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Stable Carbon Isotope Discrimination by Form IC RubisCO from *Rhodobacter sphaeroides*

Phaedra Thomas

*University of South Florida*

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Stable Carbon Isotope Discrimination by Form IC Rubisco
from *Rhodobacter sphaeroides*

by

Phaedra Thomas

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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Stable Carbon Isotope Discrimination by Form IC RubisCO from
Rhodobacter sphaeroides

Phaedra J. Thomas

ABSTRACT

Variations in the relative amounts of $^{12}\text{C}$ and $^{13}\text{C}$ in microbial biomass can be used to infer the pathway(s) autotrophs use to fix and assimilate dissolved inorganic carbon. Discrimination against $^{13}\text{C}$ by the enzymes catalyzing autotrophic carbon fixation is a major factor dictating the stable carbon isotopic composition ($\delta^{13}\text{C} = \{[^{13}\text{C}/^{12}\text{C}_{\text{sample}}/^{13}\text{C}/^{12}\text{C}_{\text{standard}}] - 1\} \times 1000$) of biomass. Six different forms of ribulose 1,5-bisphosphate carboxylase/oxygenase or RubisCO (IA, IB, IC, ID, II, and III), the carboxylase of the Calvin-Benson-Bassham cycle (CBB), are utilized by algae and autotrophic bacteria that rely on the CBB cycle for carbon fixation. To date, isotope discrimination has been measured for form IA, IB, and II RubisCOS. Isotopic discrimination, expressed as $\epsilon$ values ($=\{[^{12}\text{k}/^{13}\text{k}] - 1\} \times 1000; ^{12}\text{k}$ and $^{13}\text{k}$ = rates of $^{12}\text{C}$ and $^{13}\text{C}$ fixation) range from 18 to 29‰, explaining the variation in biomass $\delta^{13}\text{C}$ values of autotrophs that utilize these enzymes. Isotope discrimination by form IC RubisCO has not been measured, despite the presence of this enzyme in many proteobacteria of ecological interest, including marine manganese-oxidizing bacteria, some nitrifying and nitrogen-fixing bacteria, and extremely metabolically versatile organisms such as Rhodobacter sphaeroides. The purpose of this work is to determine the $\epsilon$ value for the
form IC RubisCO enzyme from *R. sphaeroides*. Under standard conditions (pH 7.5 and 5 mM DIC), form IC RubisCO had an ε value of 22.9‰. Sampling the full phylogenetic breadth of RubisCO enzymes for isotopic discrimination makes it possible to constrain the range of δ¹³C values of organisms fixing carbon via the Calvin-Benson-Bassham cycle. These results are helpful for determining the degree to which CBB cycle carbon fixation contributes to primary and secondary productivity in microbially-dominated food webs.
Chapter One

Introduction

The Calvin Cycle

The Calvin-Benson-Bassham (CBB) cycle is the most common carbon-fixing pathway for autotrophic organisms. It is present in plants, algae, cyanobacteria, and proteobacteria, and has recently been found in firmicutes (3). The CBB cycle consists of the dark reaction of photosynthesis in which CO$_2$ is incorporated into organic compounds for the biosynthetic needs of the organism. It consists of three stages: carbon fixation, reduction, and regeneration (1).

RubisCO (Ribulose 1,5-bisphosphate carboxylase/oxygenase) is the carbon-fixing enzyme in the Calvin cycle (48). The RubisCO reaction involves the catalytic conversion of one molecule of ribulose 1,5-bisphosphate (RuBP), via carboxylation, into two molecules of phosphoglyceric acid (PGA) (Figure 1). This comprises the carbon fixation step of the Calvin cycle. The products of the RubisCO reaction can be shuttled into other metabolic pathways and used to form amino acids and other precursors for biosynthesis. RubisCO is a relatively nonselective enzyme that can utilize both carbon dioxide and oxygen as substrates (18).

