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Kinetic isotope effect and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm *Riftia pachyptila*

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Abstract

It has been hypothesized that the $\delta^{13}C$ values of chemoautotrophs at deep-sea hydrothermal vents, which cluster in two groups around −11‰ and −30‰, are due to variation in isotopic discrimination by different forms of RubisCO. The most enriched $\delta^{13}C$ values are from the vestimentiferan tubeworm *Riftia pachyptila,* whose bacterial endosymbionts provide essentially all of its organic carbon via CO_2 fixation by a form II RubisCO. The kinetic parameters of purified *R. pachyptila* symbiont RubisCO were determined to assess the degree to which the $\delta^{13}C$ values of tubeworm biomass are due to isotopic fractionation during CO_2 fixation by this enzyme. Like most form II enzymes, the K_{CO_2} is high, at 240 μmol L^{-1}, whereas the CO_2/O_2 specificity factor (Ω) is low, at 8.6. The ε value, which is proportional to the degree of isotopic discrimination by the enzyme, was determined to be 19.5‰, lower than that for form I RubisCO. This low ε value supports the hypothesis that the degree of isotopic fractionation during CO_2 fixation appears to be an important influence on the $\delta^{13}C$ values of *R. pachyptila* biomass. Our results indicate that it is necessary to consider RubisCO ε values in interpreting $\delta^{13}C$ values from autotrophs.

At deep-sea hydrothermal vents, invertebrates with chemoautotrophic bacterial symbionts provide a substantial portion of the primary productivity. A diverse array of hosts, including vestimentiferan tubeworms, bivalves, gastropods, and shrimp, carpet areas of sulfide-bearing hydrothermal fluid emission in the deep sea at sites throughout the Pacific, Atlantic, and Indian Oceans (Tunnicliffe 1991; VanDover 1995; VanDover et al. 2001). Because the hosts position themselves between sources of hydrothermal fluid and bottom water, their chemoautotrophic symbionts have access to the substrates (e.g., oxygen and sulfide) necessary to fuel chemoautotrophic CO_2 fixation via the Calvin cycle (Cavanaugh 1994; Scott and Fisher 1995).

Despite a shared reliance on carbon fixed by symbiotic bacteria, these symbioses fall into two clusters with respect to their stable carbon isotope compositions ($\delta^{13}C = \{([R_{sample}/R_{std}] - 1 \times 10^3\}$, where $R = {}^{13}C/{}^{12}C$). Clams and mussels harboring chemoautotrophic endosymbionts have $\delta^{13}C$ values from −27‰ to −35‰ and form the more isotopically depleted −30‰ group. Vestimentiferan tubeworms, bacterial epibionts, and mats with $\delta^{13}C$ values from −9‰ to −16‰ comprise the more enriched −11‰ group (Childress and Fisher 1992; Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996).

These two clusters of $\delta^{13}C$ values generally are correlated with the form of the Calvin cycle CO_2 fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO)

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present in the symbionts. Thus, we hypothesized that form I enzymes from members of the −30‰ group discriminate against $^{13}CO_2$ more than do the form II enzymes of the −11‰ group (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996). Form I RubisCO, present in most of the members of the −30‰ group, consists of eight large and eight small subunits. Form II RubisCO, the form occurring in all members of the −11‰ group tested to date (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996), comprises multiple dimers of a single subunit that are evolutionarily related to the large subunits of form I RubisCO (Tabita 1999). Both form I and form II RubisCOs catalyze the carboxylation and oxygenation of ribulose 1,5-bisphosphate (RuBP), with CO_2 and O_2 as substrates. The oxygenation reaction competes with carboxylation, resulting in added energy consumption to regenerate the RuBP necessary for the RubisCO reaction. The relative rates of the carboxylase and oxygenase reactions catalyzed by RubisCO are expressed as CO_2/O_2 substrate specificity factors, or Ω values (Harpel et al. 1993). The relative rates of RubisCO-catalyzed carboxylation to oxygenation (v_c/v_o) at various CO_2 and O_2 concentrations can be predicted from the Ω value by the relation $v_c/v_o = \Omega \times [CO_2]/[O_2]$. Although they catalyze the same reactions, form I and form II RubisCOs differ in their kinetic parameters. Form I RubisCOs generally have higher affinities and specificity factors than do form II enzymes and therefore tend to function more efficiently under low CO_2, high O_2 conditions (Horken and Tabita 1999).

