discrimination between enzymes arise from differences in the structures of the transition states.

The degree to which RubisCO discriminates against $^{13}CO_2$ during fixation is expressed as an **ε**-value, equal to $\{(R_s/R_p) - 1\}*1000$, where $R_s = {}^{13}C/{}^{12}C$ of the CO_2 substrate, $R_p = {}^{13}C/{}^{12}C$ of the carbon fixed, and $R_s/R_p = \alpha =$ kinetic isotope effect (Guy *et al*., 1993; Hayes, 1993). **ε** values for only four RubisCOs have been determined using high-precision methods (Roeske and O'Leary, 1984; Roeske and O'Leary, 1985; Guy *et al*., 1993; Robinson *et al*., 2003). Form IB RubisCO from spinach ($\varepsilon = 26$–30.3‰) and the freshwater cyanobacterium *Synechococcus* 6301 ($\varepsilon = 22$‰) fractionate more than form IIs from vestimentiferan tubeworm symbionts ($\varepsilon = 19.5$‰) and the photosynthetic α-Proteobacterium *Rhodospirillum rubrum* ($\varepsilon = 18$–22‰). Most chemoautotrophic symbionts of bivalves with more negative $\delta^{13}C$ values have form IA RubisCO, whereas symbionts of vestimentiferan tubeworms and other organisms with more positive $\delta^{13}C$ values use form II RubisCO (Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996). Based on this correlation, it appears that a major factor driving the two clusters of $\delta^{13}C$ values of chemoautotrophic symbioses is the degree of isotopic discrimination by RubisCO, with form I-utilizing symbioses having lower biomass $\delta^{13}C$ values due to use of a more isotopically selective form of RubisCO (Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996).

However, despite the ecosystem-level relevance of chemoautotrophic symbioses and marine picophytoplankton (Robinson and Cavanaugh, 1995; Cavanaugh and Robinson, 1996; Shimada *et al*., 1995; Watson and Tabita, 1997), **ε**-values for form IA RubisCOs have not been measured; thus, the degree to which they may influence biomass $\delta^{13}C$ values can not be evaluated. Given that the $\delta^{13}C$ values of chemoautotrophic bacteria with form IA RubisCO are up to 16‰ more negative than phytoplankton, we hypothesized that the **ε**-value of this form of the enzyme should be higher than that of the forms IB/D enzymes present in most cyanobacteria and eukaryotic phytoplankton.

The chemoautotrophic symbiosis *Solemya velum* is a member of the more isotopically depleted cluster, with $\delta^{13}C$ values from −30 to −34‰, and symbionts that encode and express form IA RubisCO (Cavanaugh *et al*., 1988; Conway *et al*., 1989; Robinson and Cavanaugh, 1995; Schwedock *et al*., 2004). This protobranch clam inhabits burrows in reducing silty sediments on the east coast of the United States (Yonge, 1939; Levinton, 1977). Chemoautotrophic γ-Proteobacteria inhabit *S. velum* gill epithelial cell bacteriocytes. Carbon fixation by these bacteria is fuelled by sulfide oxidation, which the host clam facilitates by accessing both sulfide-bearing interstitial water and oxic seawater (Cavanaugh, 1983; Cavanaugh *et al*., 1988; Eisen *et al*., 1992).

Multiple lines of evidence suggest that the major input of organic carbon for this organism is from symbiont chemoautotrophy. In contrast to other protobranch clams, which gather and consume detritus, *S. velum* have labial palps that are so tiny that they do not reach outside of their shells (Yonge, 1939; Levinton, 1977). Suspension feeding by this clam is limited to cells less than 1 μm in diameter at rates less than half of that measured by similarly sized filter-feeding clams from the same habitat (Krueger *et al*., 1992). Instead of conventional bivalve feeding strategies, *S. velum* constructs and ventilates a Y-shaped burrow in reducing sediments to obtain the redox substrates necessary for symbiont chemoautotrophy. The clam positions itself approximately 6 cm below the sediment surface, at the juncture between the U-shaped bend of the upper portion of the burrow and lower downward-pointing burrow 'stem'. It obtains oxygen by pumping seawater through the bend, and sulfide via a ventral incurrent siphon from the stem (Stanley, 1970; Cavanaugh, 1983). Lipid profiles and carbon and nitrogen stable isotope values of symbionts and host tissues are similar, indicating that the symbionts serve as the major source of organic carbon for the clam (Conway *et al*., 1989; Conway and McDowell Capuzzo, 1991).

Another factor that could potentially contribute to the negative $\delta^{13}C$ values measured in *S. velum* biomass is environmental dissolved inorganic carbon (DIC; the sum of dissolved CO_2, HCO_3^-, and CO_3^{-2}) that is isotopically depleted. Sediments in Buzzards Bay near where *S. velum* are collected have a high input of DIC as the result of organic carbon mineralization by aerobic and anaerobic heterotrophic bacteria (McNichol *et al*., 1988). This input

of CO_2 from biomass decomposition will bring the isotopic composition of porewater DIC to more negative $\delta^{13}C$ values. Dissolved inorganic carbon $\delta^{13}C$ values measured in interstitial water from sediments from other marine locations with a substantial input of labile organic matter range from −6 to −21‰ (Presley and Kaplan, 1968; McCorkle, 1988).