There are four main forms of RubisCO, form I, II, III, and IV that differ substantially in their amino acid sequences (see below; 48). Form I RubisCO is
present in cyanobacteria, proteobacteria, and most plastids, and can be further subdivided, based on amino acid sequences, into forms IA, IB, IC, and ID. Form II is found in some proteobacteria and dinoflagellates, and the Form III enzyme is present in some archaea. Form IV RubisCO is widespread in bacteria and is not active as a carboxylase (49).

Catalytically active form I RubisCO consists of eight large subunits (encoded by the \textit{cbbL} gene) and eight small subunits (encoded by the \textit{cbbS} gene), while form II and III consist of a single type of subunit that is homologous to form I large subunits (48). The amino acid sequences of form I large subunits and II RubisCOs are quite divergent, with only ~23\% sequence similarity (34). Within the form I RubisCOs, the amino acid sequences of the large subunits of form IA and IB RubisCOs are approximately 80\% similar, as are the form IC and ID RubisCOs, while the IA/IB cluster has only
approximately 60% sequence similarity with the IC/ID cluster (34, 48). Consistent with these differences in sequence, each form has different specificities for CO$_2$ and O$_2$. Form I RubisCOs have a higher specificity for CO$_2$ than O$_2$, compared to the form II enzymes (48, 49). However, despite these differences in the primary structures of the four main forms, the active site responsible for the carboxylation of CO$_2$ and the oxygenation of O$_2$ is conserved across the various RubisCOs (48).

Other Autotrophic Pathways

There are three other autotrophic pathways present in microorganisms in addition to the CBB cycle. In the reverse citric acid cycle (rTCA), acetyl-CoA is formed by splitting citrate that was produced by reversing the citric acid cycle so that it operates in a carboxylating, reductive direction (20). The acetyl-CoA is reductively carboxylated to pyruvate, which is shuttled into other central metabolic pathways (20). The three key enzymes responsible for the reverse rotation of the oxidative citric acid cycle are ATP citrate lyase, 2-oxoglutarate: ferredoxin oxidoreductase, and fumarate reductase (20).

The acetyl-CoA pathway (AcCoA) produces acetyl-coA via sequential reduction of carbon dioxide. This molecule is reduced to the level of a methyl group while attached to a cofactor, followed by acetyl-CoA synthase-mediated condensation of this methyl group with a second carbon that has been reduced from the level of carbon dioxide to carbon monoxide via carbon monoxide dehydrogenase (CODH) (40). The 3-hydroxypropionate cycle (3-HPP), which is the most recently discovered pathway for carbon fixation, functions by carboxylating acetyl-CoA and converting it to propionyl-CoA with 3-hydroxypropionate as an intermediate (52). The key enzyme for this cycle
is malonyl-CoA reductase, which reduces malonyl-CoA, an initial product of acetyl-CoA carboxylation, to 3-hydroxypropionate (19). Propionyl-CoA undergoes carboxylation, forming malyl-CoA, which further divides into acetyl-CoA and glyoxylate (52).

Autotrophic pathways cannot be predicted based on the host organism’s phylogeny (Table 1). The rTCA cycle is found in a variety of autotrophic bacteria and archaea, Chlorobium sp., sulfur-reducing Crenarchaeota (Thermoproteus and Pyrobaculum), sulfate-reducing bacteria (Desulfovibrio), as well as microaerophilic and hyperthermophilic hydrogen-oxidizing bacteria like Aquifex sp. and Hydrogenobacter sp. (20). The acetyl-CoA pathway occurs in autotrophic sulfate-reducing bacteria, methanogens, and acetogenic bacteria (11). The acetyl-CoA pathway has also been studied in Spirochaetes like Treponema primitia (14). The 3-hydroxypropionate cycle operates in the green nonsulfur bacterium, Chloroflexus aurantiacus and autotrophic Crenarchaeota (19).