Theoretically, all RubisCO enzymes catalyze the fixation of $^{12}CO_2$ more rapidly than they catalyze the fixation of $^{13}CO_2$ (Purich and Allison 2000); however, RubisCO enzymes differ in their kinetic isotope effects, i.e., the relative rates of $^{12}CO_2$ to $^{13}CO_2$ fixation ($^{12}k/^{13}k$). For simplicity, the degree to which the reaction with $^{12}CO_2$ is favored over that with $^{13}CO_2$ is expressed as an ε value ($\varepsilon = [(R_s/R_p) - 1] \times 10^3$, where $R_s = {}^{13}C/{}^{12}C$ of the CO_2 substrate, $R_p = {}^{13}C/{}^{12}C$ of the product, i.e., carbon fixed, and $R_s/R_p = {}^{12}k/{}^{13}k$; Guy et al. 1993; Hayes 1993). Prior to this study, ε values from only three RubisCOs had been measured using high-precision methods. Form I RubisCOs from spinach (*Spinacea oleracea*; ε = 30‰) and a cyanobacterium (*Synechococcus* sp.; ε = 22‰) discriminate to a greater degree than does form II RubisCO from a nonsulfur purple photosynthetic bacterium (*Rhodospirillum rubrum*; ε = 18–22‰; Roeske and O'Leary 1984, 1985; Guy et al. 1993). Thus, if form II RubisCOs generally discriminate less against $^{13}CO_2$ than do form I enzymes, the more enriched $\delta^{13}C$ values of the hydrothermal vent symbioses in the −11‰ group may be due in part to the lower degree of isotopic discrimination by form II RubisCOs during CO_2 fixation.

Form II RubisCO from the symbionts of the giant vestimentiferan tubeworm *Riftia pachyptila* was chosen to test this hypothesis. *R. pachyptila* is the dominant member of the macrofauna of many hydrothermal vent communities at the East Pacific Rise and Galapagos Rift (Fisher 1996). This symbiosis is distinctive for its rapid growth rates, up to 1 m yr^{-1}, and massive biomass densities, with hundreds of individuals per square meter (Fisher 1996). These surprising growth rates appear to be fueled entirely by chemoautotrophic CO_2 fixation by endosymbiotic γ Proteobacteria (Childress and Fisher 1992; Cavanaugh 1994). The CO_2, H_2S, O_2, and other nutrients necessary for symbiont chemoautotrophy are acquired by the tubeworm from the vent environment with a gill-like structure, the obturaculum, and delivered to the symbionts by a well-developed vascular system (Childress and Fisher 1992; Scott and Fisher 1995). The symbionts are contained by membrane-bound vacuoles within bacteriocytes in the trophosome, a large organ that fills the tubeworm's body cavity (Cavanaugh 1994; Scott and Fisher 1995). The symbionts' intracellular location is likely to be a high-CO_2, low-O_2 habitat. The overall concentration of blood dissolved inorganic carbon (DIC; the sum of the concentrations of CO_2, HCO_3^-, and CO_3^{-2}) averages about 30 mmol L^{-1} (Goffredi et al. 1997). Because the blood pH is about 7.4, the CO_2 concentration can exceed 1 mmol L^{-1}, and blood O_2 concentrations are low (Childress and Fisher 1992; Goffredi et al. 1997). Immunological and phylogenetic analyses have confirmed that the symbiont RubisCO is a form II (Robinson and Cavanaugh 1995; Robinson et al. 1998). The kinetic parameters of the *R. pachyptila* symbiont RubisCO were thus measured to determine whether they are similar to those from other form II enzymes. Further, the ε value of RubisCO from this ecosystem-structuring primary producer was determined to evaluate the degree to which the $\delta^{13}C$ values of *R. pachyptila* biomass are due to the degree of isotopic discrimination during CO_2 fixation.

Materials and methods

Specimens—R. pachyptila were collected from the 9°N hydrothermal vent field (9°50′N, 104°17′W, 2,600 m depth) during DSV ALVIN dives 3062–3074 in April 1996. The worms were transported to the surface in an insulated container to maintain a temperature of 2°C. On board ship, the symbiont-containing trophosomes were dissected, flash frozen in liquid nitrogen, and stored at −80°C until use.