Two hypotheses were tested to infer the roles of RubisCO fractionation and environmental DIC in generating the isotopically depleted $\delta^{13}C$ values of *S. velum* biomass: (i) form IA RubisCO of *S. velum* symbionts discriminates against ^{13}C more than other RubisCO enzymes, and (ii) dissolved inorganic carbon from *S. velum*'s habitat has a more negative $\delta^{13}C$ value than is typical for overlying seawater. In order to determine the degree to which *S. velum* biomass $\delta^{13}C$ values are affected by these enzymatic and environmental factors, symbiont form IA RubisCO was purified and characterized with respect to its kinetic parameters, including its ε-value, and the $\delta^{13}C$ of interstitial DIC from its habitat was determined.

Results

RubisCO temperature and pH optima

Solemya velum symbiont RubisCO was active at a broad range of pH values (6.5–9) and temperatures (5–35°C) (Fig. 1). Previous determinations of kinetic isotope effects for spinach have been conducted at pH 8.5, 25°C (Roeske and O'Leary, 1984; Guy *et al*., 1993). As *S. velum* RubisCO demonstrated a high activity under these conditions, the kinetic isotope effect for this enzyme was measured at this pH and temperature to facilitate comparisons with spinach RubisCO.

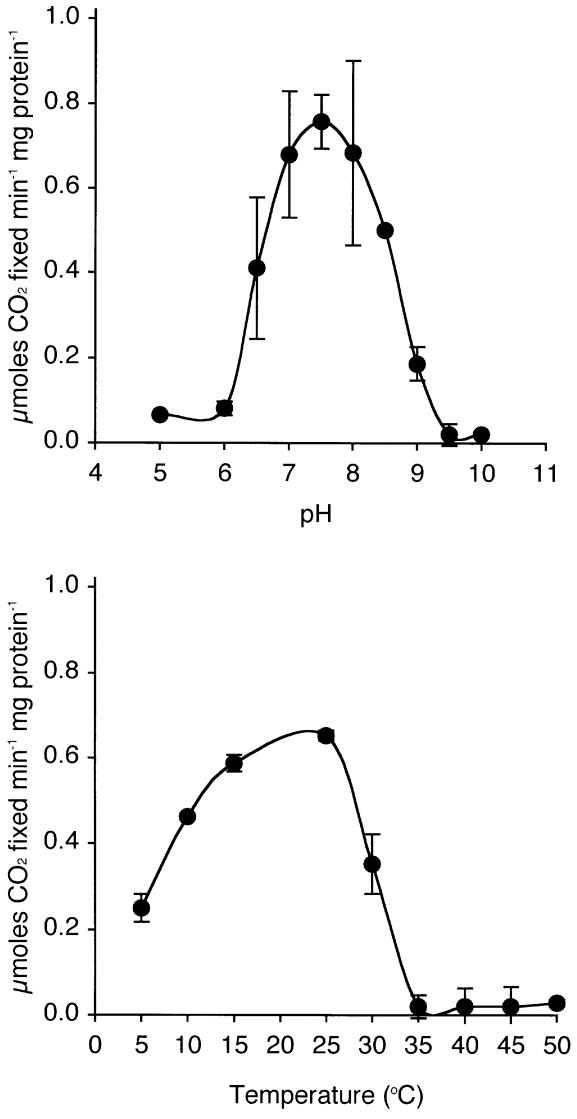

Fig. 1. *S. velum* RubisCO pH and temperature optima. Carbon fixation rates were measured for purified RubisCO at a range of pH and temperatures using an isotope disequilibrium technique. Error bars are the standard deviations of the carbon fixation rates ($n = 3$ timepoints per run).

Kinetic isotope effects

The precision both of data within the experiments (Fig. 2), and of the ε-values between experiments (Table 1) was high. Accordingly, 95% confidence intervals for ε-values from datasets combined either using a regression with dummy variables or with a Pitman estimator were quite similar (Table 2), which is consistent with the results from Scott *et al*. (2003). Using a Pitman estimator is a more reliable method than using dummy variables for combining smaller datasets such as these, which had 4–6 timepoints (Scott *et al*., 2003); accordingly, the following discussion is based on ε-values calculated with Pitman estimators. RubisCO from *S. velum* symbionts has an ε-value of 24.4‰ (Table 2; Fig. 2). The ε-value for spinach RubisCO measured here (28.2‰; Table 2) is within the range of previously published values using high-precision methods (26.4–30.3‰; Roeske and O'Leary, 1984; Guy *et al*., 1993).