The autotrophic pathways presented in Table 1 differ in two important ways: their sensitivity to oxygen (O₂) and their energetic requirements. The rTCA cycle and the acetyl-CoA pathway are sensitive to oxygen, due to the extreme oxygen sensitivity of pyruvate: ferredoxin oxidoreductase, which is responsible for carboxylating acetyl-CoA (rTCA and acetyl-CoA pathways), as well as CODH (acetyl-CoA pathway), which explains why these alternative methods of fixing CO₂ predominate in anaerobic environments (4, 50). The Calvin cycle and the 3-hydroxypropionate cycle are both less sensitive to oxygen (Thauer, 2007).
Table 1. The distribution of autotrophic pathways among the domains of life.
*Only divisions with autotrophic members are listed. †It has not been determined whether *Treponema primitia* is an autotroph. Abbreviations: Calvin-Benson-Bassham cycle = CBB, reductive citric acid cycle = rTCA, acetyl-CoA pathway = Ac-CoA, and 3-hydroxypropionate cycle = 3-HPP.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Division*</th>
<th>Pathway(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td>CBB, rTCA</td>
</tr>
<tr>
<td></td>
<td>Cyanobacteria</td>
<td>CBB</td>
</tr>
<tr>
<td></td>
<td>(Eukarya - Plants)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroflexi</td>
<td>3-HPP</td>
</tr>
<tr>
<td></td>
<td>Chlorobi</td>
<td>rTCA</td>
</tr>
<tr>
<td></td>
<td>Aquificae</td>
<td>rTCA</td>
</tr>
<tr>
<td></td>
<td>Firmicutes</td>
<td>CBB, Ac-CoA</td>
</tr>
<tr>
<td></td>
<td>Planctomycetes</td>
<td>Ac-CoA</td>
</tr>
<tr>
<td></td>
<td>Spirochaetes</td>
<td>Ac-CoA†</td>
</tr>
<tr>
<td>Archaea</td>
<td>Crenarchaeota</td>
<td>rTCA, 3-HPP</td>
</tr>
<tr>
<td></td>
<td>Euryarchaeota</td>
<td>Ac-CoA, CBB?</td>
</tr>
</tbody>
</table>

All of the carbon fixation pathways discussed require NADPH, ferredoxin, or other intracellular electron-shuttling cofactors as electron donors, except the acetyl-CoA pathway, which uses H₂ as its donor and is the only known autotrophic pathway that yields metabolic energy instead of consuming it. This is because it directly couples the removal of electrons from H₂, and transfers these electrons to CO₂ for the creation of membrane potential (11). The Calvin cycle is the most energetically expensive method of fixing carbon, due to multiple dephosphorylation events necessary to regenerate RuBP
from 3-PGA, followed by the rTCA cycle, and acetyl-CoA pathway (28). Autotrophs that are primarily found in low-oxygen environments often use alternatives to the CBB cycle because they demand less energy to synthesize an equivalent amount of biomass. However, in oxic environments, the CBB cycle dominates due to its relative stability under these conditions (28).

**Elucidating autotrophic pathways from contemporary and ancient environmental samples**

It is interesting to resolve which pathways are in operation in contemporary and ancient environments because it gives us insight into the history of carbon cycling. Identifying the autotrophic pathways operating in ancient environments can help clarify whether the CBB cycle has always been the dominant carbon-fixing pathway.

In order to determine which pathways are operating in a particular contemporary environment, enzyme and nucleic acid assays can be used. If sufficient biomass is present, assays for enzymes diagnostic for the different pathways can be used; e.g., RubisCO for the CBB cycle (48), ATP citrate lyase for the rTCA cycle (20), CODH for the acetyl-CoA pathway (40), and malonyl-CoA reductase for the 3-hydroxypropionate cycle (19). If insufficient biomass is available for enzyme assays, nucleic acid-based approaches (e.g., Southern & Northern blots; PCR) can be utilized to identify which autotrophic CO₂-fixing pathways are in operation. The *cbbL* or *cbbM* genes for form I or II RubisCO can be used as indicators of Calvin Cycle presence in a target organism; genes encoding the enzymes diagnostic for other autotrophic pathways, (see above) can be utilized as markers for the presence of those pathways.
However, enzyme assays and nucleic acid-based techniques do not work for fossil samples, as these macromolecules are not present in appreciable quantities. Instead, isotope measurements can be used, since $^{12}\text{C}$ and $^{13}\text{C}$ contents persist throughout geological time. Stable carbon isotope compositions of organic compounds extracted from fossil sediments can help identify which autotrophic pathway dominated the input of carbon into a particular ecosystem (42, 27).