RubisCO purification—RubisCO was purified from *R. pachyptila* trophosomes by high-performance liquid chromatography (HPLC), using a modification of previously published protocols (Chene et al. 1992). Trophosome samples pooled from four individuals were ground to a fine powder in liquid nitrogen using a mortar and pestle and homogenized in a ground-glass tissue grinder containing three times the volume (w : v) of grinding buffer (20 mmol L^{-1} Tris HCl, pH 8.0, 50 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} $NaHCO_3$, 1 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA], 10 mmol L^{-1} β-mercaptoethanol, 1 mmol L^{-1} phenylmethylsulfonyl flouride [PMSF]). Homogenate was sonicated on ice using three 15-s bursts and was then centrifuged at 20,000 × g for 20 min. The supernatant was incubated at 50°C for 10 min, placed on ice for 10 min, and centrifuged at 20,000 × g for 10 min to remove denatured proteins and the remaining cellular debris (Hernandez et al. 1996). The resulting extract was filtered through a 0.4-μm low-protein-binding syringe filter (Amicon) and desalted into RubisCO buffer A (similar to grinding buffer but with 10 mmol L^{-1} $MgCl_2$ and no PMSF) using a Pharmacia Biotech PD-10 column.

Desalted extract was loaded onto a 20-ml Q-Sepharose Fast-Flow column (Pharmacia) equilibrated with RubisCO

buffer A and run at a flow rate of 8 ml min^{-1} using Rainin biocompatable HPLC pumps. The column was developed with a linear gradient of 0–0.5 mol L^{-1} NaCl in RubisCO buffer A with the RubisCO eluting at approximately 0.3 mol L^{-1} NaCl. Fractions containing RubisCO (detected by spot Westerns) were pooled, concentrated in a Centriplus 30 centrifugal concentrator (Amicon), desalted into RubisCO buffer A, and loaded onto a Pharmacia Mono Q 5/5 column. The column was run at a flow rate of 1 ml min^{-1} and developed as above, with RubisCO eluting at an identical NaCl concentration. Fractions containing RubisCO were pooled, desalted into 10 mmol L^{-1} Tris-citrate buffer (10 mmol L^{-1} Tris citrate, pH 7.3, 10 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} $NaHCO_3$, 1 mmol L^{-1} EDTA), and loaded onto a Mono Q 5/5 column equilibrated with this buffer. The RubisCO was eluted with a 40-ml linear gradient comprised of 10–100 mmol L^{-1} Tris-citrate buffer, with RubisCO eluting near the center of the gradient.

Purified RubisCO was pooled, concentrated in a Centricon 30 centrifugal concentrator (Amicon), and stored at −80°C in RubisCO storage buffer (50 mmol L^{-1} Bicine, pH 8.0, 10 mmol L^{-1} $MgCl_2$, 66 mmol L^{-1} $NaHCO_3$, 1 mmol L^{-1} EDTA, 10 mmol L^{-1} β-mercaptoethanol, 20% v : v glycerol).

Spot Westerns, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blotting—RubisCO purification was monitored by spot Westerns, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE), and Western blotting. To track RubisCO during purification, spot Westerns (Harlow and Lane 1988) were performed on fractions collected from the HPLC columns during gradient development. Aliquots of each fraction (1 μl) were spotted onto a nitrocellulose membrane (BioRad) and allowed to dry. Membranes were then incubated with antiserum specific for form II RubisCO (α-RrFII, raised against the *R. rubrum* RubisCO; Robinson and Cavanaugh 1995) at a dilution of 1 : 3,000, and developed by the alkaline phosphatase conjugated 2° antibody system according to the manufacturer's specifications (BioRad). Reactive fractions were collected and either subjected to further purification or stored at −80°C in RubisCO storage buffer.

The relative purity of the RubisCO at each stage of the purification process was confirmed using SDS-PAGE. Equivalent amounts of protein from each sample, quantified using the Bradford assay (Robinson and Cavanaugh 1995) with IgG as a standard, were diluted 1 : 1 in SDS-PAGE sample buffer (Harlow and Lane 1988) and heated to 100°C for 5 min before electrophoresis. Proteins were separated on a 10% SDS–polyacrylamide gel (Robinson and Cavanaugh 1995) and were stained with Coomassie blue to monitor purity or were transferred to polyvinylidene fluoride membrane (Millipore) by semidry electrophoresis. Membranes were incubated with α-RrFII as described for the spot Westerns.