Environmental DIC

The concentration and stable isotope composition of DIC in the interstitial water from the core samples departed from those measured at the sediment surface (Fig. 3). The DIC and CO_2 concentrations rose from values of 2.28 and 0.03 mM at the sediment surface to maximum values of 4.97 and 0.21 mM at 5–15 cm depth, while the $\delta^{13}C$ of DIC and pH decreased with depth in the sediment from +1.2‰ and 7.86 at the surface to −5.9‰ and 7.28 between 5 and 15 cm. The broad range in these environmental DIC and pH values likely reflects the substantial horizontal and vertical heterogeneity in interstitial water chemistry that one would expect in the metabolically active and patchy

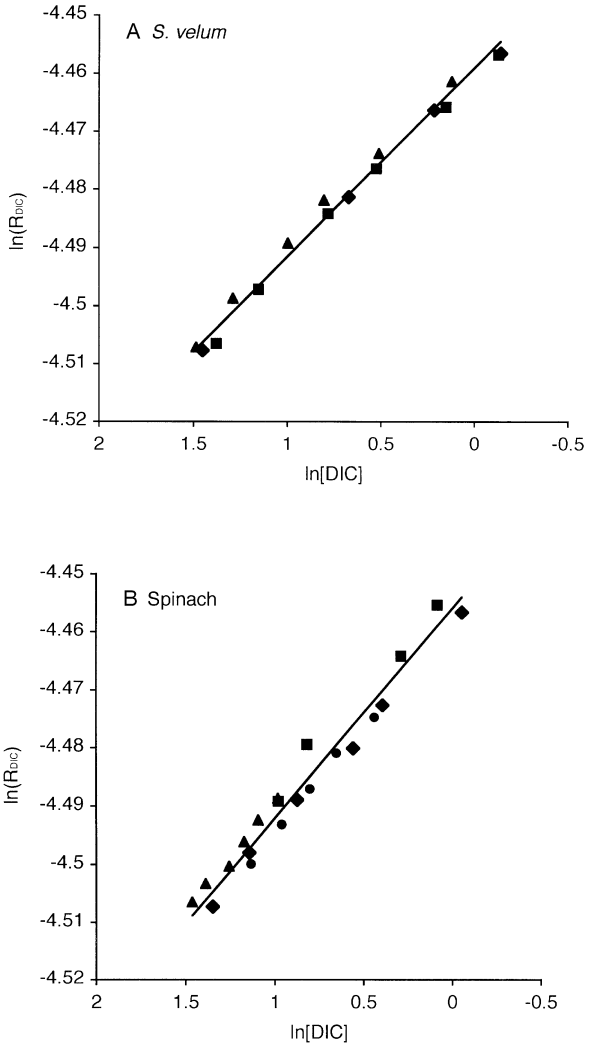

Fig. 2. Isotopic enrichment of DIC as CO_2 is consumed by purified RubisCO from (A) *S. velum* symbionts and (B spinach. Each of the independent experiments is plotted with a different symbol, and the line corresponds to (A) $\varepsilon = 24.4‰$ and (B) $\varepsilon = 28.2‰$ R_{DIC} and [DIC] are the isotope ratio and concentration of the DIC, respectively, present in the reaction.

eelgrass community from which these samples were taken.

Discussion

In contrast to our expectations, *S. velum* symbiont form IA RubisCO has an ε-value that falls within the range of ε-values of form IB RubisCOs (22–29‰; Guy *et al*., 1993). This higher degree of isotope discrimination, compared with form II enzymes (18–22‰; Guy *et al*., 1993; Robinson *et al*., 2003) clearly contributes to the difference in biomass $\delta^{13}C$ values between symbioses with either of these two forms of RubisCO. However, based solely on clam biomass $\delta^{13}C$ values, it was expected that the ε-value for the *S. velum* symbiont RubisCO would be >30‰.

Table 1. RubisCO ε values determined for individual experiments.

Organism	Expt. No.	ε ± std.dev (‰)
S. velum symbionts	1	24.3 ± 0.9
	2	24.3 ± 1.0
	3	24.9 ± 1.0
Spinach	1	29.2 ± 2.0
	2	28.8 ± 0.9
	3	27.2 ± 1.1
	4	27.5 ± 3.2

Clearly, other factors, such as environment and morphology, are also contributing to the more isotopically depleted $\delta^{13}C$ values of *S. velum* biomass. Interstitial water collected from depths in the sediment where *S. velum* inhabits its burrows has elevated concentrations of isotopically depleted DIC (Fig. 3), probably resulting from the decomposition of organic matter (Presley and Kaplan, 1968; McCorkle, 1988). Both an increased rate of CO_2 supply to the symbionts due to high concentrations of DIC (and thus, CO_2) as well as isotopically depleted source DIC will result in fixed carbon with more negative $\delta^{13}C$ values. The actual concentration and isotopic composition of DIC in the water the clams draw through their mantle cavity is likely to be intermediate between the values measured in interstitial water and at the sediment surface, because they pump water both from below, through a ventral incurrent siphon to obtain sulfide, and above, through the U-shaped bend in their burrows to obtain oxygen (Stanley, 1970; Cavanaugh *et al*., 1983). As the measurements of interstitial water chemistry were made in August, when rates of decomposition would be particularly high due both to elevated temperatures and seasonally high rates of organic carbon input from the eelgrass community, during the winter it is likely that the concentration of interstitial DIC is lower, and its $\delta^{13}C$ is higher.