**Stable carbon isotope ratios and $\delta^{13}\text{C}$ values**

Three isotopes of carbon exist: two stable isotopes ($^{12}\text{C}$ and $^{13}\text{C}$), and radioactive $^{14}\text{C}$. $^{12}\text{C}$ is more abundant in nature than $^{13}\text{C}$, which is ~1‰ of all carbon. The relative amounts of $^{12}\text{C}$ and $^{13}\text{C}$ in a sample are expressed as $\delta^{13}\text{C}$ values, in parts per thousand (‰): $\delta^{13}\text{C} = (\{R_{\text{sample}}/R_{\text{standard}}\} - 1) \times 1000$, where the limestone, PeeDee Belemnite, is the standard (31, 27), and $R = ^{13}\text{C}/^{12}\text{C}$. A more negative $\delta^{13}\text{C}$ value indicates that there is less $^{13}\text{C}$ in the sample (it is ‘isotopically depleted’). Conversely, a more positive $\delta^{13}\text{C}$ indicates that more $^{13}\text{C}$ is present in the sample (it is ‘isotopically enriched’) (31).

$\delta^{13}\text{C}$ values vary greatly. The $\delta^{13}\text{C}$ value of atmospheric CO$_2$ is ~$-8$‰, while the $\delta^{13}\text{C}$ of inorganic carbon dissolved in seawater is between ~$2$‰ and ~$1$‰ (41, 21, 23). Biomass $\delta^{13}\text{C}$ values are more isotopically depleted than these inorganic carbon sources, due to the relative weakness of bonds to $^{12}\text{C}$, compared to $^{13}\text{C}$. Since the zero-point energy of bonds to $^{13}\text{C}$ is lower than the zero-point energy of bonds to $^{12}\text{C}$, compounds containing $^{12}\text{C}$ tend to react more quickly than those containing $^{13}\text{C}$ (Figure 2) (29). As a result, autotrophs fix $^{12}\text{CO}_2$ more rapidly than $^{13}\text{CO}_2$ and their biomass has more negative $\delta^{13}\text{C}$ values (6). Since autotroph biomass is isotopically depleted relative to source CO$_2$,
so is the biomass of the heterotrophs that consume them (17).

![Zero-point energy diagram of 12C - and 13C - carbon dioxide.](image)

The δ^{13}C of C3 plants that use the CBB cycle is -18-30‰; this great degree of isotope depletion relative to atmospheric CO₂ is largely due to substantial fractionation by RubisCO during carbon fixation (17). The δ^{13}C of C4 plants is -8‰ to -20‰. These values are more positive than C3 plants because the enzymes responsible for the initial fixation of carbon into C4 compounds do not fractionate to the same degree as RubisCO does (17). The δ^{13}C value of marine photoautotrophs such as algae is between -18 and -28‰, but is generally more toward the isotopically enriched end of this range (13). They can be isotopically enriched due to a variety of factors, including carbon-concentrating mechanisms (CCMs) and diffusive limitation (DL) (22).

In the case of both CCMs and DL, the isotopic enrichment of intracellular biomass is due to isotopic enrichment of intracellular CO₂. This isotopic enrichment of intracellular CO₂ is due to low rate of CO₂ efflux from the cells, compared to the rate of
influx and fixation. In the case of a cell with a CCM, the rate of influx is high, due to the activities of multiple bicarbonate transporters. Intracellular bicarbonate is converted to CO₂ by carbonic anhydrase. The majority of CO₂ is fixed by RubisCO. The CO₂ remaining is isotopically enriched. Cells with CCMs typically have mechanisms to prevent the efflux of this pool of intracellular CO₂, which prevents the isotopic signature from CO₂ fractionation by RubisCO from being wiped away by rapid exchange of intracellular CO₂ with extracellular dissolved inorganic carbon (22). Likewise, cells experiencing DL have a very low rate of CO₂ efflux, since diffusive limitation of CO₂ supply to the cell results in extraordinarily low intracellular concentrations of CO₂ (22).