Kinetic parameters—The K_{CO_2} and specific activity for the purified *R. pachyptila* symbiont RubisCO were determined in two independent experiments as previously described (Horken and Tabita 1999). To measure the K_{CO_2}, activated RubisCO was added to an oxygen-free reaction mixture containing RuBP and DIC, with the concentration of CO_2 ranging from 5 to 1,200 μmol L^{-1} and a specific activity of ~50 mCi $mmol^{-1}$. After a 30-s incubation, reactions were terminated by adding proprionic acid, and the amount of acid-stable (''fixed'') carbon was quantified by scintillation counting. The direct plot method was used to estimate the K_{CO_2} value (Horken and Tabita 1999).

Specificity factors (Ω) were determined in three separate experiments by incubating RubisCO at a range of CO_2 concentrations with a headspace of either 100% N_2 or air (Harpel et al. 1993). The reaction was begun by adding [1-^{3}H]RuBP. The amounts of ^{3}H-phosphoglycerate and ^{3}H-phosphoglycolate produced by the carboxylase and oxygenase reactions, respectively, were quantified via anion exchange chromatography with an online radioactivity detector hooked up to an HPLC system (Harpel et al. 1993).

Isotope fractionation—The isotope fractionation (ε) of the *R. pachyptila* symbiont RubisCO was measured by the substrate depletion method (Guy et al. 1993). Because the amount of RubisCO that could be purified from the trophosomes was very limited, it was not possible to measure isotope fractionation by the enzyme under a range of conditions (e.g., temperature, pH). For the measured value of ε to reflect isotopic fractionation of the enzyme in situ, the temperature and pH chosen for determining the isotope fractionation were similar to the temperature of the tubeworm's hydrothermal vent habitat (2–30°C; Fisher 1996) and the pH of the trophosome and the vascular blood (7–7.5; Childress and Fisher 1992). The RubisCO reaction was conducted in a sealed vessel, and both the concentration and isotope ratio ($^{13}C/^{12}C$) of the substrate DIC were measured as it was consumed by the RubisCO reaction.

Vials used to sample the reaction, cuvettes used to quantify the DIC, and reaction buffer (100 mmol L^{-1} Bicine, pH 8.0, with NaOH, 5 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} dithiothreitol) were sparged with CO_2-free N_2 for at least 14 h prior to use and sealed beneath a N_2 headspace. RuBP was enzymatically synthesized and purified according to previously published protocols (Wong et al. 1979). *R. pachyptila* symbiont RubisCO (0.5 mg) was desalted into reaction buffer using a Pharmacia PD-10 column to remove glycerol and DIC from the RubisCO storage buffer. RubisCO was then activated on ice for 1 h in reaction buffer containing 5 mmol L^{-1} $NaHCO_3$ in a volume of 3.5 ml.

Reactions were performed in 60-ml syringes in the absence of a gas phase to avoid complications due to the equilibrium of dissolved and gaseous CO_2. Reaction buffer was drawn into the syringes, and $NaHCO_3$ was added to a concentration of 5 mmol L^{-1}. Bovine erythrocyte carbonic anhydrase (30 μg ml^{-1}; Sigma) maintained CO_2 and HCO_3^- at chemical and isotopic equilibrium. Enough RuBP was added for the reaction to proceed to a maximum of 70% completion (150–180 μmoles). The reaction mix was maintained at 30°C. The DIC concentration present in the reaction mix initially and over the course of the reaction was determined spectrophotometrically with a coupled phosphoenolpyruvate carboxylase/malate dehydrogenase system that oxidizes 1 mole of NADH per mole of DIC present (Peled 1985).