Low clam biomass $\delta^{13}C$ values may also be influenced by high rates of exchange between environmental and

Table 2. RubisCO ε value averages and 95% confidence intervals (CI).

Organism	Averaging method[a]	ε (‰)	95% CI of ε (‰)
S. velum symbionts	Dummy variables	24.5	23.2–25.6
	Pitman estimator	24.4	23.3–25.6
Spinach	Dummy variables	27.7	26.2–29.2
	Pitman estimator	28.2	26.6–29.8

a. Averaging methods are as recommended in Scott *et al*. (2003). To use the dummy variable method, data from replicated experiments were combined into a single linear regression, and dummy variables were used to account for differences in the y-intercepts of the lines between experiments. To determine the ε–values using a Pitman estimator, a Matlab program (available at http://www.elsevier.com/inca/publications/store/2/1/2/) was used (Scott *et al*., 2003).

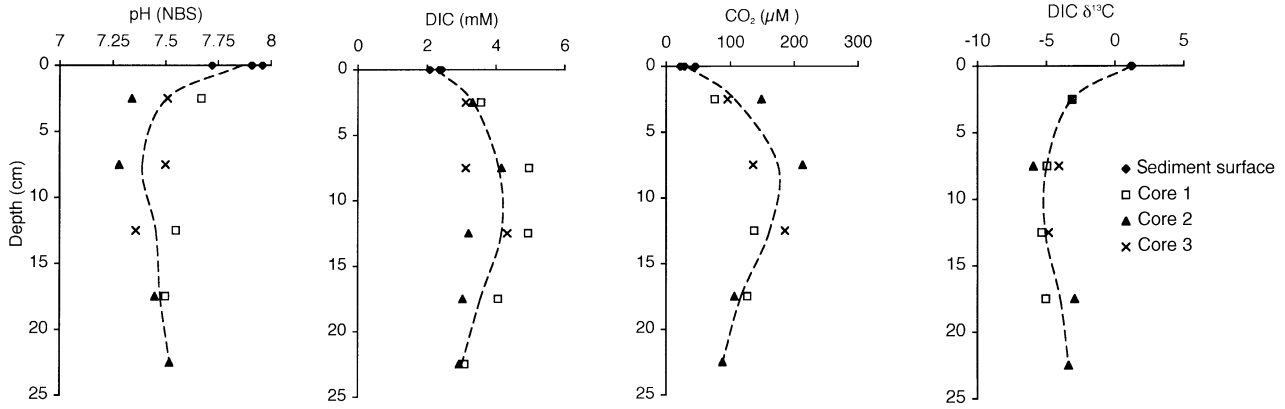

Fig. 3. Sediment pH, DIC and CO_2 concentrations, and δ^{13}C-DIC of the *S. velum* habitat. DIC and pH depth profiles were measured at the surface of and in the porewater of three cores (labelled in the graphs with different symbols). The pH values were measured with a calomel electrode calibrated with National Buffer Standards (NBS), and the CO_2 concentration was calculated from the pH and DIC concentrations with GEOSECS dissociation constants and the CO2SYS program (Lewis and Wallace, 1998).

symbiont intracellular CO_2. High rates of CO_2 exchange would effectively 'erase' the effects of carbon fixation by RubisCO and of host cellular respiration on the isotopic composition of intracellular CO_2, and bring environmental and intracellular CO_2 to near-isotopic equilibrium. To estimate the δ^{13}C of CO_2 within the symbiont cells, the symbiont RubisCO kinetic isotope effect (α) and the isotope ratio of clam biomass were used. As $\alpha = R_s/R_p$, the isotope ratio of intracellular CO_2 (R_i) was calculated from the isotope ratio of clam biomass (R_b) and the RubisCO KIE (α) with the formula $R_i = R_b \times \alpha$. This intracellular CO_2 pool was estimated to have a δ^{13}C of -6 to -10‰, which overlaps with the δ^{13}C of environmental CO_2 (–8 to -14‰, calculated from environmental DIC values using equilibrium constants from Mook *et al*., 1974).