The differences in δ¹³C values among C3, C4, and marine organisms make it possible to trace carbon through food webs (17), drawing on the adage that you are what you eat.

The ¹³C-content of fossil organic carbon has been used as evidence for biological carbon fixation. In some fossil samples, both organic and inorganic carbon are preserved, providing a means to measure isotope discrimination between inorganic (δ¹³C = ~2‰) and organic carbon (δ¹³C = -25‰), billions of years ago (42). The level of isotopic discrimination between organic and inorganic carbon in these samples cannot be achieved by a/biological processes, it is only possible via enzyme discrimination during autotrophic carbon fixation (42). Based on the limited data available from cultures, the level of isotope discrimination observed in these fossil biomass samples is consistent with the CBB cycle and acetyl-CoA pathway. However, hypotheses about which autotrophic pathways may be operating are weakened by limited sampling; it is still not possible to predict, with confidence, the δ¹³C values expected for each pathway.
Enzymatic isotope discrimination

The $\varepsilon$ value is a measure of isotope discrimination by an enzyme; in this case, RubisCO. Biomass $\delta^{13}C$ values from organisms using the CBB cycle are almost always more negative than source CO$_2$ (39), but different RubisCO enzymes fractionate to varying degrees due to slight differences in the structure of their active site (15). Isotope fractionation is described as a discrimination factor ($\varepsilon$; = $\left\{ \frac{[12k]}{[13k]} \right\} - 1$ X 1000, where $^{12}k$ and $^{13}k$ = the rates of $^{12}C$ and $^{13}C$ fixation (15). Epsilon values ($\varepsilon$) for enzymes are calculated by measuring the change in the isotopic composition of the substrate pool as an enzyme reaction progresses. More isotopically selective enzymes will leave behind relatively more $^{13}CO_2$, while less isotopically selective enzymes (with smaller $\varepsilon$ values) will leave behind less $^{13}CO_2$.

For RubisCO enzymes, the isotopic composition of dissolved inorganic carbon (DIC) is monitored; changes in the $\delta^{13}C$ of DIC can be described using the Rayleigh distillation equation: $(R/R_1) = (C/C_1)^{1/\alpha}$, where $R$ is the isotope ratio of the DIC, $C$ is the DIC concentration, and both $R_1$ and $C_1$ represent the corresponding quantities present at the beginning of the experiment (45). The $\alpha$ is $R_r/R_p$; $R_r$ is the isotope ratio of available reactant, and $R_p$ is the isotope ratio of the product, and is equal to $^{12}k/^{13}k$ (45). $\varepsilon$ values should affect the $\delta^{13}C$ values of autotroph biomass. Large $\varepsilon$ values should result in more negative biomass $\delta^{13}C$ values, while small $\varepsilon$ values should result in more positive biomass $\delta^{13}C$ values, because a less isotopically selective enzyme should fix more $^{13}CO_2$. The $\varepsilon$ values for forms IA, IB, and II RubisCO enzymes are currently known ($\varepsilon = 18-29\%$). However, the $\varepsilon$ values for forms IC and ID RubisCOs have not been measured,
which makes it impossible to predict the range of $\delta^{13}C$ values expected for organisms using the CBB cycle, which really limits the interpretation of the $\delta^{13}C$ values from contemporary and ancient samples.

The purpose of this research is to determine $\varepsilon$ values for the form IC RubisCO enzymes. Form IC RubisCOs have not been explored yet and knowing their $\varepsilon$ values will impact the fields of microbial ecology and biogeochemistry because it will help to constrain the range of $\delta^{13}C$ values expected for organisms using the CBB cycle.
Chapter Two