The reaction was initiated by adding the activated RubisCO to the reaction mix. A 10-ml portion was imme-

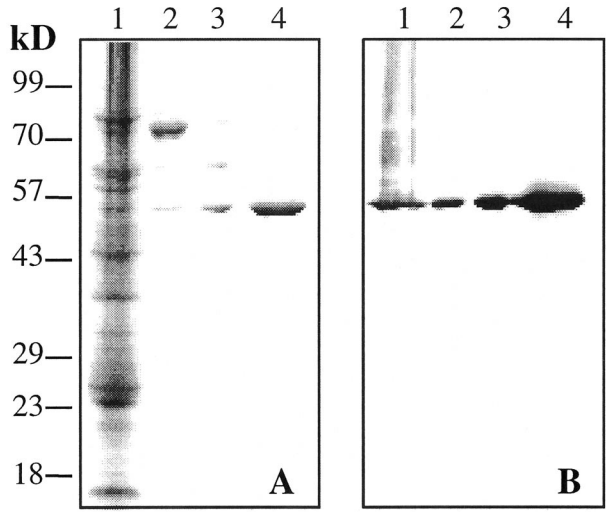

Fig. 1. SDS-PAGE (A) and Western blot (B) using antiserum against *R. pachyptila* symbiont form II RubisCO during its purification from trophosome. Lane 1: trophosome crude extract; lane 2: as eluted from the Q-Sepharose column; lane 3: as eluted from the Mono Q column, pH 8.0; lane 4: as eluted from the Mono Q column, pH 7.3. Numbers to the left refer to the positions of molecular weight markers (Gibco).

diately taken and quenched with 1 ml concentrated H_3PO_4 for the determination of the initial $\delta^{13}C$ value of the DIC. The reaction was allowed to proceed until approximately 30% of the DIC had been consumed, and then an additional 0.5 mg of desalted RubisCO was added. Control aliquots were taken for $\delta^{13}C$ value measurements both before and after addition of the fresh enzyme to ensure that no DIC had contaminated the reaction mix with the addition. Subsamples of 2.5–3 ml were taken throughout the reaction. The DIC in the quenched aliquots was purified by cryodistillation on a high-vacuum line (O'Leary 1980). Isotope ratios of the DIC were measured on a Finnigan Delta S isotope ratio mass spectrometer.

The value of ε (equivalent to Δ of Guy et al. 1993) was calculated from the change in the isotopic composition of the CO_2 as it was consumed by *R. pachyptila* symbiont RubisCO. ε is equal to $[(R_s/R_p) - 1\} \times 10^3$, where the kinetic isotope effect $= R_s/R_p = k^{12}/k^{13}$. ε is the slope of the line with $x = -\ln(f \times R/R_0)$ and $y = -10^3 \times \ln(R_0/R)$, where R is the isotope ratio of the CO_2 present at some time point, and R_0 is the isotope ratio of the CO_2 initially present. Values for ε were calculated for each of the four independent experiments, and the mean and SD of ε were estimated from these four values.

Results

RubisCO purification—Form II RubisCO (4 mg) was successfully purified to homogeneity from 100 g of *R. pachyptila* trophosome. SDS-PAGE and Western blot analyses indicate that the purity was >90% following the final pass through the Mono Q column (Fig. 1). The enzyme has subunits with an estimated molecular mass of 52 kDa, consistent with the molecular mass of 50.6 kDa predicted from the sequence of the *R. pachyptila* symbiont RubisCO gene (Robinson et al. 1998). The specific activity of the purified enzyme was 0.5–0.6 μmol CO_2 min^{-1} mg^{-1}.

Table 1. Isotopic discrimination by *Riftia pachyptila* symbiont RubisCO. Results from the four independent determinations are shown here. $\varepsilon = (^{12}k/^{13}k - 1) \times 1{,}000$.

Experiment	ε (‰)
1	19.2
2	17.9
3	18.5
4	22.5
Mean ± SE	19.5 ± 1.0

Kinetic parameters—*R. pachyptila* symbiont RubisCO had a high K_{CO_2} (mean ± SD: 239 ± 28 μmol L^{-1}; $n = 2$) and a low specificity factor (mean ± SE: $\Omega = 8.6 \pm 0.9$; $n = 3$).