The similarity in the isotopic composition of environmental and intracellular CO_2 is likely the result of *S. velum* morphology and behaviour. *Solemya velum*, like other protobranch bivalves, actively pumps water through its mantle cavity, both anterior to posterior through the upper portion of its Y-shaped burrow, and through a ventral incurrent siphon (Owen, 1961). *Solemya velum* is capable of water pumping rates that are among the highest known for a clam its size (C. Cavanaugh, D. Kreuger S. Gallager, unpubl. data). The path between the CO_2 in the water being pumped through the mantle cavity and the symbiont cytosol is a short one: the CO_2 must only transit a thin intercalary cell before entering the apical end of a bacteriocyte, where the symbionts are located (Cavanaugh, 1983). Rapid flushing of mantle cavity water, and symbiont juxtaposition to this pool, would act in concert to decrease the boundary layer between environment and symbiont CO_2 pools and to maintain them in near-isotopic equilibrium.

These same factors also apply to other bivalves with chemoautotrophic symbionts. All actively pump seawater through their mantle cavities; all house symbionts in their gills within a short diffusive distance from their seawater source of CO_2 (Fisher, 1990; Cavanaugh and Robinson, 1996). CO_2 exchange between the symbionts and the environment is facilitated by this juxtaposition, and may explain why all have similar δ^{13}C values, despite a diversity of habitats, from coastal reducing sediments to deep-sea hydrothermal vents and hydrocarbon seeps (Fisher, 1990). Evaluating the significance of high rates of exchange for δ^{13}C values of other bivalve-chemoautotroph symbioses awaits measurement of ε-values of RubisCOs from these associations, as well as a greater understanding of CO_2 transport both by the clam host and the symbionts, and of carbon translocation between the symbionts and the host.

Given that the few ε-values measured from form IA, IB, and II RubisCO enzymes span >10‰, it is apparent that more ε-values need to be collected, particularly for the form IA and ID enzymes found in marine phytoplankton, the form IC enzymes in Proteobacteria, and form III RubisCOs from Archaea to include the entire breadth of phylogenetic and catalytic diversity of this ancient enzyme. Knowing the full range of ε-values for RubisCOs is key to unlocking the substantial heterogeneity of δ^{13}C values in contemporary and fossil biomass.

Experimental procedures

Reagents/Enzymes

All reagents were purchased from Sigma and used directly, except for $MgCl_2$ and ribulose 1,5-bisphosphate. Precise Mg^{2+} concentrations are critical for ε–value determination, as isotope discrimination by some RubisCO enzymes is sensitive to the concentration of this ion (Guy *et al*., 1993). Given the hygroscopic nature of $MgCl_2$, a concentrated stock solution (~0.5 M) was generated by dissolving $MgCl_2$ in distilled deion-

ized water and standardized gravimetrically by precipitating and quantifying Cl^- ions with $AgNO_3$ (Potts, 1987). The Mg^{2+} concentration in these standardized solutions was routinely 10–15% lower than those based on the weight of $MgCl_2$, as the result of variable water content.

Ribulose 1,5-bisphosphate (RuBP) that is available commercially is contaminated with inhibitory isomers which accumulate over time (Paech *et al.*, 1978) and is unsuitable for long-term incubations, such as those necessary for ε-value determinations. RuBP was therefore enzymatically synthesized from ribose 5-phosphate using spinach phosphoriboisomerase (PRI; Sigma P9752) and purified phosphoribulokinase (PRK; see below; Sigma P9877), precipitated as a barium salt, and used within 4 days to minimize the accumulation of inhibitory isomers (Horecker *et al.*, 1958). On the day of an experiment, Ba^{2+}RuBP was resuspended in 0.5 M HCl, and sufficient concentrated H_2SO_4 was added to precipitate most of the Ba^{+2}. The remainder was removed with a cation exchange column (SP-sepharose; Pharmacia). RuBP was quantified by absorbance at 280 nm (Rice and Pon, 1978). Carbon fixation by spinach RubisCO was substantially faster in long-term incubations with this freshly prepared RuBP than with the commercially available compound (K. Scott, unpubl. data).

Spinach RubisCO (Sigma R8000), used as a control for the ε determinations (see below), and PRK, for synthesizing RuBP (see above), were purified because the RubisCO was contaminated with pigments and other debris, and the PRK was contaminated with RubisCO. Enzymes were dissolved in gel filtration buffer (20 mM TRIS, pH 8, 0.15 M NaCl, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 1 mM EDTA, 1 mM β-mercaptoethanol) and injected into a gel filtration column (Superdex 200, Pharmacia) equilibrated with this buffer. Spinach RubisCO was purified immediately before use, whereas purified PRK was added to PRK storage buffer (40 mM bicine, pH 8, 20 mM $MgCl_2$, 10 mM dithiothreitol, and 20% glycerol by volume), frozen in liquid nitrogen, and stored at −80°C until use.