Isotope Discrimination by Form IC RubisCO

It is of interest to ascertain which carbon-fixing pathways are operating in contemporary and ancient microbially-dominated habitats, as the pathways differ in energetic expense and cofactor requirements (11), which in turn influence the ecology of the organisms. It is not possible to collect biochemical and/or nucleic acid-based evidence for pathway presence in the case of fossil biomass samples. Variations in the relative amounts of $^{12}\text{C}$ and $^{13}\text{C}$ in autotrophic microbial biomass, expressed as $\delta^{13}\text{C}$ values ($= \{[R_{\text{sample}}/R_{\text{standard}}] - 1\} \times 1000; R_{\text{sample}} = ^{13}\text{C}/^{12}\text{C}_{\text{sample}}, R_{\text{standard}} = ^{13}\text{C}/^{12}\text{C}_{\text{PeeDeBelemnite}}$), can be used to gather information about the metabolic pathway(s) used by these organisms. For autotrophs, $\delta^{13}\text{C}$ values can also be used to ascertain the rate of CO$_2$ exchange between the cell and the environment, to determine the source of the CO$_2$, and to elucidate environmental factors influencing carbon fixation (17). Indeed, the broad range of $\delta^{13}\text{C}$ values collected for autotrophic microorganisms ($\delta^{13}\text{C} = -8\%$ to $-35\%$) has been utilized as evidence for the interplay of these factors. However, in order for the influence of the above factors to be rigorously evaluated, the baseline fractionation by the carboxylase(s) responsible for carbon fixation must be measured (8, 11, 13, 17, 33, 43).
Autotrophs that utilize the Calvin-Benson-Bassham cycle (CBB autotrophs), have a diversity of RubisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzymes that catalyze the carboxylation of ribulose 1,5-bisphosphate (RuBP) to form two molecules of phosphoglyceric acid (PGA; 47). RubisCO exists in six different forms that are catalytically active as carboxylases (IA, IB, IC, ID, II, and III; Figure 3). Form I RubisCO is present in cyanobacteria (IA, IB), some proteobacteria (IA, IC), most chloroplasts (IB, ID) (48), and the firmicute *Sulfobacillus acidophilus* (IC/ID) (3).

Catalytically active form I RubisCO consists of eight large subunits (encoded by the *cbbL* gene) and eight small subunits (encoded by the *cbbS* gene). Form II is found in proteobacteria and some dinoflagellates, and form III is present in some archaea (48). Both form II and III enzymes consist of a single type of subunit, evolutionarily related to the large subunits of form I RubisCO (48).

Given the divergent forms of RubisCO, it is not surprising that RubisCO enzymes discriminate against \(^{13}\)CO\(_2\) to different degrees. Isotope discrimination \((\varepsilon = \{\frac{[^{12}k]}{[^{13}k]} - 1\} \times 1000; \ [^{12}k] \text{ and } [^{13}k] = \text{rates of } ^{12}\text{C and } ^{13}\text{C fixation}\) (15) has been measured in a limited number of form IA, IB, and II enzymes, and ranges from 18 to 29‰ (44, 15, 36, 37, 38, 46). Since the \(\varepsilon\) value is roughly equal to the difference between the \(\delta^{13}\text{C}\) of the CO\(_2\) source from which the RubisCO is drawing (intracellular CO\(_2\)) and the \(\delta^{13}\text{C}\) of the CO\(_2\) that it fixes (15, 44), heterogeneity in RubisCO \(\varepsilon\) values is likely to be responsible for at least 10‰ of the \(\delta^{13}\text{C}\) scatter observed in CBB autotrophs. Prior to this study, it was impossible to predict whether form IC RubisCO enzymes would have \(\varepsilon\) values similar to those measured for form IA, IB, & II RubisCOs.
Our long-term objective is to sample the full phylogenetic breadth of RubisCO enzymes and be able to constrain the $\delta^{13}$C values expected for CBB autotrophs from ancient and contemporary ecosystems. Isotope discrimination by form IC RubisCO has not been measured, despite the presence of this enzyme in many proteobacteria of ecological interest, including marine manganese-oxidizing bacteria (5, 32), some nitrifying and nitrogen-fixing bacteria, and soil microorganisms like the extremely metabolically versatile bacterium, *Rhodobacter sphaeroides* (48; Fig.3).