Isotope fractionation—The kinetic isotope effect of the *R. pachyptila* symbiont RubisCO was 1.0195; $\varepsilon = 19.5‰ \pm 1.0‰$ (mean ± SE, $n = 4$; Table 1; Fig. 2). The reaction conditions for determining the isotope fractionation for this enzyme were chosen to reflect those present in situ; however, other parameters that may affect isotopic discrimination could be examined in the future. For example, *Riftia rubrum* RubisCO demonstrates a 3.4‰ range in ε when the concentration of Mg^{+2} is increased from 2 to 25 mmol L^{-1} (Guy et al. 1993). Determining the effect of temperature and pH would also be of interest, although neither has been shown

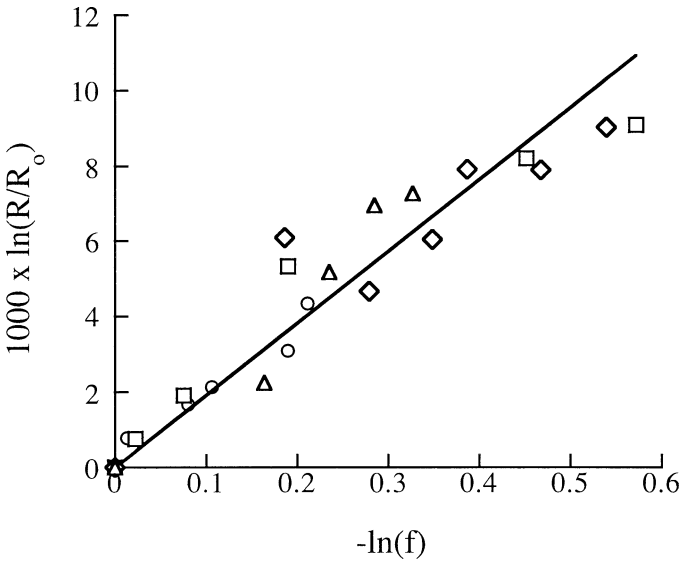

Fig. 2. Changes in the isotope ratio of CO_2 as it is consumed by *R. pachyptila* symbiont RubisCO. Results from four experiments are plotted, each with different symbols. R_0 is the isotope ratio of the CO_2 initially present in the reaction, R is the isotope ratio of the CO_2 at a particular time point, and f is the fraction of the initial CO_2 present at that time point ($=[CO_2]/[CO_2]_0$). The slope of the line $10^3 \times \ln(R/R_0)$ versus $-\ln(f)$ is equal to $\varepsilon/(1 + \varepsilon/1{,}000)$. The line corresponding to $\varepsilon = 19.5‰$ is shown.

to have a substantial effect on fractionation by form I RubisCOs (Christeller and Laing 1976; Roeske and O'Leary 1984).

Discussion

The CO_2 fixing enzyme of *R. pachyptila* symbionts appears to be a ''typical'' form II RubisCO. The *R. pachyptila* symbiont RubisCO K_{CO_2} value of 239 μmol L^{-1} falls within the range of 100–250 μmol L^{-1} observed for form II enzymes from free-living bacteria (Horken and Tabita, 1999). Similarly, the specificity factor ($\Omega = 8.6$) is comparable to values measured for other form II RubisCOs ($\Omega = 10$–15) and is substantially lower than those of form I enzymes ($\Omega = 25$–240; Tabita 1999). The K_{CO_2} and specificity factor of the *R. pachyptila* symbiont RubisCO indicate that this RubisCO catalyzes CO_2 fixation most effectively in high-CO_2, low-O_2 environments, as shown for other form II enzymes. The isotope fractionation of *R. pachyptila* symbiont RubisCO ($\varepsilon = 19.5$‰) is close to that of the form II enzyme from *R. rubrum* ($\varepsilon = 18$–22‰) and is lower than ε values from the form I enzymes (22–30‰; Guy et al. 1993), supporting the hypothesis that isotope fractionation by this enzyme plays a role in determining the $\delta^{13}C$ of *R. pachyptila* biomass.