The purity of PRK and spinach RubisCOs was monitored during their preparation using SDS-PAGE gels (Ausubel *et al.*, 1994). Enzyme identities were confirmed by their molecular weights (from Coomassie blue-stained gels) and activity measurements. During purification, samples were assayed radiometrically for RubisCO and PRK activity (Beudeker *et al.*, 1980; Tabita, 1980; Cavanaugh, 1983). The identity of the RubisCO enzymes was further confirmed with Western blots using antisera specific for form I RubisCO (Robinson and Cavanaugh, 1995). Proteins were quantified using a kit based on the Bradford assay (Bio-Rad Laboratories).

S. velum *symbiont RubisCO*

Solemya velum clams are tiny (~1 cm long); thus, to obtain sufficient RubisCO for kinetic isotope effect determinations, it was necessary to clone and express the symbiont RubisCO genes (cbbL and cbbS) in *E. coli* (Schwedock *et al.*, 2004). RubisCO was co-expressed with the molecular chaperonins GroES and GroEL, and purified by NH_4SO_4 precipitation from *E. coli* cell-free extracts (Schwedock *et al.*, 2004). Enzyme identity and activity were monitored during purification as described above for spinach RubisCO. Purified *S. velum* symbiont RubisCO was desalted into RubisCO storage buffer (50 mM Bicine, pH 8.0, 10 mM $MgCl_2$, 66 mM $NaHCO_3$, 1 mM EDTA, 10 mM β–mercaptoethanol, 20% v:v glycerol), frozen in liquid nitrogen, and stored at −80°C.

RubisCO pH and temperature optima

In order to establish that *S. velum* symbiont RubisCO activity would be high in experiments to measure its ε-value, its response to pH and temperature was determined by incubating the enzyme under the appropriate conditions and measuring its activity radiometrically (Beudeker *et al.*, 1980; Cavanaugh, 1983). Complicating these experiments is the sensitivity of the concentration of CO_2 to both of these parameters. To maintain a similar CO_2 concentration at all pH and temperatures tested, an isotopic disequilibrium method was used, which capitalizes on the slow rate of interconversion of CO_2 and HCO_3^- (Cooper and Filmer, 1969). For this technique, a $^{14}CO_2$ solution was prepared by bubbling distilled water with CO_2 gas until saturated (~40 mM, pH 4), sealing it in a glass vial under a CO_2 headspace, injecting $NaH^{14}CO_3$ to a final concentration of 100 μCi ml^{-1} (specific activity = 33 μCi/mmol) and allowing it to equilibrate for 20 min before use. When this $^{14}CO_2$ solution is added to a solution at near-neutral pH, as these assays are, the CO_2 slowly hydrates to form HCO_3^-. Because the half-hydration time of CO_2 under these conditions is approximately 30 s (Asada, 1982), the assay timecourse is kept within this time-frame.

To determine the enzyme's optimum pH, the assay buffer (100 mM bicine pH 6–10, 30 mM $MgCl_2$, 1 mM dithiothreitol) was prepared with water treated to minimize the concentration of O_2 and CO_2. To remove DIC, distilled water (100 ml) was acidified with a drop of 12 N HCl and bubbled with N_2 gas for 10 min before the buffer salts were added. Buffer was brought to the appropriate pH by adding 10 N NaOH that had been freshly prepared with distilled deionized water, which limited the introduction of DIC with this basic solution. One ml of assay buffer was added to 2 ml glass vials primed with stir bars and sealed under a N_2 headspace with gastight septa to prevent equilibration with atmospheric CO_2 and O_2. Using a Pharmacia NAP-5 column, purified *S. velum* symbiont RubisCO was desalted from storage buffer into assay buffer adjusted to pH 8 and supplemented with 5 mM $NaHCO_3$. RubisCO was activated on ice for one hour before use. Assays were conducted at ~20°C. To begin an assay, 10 μl (15 μg) of RubisCO were added to the 1 ml portions of assay buffer. Fifteen seconds later, RuBP was added to a final concentration of 0.4 mM. After another 15 s, 10 μl of $^{14}CO_2$ solution was injected to initiate the reaction. Samples (200 μl) removed at 10 s intervals over a 30 s timecourse, were immediately injected into scintillation vials containing 200 μl of 60°C glacial acetic acid, sparged gently with air for 30 s, and left at 60°C for approximately 20 min before scintillation cocktail was added. At the end of the incubation, to measure the specific activity of the ^{14}C in each incubation, 10 μl samples were injected into 3 ml scintillation cocktail containing 50 μl phenylethylamine to trap the $NaH^{14}CO_3$. Specific activities and acid-stable ^{14}C were measured via scintillation counting.

To measure the temperature optimum for the enzyme, vials containing assay buffer at pH 8 were brought to the appro-

priate temperature (5–50°C) before beginning the assay as described above.