*Rhodobacter sphaeroides* is an $\alpha$-proteobacterium capable of nonoxygenic photolithoautotrophic growth and photoheterotrophic growth (35). This organism has two RubisCO enzymes: a form IC RubisCO and a form II RubisCO (12). In this organism, the two forms of RubisCO are differentially expressed in response to a variety of growth conditions, e.g. CO$_2$ concentration (10). In order to be able to detect the isotope signature of organisms using form IC RubisCO for carbon fixation, we measured the $\varepsilon$ value for *R. sphaeroides* RubisCO using the high-precision substrate depletion method (15, 36, 43).
Rhodobacter sphaeroides ATCC17025
Stappia aggregata IAM12614
Paracoccus denitrificans PD1222
Xanthobacter autotrophicus Py2
Xanthobacter autotrophicus
Methylibium petroleiphilum PM1
Ralstonia eutropha H16
Ralstonia eutropha
Burkholderia xenovorans LB400
Burkholderia phymatum STM815
Bradyrhizobium japonicum USDA110
Bradyrhizobium japonicum
Oligotropha carboxidovorans
Bradyrhizobium sp ISTAI1
Nitrobacter hamburgensis X14
Rhodopseudomonas palustris CGA009
Rhodopseudomonas palustris BisB18
Aurantimonas sp SI859A1
Mnoxidizing bacterium SI859A1
Roseovarius sp HTCC2601
Acidiphilium cryptum JF5
Nitrosospira sp 40KI
Emiliania huxleyi
Porphyridium aerugineum
Phaeodactylum tricornutum
Cylindrotheca sp
Thalassiosira pseudonana
Thalassiosira nordenskioeldii
Sulfobacillus acidophilus
Nitrobacter winogradskyi
Nitrosomonas sp ENI11
Solemya velum
Prochlorococcus marinus MIT 9313
Synechococcus sp WH 8102
Chromatium vinosum
Spinacia oleracea
Trichodesmium erythraeum
Nostoc punctiforme
Synechococcus elongatus PCC 6301
Archaeoglobus fulgidus
Methanococcus jannaschii
Methanosarcina acetivorans
Thiobacillus denitrificans
Thiomicrospira crunogena
Rhodobacter capsulatus
Rhodobacter sphaeroides II
Figure 3. Minimum evolution tree of RubisCO large subunit (cbbL) nucleotide sequences. The nucleotide sequences obtained from GenBank were translated to amino acid sequences and aligned based on the amino acid sequences using BIOEDIT (16). The alignments were examined to make certain that active sites and other conserved regions were properly aligned. Nucleotide sequences were used to assemble a phylogenetic tree with MEGA 3.1 software, using the Kimura 2-parameter nucleotide model with 1000 replicates for calculating bootstrap values (24).

Experimental Procedures

Form IC enzyme was cloned from *Rhodobacter sphaeroides*, expressed in *Escherichia coli*, and purified using standard HPLC protocols (25, 18). The purity of the enzyme was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue (25).

RubisCO ε values were determined by using the substrate depletion method, in which a buffered solution of RubisCO, carbonic anhydrase (CA), ribulose 1,5-bisphosphate (RuBP), and dissolved inorganic carbon is sealed in a gastight syringe (44). The ε value is calculated from the changes in the δ^{13}C value of the dissolved inorganic carbon pool as it is consumed by RubisCO (44). RuBP was synthesized enzymatically to minimize the concentration of inhibitory isomers present in commercially-available RuBP (44, 46). Fresh RuBP was stored at –70°C and used within one week of synthesis.

Form IC RubisCO was prepared for the reaction by desalting it into RubisCO buffer (25 mM MgCl₂, 10 mM dithiothreitol, 50 mM Bicine, 5 mM NaHCO₃, pH 7.5), using PD-10 columns (Amersham Biosciences, NJ). 20 mL of reaction buffer (RubisCO buffer without NaHCO₃) was sparged with N₂ and 1 mg of bovine carbonic anhydrase (CA) was added. This solution was filter-sterilized and loaded into a glass gastight syringe sealed with a septum (44). Filter-sterilized NaHCO₃ (final concentration of 5
mM) and RubisCO (~1 mg/mL) were added to the reaction syringe and activated for 10-15 minutes. The reaction was started by injecting fresh RuBP (~5 mM) into the syringe, and was maintained at 25°C.