The K_{CO_2} of *R. pachyptila* symbiont RubisCO and the scarcity of this enzyme in the symbionts are consistent with a high-CO_2 environment in the trophosome. Despite measures taken to minimize and track losses during purification, 100 g of trophosome (equivalent to approximately 15–30 g of symbionts; Childress and Fisher 1992) yielded only 4 mg of RubisCO. Furthermore, analyses of trophosome samples from numerous *R. pachyptila* by SDS-PAGE and Western blots indicate that the concentration of RubisCO is very low, <1% of trophosome protein (Fig. 1). *R. rubrum* compensates for the low affinity of its form II RubisCO by massively overexpressing this enzyme, to 50% of total soluble protein, when grown autotrophically under low-CO_2 conditions (Sarles and Tabita 1983). The amount of RubisCO detected here suggests that the symbionts have a high concentration of intracellular CO_2. The CO_2 concentration in both the vent habitat and blood from freshly collected *R. pachyptila* can reach 1.8 mmol L^{-1} (Goffredi et al. 1997), and carbonic anhydrase activity is present in the trophosome, which could speed the transfer of CO_2 through the bacteriocyte cytosol to the symbionts (Kochevar et al. 1993). However, the affinities of both *R. pachyptila* symbiont RubisCO ($K_{CO_2} = 239$ μmol L^{-1}) and intact symbiont cells ($K_s = 50$ μmol L^{-1}) are considerably lower than 1.8 mmol L^{-1} (Scott et al. 1999), suggesting that the concentration of CO_2 may be lower at the symbiont cell surface and within the symbiont cytosol than in the blood. Further study will be necessary to resolve this discrepancy. However, it is apparent that *R. pachyptila* symbionts, protected as they are from short-term fluctuations in the concentration of CO_2 by their intracellular location, can avoid the necessity of having genes for both form I and form II RubisCOs for expression in response to changes in the CO_2 concentration, as many free living bacteria do (Tabita 1999).

The low specificity for CO_2 versus O_2 of *R. pachyptila* symbiont RubisCO is not likely to diminish symbiont carbon fixation rates; trophosome dissolved $CO_2 : O_2$ ratios are high. Oxygen concentrations measured in seawater samples among *R. pachyptila* range from undetectable to 110 μmol L^{-1} (Childress and Fisher 1992). Assuming O_2 in the blood is at equilibrium with environmental O_2, and blood CO_2 is 1.8 mmol L^{-1} (Goffredi et al. 1997), the $CO_2 : O_2$ ratio is 17. If the ratio of CO_2 to O_2 is similar in the symbiont cytosol, a RubisCO specificity factor of 8.6 would result in the rate of carboxylation outpacing oxygenation by 144 : 1. The O_2 concentration within the symbiont cells is likely to be much lower; *R. pachyptila* blood sequesters O_2 with extremely high-affinity, high-capacity hemoglobins, and both the host tubeworm and the symbionts consume O_2 (Childress and Fisher 1992). Thus, the carboxylation reaction is probably even more rapid relative to oxygenation than calculated here.

The low ε value of 19.5‰ for *R. pachyptila* symbiont RubisCO supports the hypothesis that the degree of isotopic fractionation during CO_2 fixation is an important influence on the $\delta^{13}C$ values of *R. pachyptila* biomass. *R. pachyptila* have more isotopically enriched biomass than do vent chemoautotrophic symbioses relying on form I RubisCO for carbon fixation, and *R. pachyptila* symbiont form II RubisCO discriminates less than do form I RubisCO enzymes. This finding is consistent with the hypothesis that heterogeneity in RubisCO ε values is at least in part responsible for the bimodal distribution of biomass $\delta^{13}C$ values from hydrothermal vent organisms. Other symbioses and bacterial mats from vent environments also have enriched $\delta^{13}C$ values and have been found to express a form II RubisCO (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996), suggesting that the enzymes from these species will also discriminate against $^{13}CO_2$ less than form I RubisCO does. That so many of these hydrothermal vent organisms have form II RubisCO is consistent with the high $CO_2 : O_2$ ratio of their environment, which is an ideal habitat for low-affinity, low-specificity form II RubisCOs.