Kinetic isotope effect

The kinetic isotope effect (KIE) of the RubisCO of *S. velum* symbionts and of *Spinacea oleracea* (spinach, as a positive control) were measured using the substrate depletion method, in which the concentration and $\delta^{13}C$ of the CO_2 are measured as it is consumed by the RubisCO reaction (modified from Guy *et al*., 1993). Isotope discrimination by both enzymes was determined at the same pH (8.5) and temperature (25°C). The KIE assay buffer (50 mM bicine, pH 8.5, 25 mM $MgCl_2$, 1 mM dithiothreitol) was prepared with N_2-sparged distilled water to minimize CO_2 and O_2 concentrations as above. Bovine erythrocyte carbonic anhydrase (40 μg ml^{-1}), which catalyses the interconversion of CO_2 and HCO_3^-, was added to maintain DIC at chemical and isotopic equilibrium, and the buffer was filter-sterilized under vacuum to minimize outgassing during the reaction. KIE buffer was brought to 5 mM $NaHCO_3$ by adding the appropriate amount of a filter-sterilized stock $NaHCO_3$ solution, and was loaded into a heat-sterilized, septum-sealed 25 ml glass gastight syringe. RubisCO (5–10 mg) was desalted with a NAP-5 column (Pharmacia) equilibrated with KIE assay buffer and filter-sterilized. RubisCO was then injected into the KIE assay syringe and activated for 10 min at 25°C. During RubisCO activation, in preparation for removing samples from the reaction mix, the Teflon plungers of six 10 ml gastight syringes were coated with high vacuum grease to improve their seal. The sampling syringes were then primed with 1 ml 85% H_3PO_4 which had been bubbled with N_2 for 10 min to remove CO_2, and sealed with Teflon mininert valves.

The reaction was begun by injecting approximately 100 μmoles of filter-sterilized RuBP into the assay syringe, and progressed until all the RuBP was consumed. The concentration of dissolved inorganic carbon (DIC) was monitored by injecting 50–300 μl portions of the reaction mix into a Capni-Con 5 blood gas analyzer (Cameron Instruments). As the DIC was consumed by RubisCO, 3–5 ml samples were removed at intervals from the reaction mix and injected into the sampling syringes to terminate the reaction and convert the DIC to CO_2. CO_2 was cryodistilled from the samples by injecting them into a vacuum line with dry ice/ethanol traps for water, and liquid nitrogen traps for CO_2 (O'Leary, 1980). The cryodistilled CO_2 samples were injected into an Optima gas inlet mass spectrometer to determine their $\delta^{13}C$ values. For each enzyme, three (*S. velum*) or four (spinach) independent experiments were run.

The calculations necessary to estimate ε-values from the data are similar to those in Guy *et al*. (1993). However, a more direct derivation is presented here, which also incorporates an improved method for regressing the data to calculate the most accurate, precise ε-values (Scott *et al*., 2003). Since the $\delta^{13}C$ of DIC is what is actually measured for these experiments, and CO_2 is the substrate for RubisCO, it is necessary to account for the equilibrium isotope fractionation between bicarbonate and CO_2 (Mook *et al*., 1974) when calculating the ε-values. The steps that follow account for this adjustment. The change in the concentration of the DIC in the reaction as CO_2 is consumed by RubisCO can be described as

$$HCO_3^- \underset{K_2}{\overset{K_1}{\rightleftarrows}} CO_2 \xrightarrow{k'_R} PGA \tag{1}$$

where HCO_3^- and CO_2 are the bicarbonate and carbon dioxide dissolved in the reaction buffer, and PGA is the phosphoglyceric acid product of the RubisCO reaction. The rate constants for bicarbonate dehydration and carbon dioxide hydration are k_1 and k_2, respectively, whereas k'_R is equal to $V_{max}/K_M + (CO_2)$. During the timecourse of the reaction,

$$\frac{d(\mathrm{DIC})}{dt} = \frac{d(CO_2)}{dt} + \frac{d(HCO_3^-)}{dt} \tag{2}$$

Based on equation 1,

$$\frac{d(CO_2)}{dt} = k_1(HCO_3^-) - k_2(CO_2) - k'_R(CO_2) \tag{3}$$

and

$$\frac{d(HCO_3^-)}{dt} = k_2(CO_2) - k_1(HCO_3^-) \tag{4}$$

Therefore,

$$\frac{d(\mathrm{DIC})}{dt} = -k'_R(CO_2) \tag{5}$$

Since, at pH 8.5, CO_3^{-2} is only ~1.7% of the DIC present,

$$k_{eq} = \frac{(CO_2)}{(H^+)(\mathrm{DIC} - CO_2)} \tag{6}$$

where K_{eq} is the inverse of the dissociation constant for carbonic acid. Rearranging 6, one obtains

$$(CO_2) = \frac{K_{eq}(H^+)(\mathrm{DIC})}{1 + K_{eq}(H^+)} \tag{7}$$

Substituting equation 7 into equation 5,

$$\frac{d(\mathrm{DIC})}{dt} = \frac{-k_R K_{eq}(H^+)(\mathrm{DIC})}{1 + K_{eq}(H^+)} \tag{8}$$

which is approximately equal to $-k_R K_{eq}(H^+)(\mathrm{DIC})$ at pH 8.5. Re-writing equation 8 in terms of ^{12}C and ^{13}C, and dividing one by the other, one obtains

$$\frac{d(\mathrm{DI}^{12}\mathrm{C})}{d(\mathrm{DI}^{13}\mathrm{C})} = \frac{{}^{12}k_R\,{}^{12}K_{eq}(\mathrm{DI}^{12}\mathrm{C})}{{}^{13}k_R\,{}^{13}K_{eq}(\mathrm{DI}^{13}\mathrm{C})} \tag{9}$$

which describes the relative rates of $DI^{12}C$ and $DI^{13}C$ consumption. Rearranging equation 9,

$$\frac{d(\mathrm{DI}^{12}\mathrm{C})}{(\mathrm{DI}^{12}\mathrm{C})} \times \frac{{}^{13}k_R}{{}^{12}k_R} \times \frac{{}^{13}K_{eq}}{{}^{12}K_{eq}} = \frac{d(\mathrm{DI}^{13}\mathrm{C})}{(\mathrm{DI}^{13}\mathrm{C})} \tag{10}$$

Since

$$\frac{{}^{13}k_R}{{}^{12}k_R} = \frac{1}{\alpha_R} \text{ and } \frac{{}^{13}K_{eq}}{{}^{12}K_{eq}} = \frac{R_{CO_2}}{R_{HCO_3^-}} = \frac{1}{C} \tag{11}$$

where α_R is the kinetic isotope effect for RubisCO, and C is the equilibrium isotope effect between dissolved CO_2 and HCO_3^- (as in Guy *et al*., 1993), equation 11 can be re-written and integrated as

$$\frac{1}{\alpha} \times \frac{1}{C} \times \int \frac{d(\mathrm{DI}^{12}\mathrm{C})}{(\mathrm{DI}^{12}\mathrm{C})} = \int \frac{d(\mathrm{DI}^{13}\mathrm{C})}{(\mathrm{DI}^{13}\mathrm{C})} \tag{12}$$

which is equal to

$$\frac{1}{\alpha C} \times \ln\left(\frac{(DI^{12}C)}{(DI^{12}C)_0}\right) = \ln\left(\frac{(DI^{13}C)}{(DI^{13}C)_0}\right) \quad (13)$$

Equation 13 can be re-arranged to

$$\frac{1}{\alpha C} \times \ln\left(\frac{(DI^{12}C)}{(DI^{12}C)_0}\right) - \ln\left(\frac{(DI^{12}C)}{(DI^{12}C)_0}\right) = \ln\left(\frac{R_{DIC}}{R_{DIC_o}}\right) \quad (14)$$

By rearranging 14, one obtains the line

$$\ln(R_{DIC}) = \left(\frac{1}{\alpha C} - 1\right) \times \ln[DIC] + \ln\left(\frac{R_{DIC_0}}{[DIC_0]^{\left(\frac{1}{\alpha c}-1\right)}}\right) \quad (15)$$

which makes the approximation that $[DI^{12}C]$~[DIC], as $DI^{13}C$ is ~1% of DIC, and has been found to produce the most accurate, precise values of α (Scott *et al.*, 2003).

To generate 'average' values of ε for datasets with a level of precision similar to those reported here, either combining data from all experiments into a single regression line with dummy variables to compensate for differences in the y-intercept between experiments, or using a Pitman estimator should yield unbiased, precise estimates of ε (Scott *et al.*, 2003). Both methods were used here on the experimental datasets to see how they would perform in comparison with results from the study of Scott *et al.* (2003) which used simulated datasets. For both methods, the 95% confidence intervals for ε were calculated directly from the 95% confidence intervals of the slope of the line (equation 15).

Environmental DIC

Environmental DIC samples were collected from *S. velum* habitat (eelgrass beds near Wood's Hole, Massachusetts). Seawater samples were taken from immediately above the sediment surface before pushing three 10 cm diameter push cores through the eelgrass beds. Push cores were kept on ice and sampled within six hours of collection. Five cm portions were extruded from the cores and scooped into centrifuge tubes with minimal air headspace. Interstitial water was extracted by centrifuging the samples for 5 min at 9000 *g* and 4°C. The top aqueous layer was drawn into glass syringes, which were sealed by fitting them with needles, voiding sample until the needle dead volume was filled, and plunging the needle tips into rubber stoppers. These water samples were stored on ice and analysed within 16 h of collection.

Dissolved inorganic carbon was quantified and purified for stable isotope analyses as described above. The pH was determined with a combination calomel pH electrode after bringing the samples to 22°C, which was the seawater temperature when collected. The concentrations of CO_2 were calculated using the CO2SYS program, with GEOSECS constants and the National Buffer Standards (NBS) pH scale (Lewis and Wallace, 1998). Because of the limited amount of interstitial water present in each section, for some samples, it was not possible to conduct all the analyses described here.

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