The carbon isotope values of *R. pachyptila* biomass ($\delta^{13}C$ of −16‰ to −9‰) are 10–17‰ more enriched than expected, based on the ε value of symbiont RubisCO and the $\delta^{13}C$ value of CO_2 from their habitat (approximately −7‰; Mook et al. 1974; Childress et al. 1993). This finding indicates that factors other than isotope fractionation by RubisCO are also contributing to the ^{13}C enrichment of *R. pachyptila* biomass. Other factors have also been noted for their effects on phytoplankton $\delta^{13}C$ values. Anapleurotic carbon fixation by the tubeworm symbiosis will contribute to ^{13}C enrichment because some of the carboxylases that catalyze this process do not have ε values as large as those of RubisCO, although this is typically considered to be of minor impact on $\delta^{13}C$ values (Goericke et al. 1994). A CO_2 transport pathway, analogous to the C4 pathway in plants, has been proposed for *R. pachyptila* symbionts (Arndt et al. 1998) and is consistent with the high levels of four-carbon organic acids in tubeworm blood. However, these compounds are now believed to function primarily as part of a host response to anoxia (Arndt et al. 1998). As shown for some isotopically enriched phytoplankton, *R. pachyptila* might rely on bicarbonate transporters (Goericke et al. 1994).

However, neither the host nor the symbionts transport HCO_3^-, relying instead on CO_2 diffusion (Goffredi et al. 1997; Scott et al. 1999). CO_2 limitation appears to have some impact, because larger *R. pachyptila* have more positive $\delta^{13}C$ values than do smaller worms (Fisher et al. 1990). However, carbon limitation cannot account for the findings that even the smallest *R. pachyptila* (3–10 mg) are ^{13}C enriched ($\delta^{13}C = -16‰$ to $-14‰$). Furthermore, CO_2 limitation for the symbionts seems unlikely given the high CO_2 concentrations in vent water and *R. pachyptila* blood (Childress et al. 1993; Goffredi et al. 1997).

Another possibility is that boundary layer effects may influence biomass $\delta^{13}C$ values (Goericke et al. 1994). In this scenario, because of multiple ultrastructural barriers or long diffusive distances, the rate of exchange between the intracellular pool of CO_2 and the environmental pool is slow enough that RubisCO-catalyzed CO_2 fixation drives the intracellular CO_2 pool to more enriched $\delta^{13}C$ values. This scenario seems likely for *R. pachyptila* symbionts, because they are separated from their blood CO_2 supply by the bacteriocyte membrane and cytosol, the perisymbiont vacuole membrane, and the gram-negative cell envelope. The influence of rapid carbon fixation and multiple barriers to diffusion is suggested when the $\delta^{13}C$ of CO_2 within the symbiont cells is calculated. Because the RubisCO kinetic isotope effect ($^{12}k/^{13}k$) is equal to R_s/R_p, the isotope ratio of CO_2 within the symbiont cells is equal to $R_p \times {}^{12}k/{}^{13}k$. If the isotope ratio of *R. pachyptila* biomass is used to approximate R_p, the intracellular CO_2 $\delta^{13}C = +3‰$ to $+10‰$, substantially higher than environmental CO_2. Such ^{13}C-enriched intracellular CO_2 may be the isotopic ''fingerprint'' of the RubisCO reaction when it is occurring under circumstances where CO_2 exchange is not rapid enough to bring environmental and intracellular CO_2 pools to isotopic equilibrium.

The $\delta^{13}C$ values of marine chemo- and photoautotrophic primary producers are widely used to infer the factors influencing CO_2 fixation in situ. *R. pachyptila* symbiont RubisCO is the first carboxylase from a marine ecosystem-structuring primary producer to have its ε value measured with high precision methods. Membership of other hydrothermal vent autotrophic symbioses in the $-11‰$ or $-30‰$ groups may be influenced by the ε value of the RubisCO enzymes carried by the symbionts (Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996). Similarly, marine phytoplankton have a broad range of biomass $\delta^{13}C$ values, from $-31‰$ to $-18‰$ (Rau et al. 1989). Although some of this heterogeneity may be due to growth rate effects and environmental CO_2 concentrations (Goericke et al. 1994), marine phytoplankton carry a phylogenetically broad array of form I RubisCO enzymes, and some dinoflagellates even utilize a form II RubisCO for CO_2 fixation (Morse et al. 1995; Rowan et al. 1996; Pichard et al. 1997). Given the apparent influence of the ε value of *R. pachyptila* symbiont RubisCO on this organism's $\delta^{13}C$ value and the diversity of RubisCO enzymes found in both photo- and chemoautotrophs, it is crucial to consider enzyme-mediated fractionations when interpreting the variability of $\delta^{13}C$ values of contemporary and ancient autotrophs from all habitats, whether they are photo- or chemosynthetically sustained.

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