Reaction progress was monitored by removing samples and injecting them into a gas chromatograph (9) to measure the dissolved inorganic carbon concentration (DIC, = \( \text{CO}_2 + \text{HCO}_3^- + \text{CO}_3^{2-} \)). Over the time course of the reaction, samples were removed from the reaction syringe, acidified with 43% phosphoric acid, and injected into a vacuum line to cryodistill the DIC (44). The cryodistilled DIC samples were sent to the Boston University Stable Isotope Facility for measurement of the \( \delta^{13} \text{C} \) of the DIC.

\( \varepsilon \) values were derived from the DIC concentrations and the \( \delta^{13} \text{C} \) values of the DIC as calculated in Scott et al. (45). A modified version of the Rayleigh distillation equation was used:

\[
\ln(R_{\text{DIC}}) = \{(1/\alpha C) -1\} \times \ln[DIC] + \ln\{(R_{\text{DIC}0}/[DIC]_0) (1/\alpha C - 1)\}
\]

where

\( R_{\text{DIC}} = ^{13}C/^{12}C \) of DIC at a particular timepoint

\( R_{\text{DIC}0} = R_{\text{DIC}} \) at the first timepoint

\( [DIC] = \) concentration of DIC at a particular timepoint

\( [DIC]_0 = [DIC] \) at the first timepoint

\( C = R_{\text{HCO}_3}/R_{\text{CO}_2} \) from Mook et al., (30)

\( \alpha = k^{12}/k^{13} = \) kinetic isotope effect for \( \text{CO}_2 \)

The \( \varepsilon \) values were calculated from the slope of this line \( (\varepsilon = (\alpha - 1) \times 1000) \), and data from multiple runs were averaged using Pitman Estimators (45).
Results

As expected, carbon fixation by form IC RubisCO of *R. sphaeroides* results in isotopic enrichment of the remaining dissolved inorganic carbon (Figure 4, Figure 5).

The $\varepsilon$ value for this enzyme is 22.9‰ with a 95% confidence interval of 21.4-24.7‰ (Table 2). *Rhodobacter sphaeroides* RubisCO is less isotopically selective than spinach RubisCO ($\varepsilon = 27.5\%$ when incubated under identical conditions) (2).

![Diagram](image_url)

Figure 4. Changes in the concentration (♦) and $\delta^{13}$C (■) of dissolved inorganic carbon (DIC) vs. time for *Rhodobacter sphaeroides* RubisCO sealed in a sterile, gastight syringe in buffer with ribulose 1,5-bisphosphate and carbonic anhydrase (see Experimental Procedures for details).
Figure 5. Natural log-transformed isotope ratios (R = $^{13}$C/$^{12}$C) and concentrations of dissolved inorganic carbon (DIC) during carbon fixation by form IC RubisCO. Results from nine independent experiments are shown, and each is depicted with a different symbol. The initial isotope ratio and concentration of DIC varied slightly between experiments; for clarity, data from all experiments have been normalized to have the same initial DIC concentration and isotope ratio.

Table 2. RubisCO $\varepsilon$ values determined from nine independent experiments with one enzyme preparation of the *R. sphaeroides* enzyme

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>$\varepsilon$ value (‰)</th>
<th># of Timepoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29.6</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>24.4</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>24.1</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>21.0</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>22.7</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>24.3</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>24.7</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>22.6</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>24.1</td>
<td>7</td>
</tr>
</tbody>
</table>
Discussion

This is the first determination of an $\varepsilon$ value from a Form IC RubisCO, and it falls within the range of $\varepsilon$ values measured for other RubisCO enzymes (Table 3). Its $\varepsilon$ value is statistically indistinguishable from form IA enzymes, form IB from the cyanobacterium *Anacystis nidulans*, as well as from form II RubisCO from *Rhodospirillum rubrum*. However, *R. sphaeroides* RubisCO has an $\varepsilon$ value significantly higher than that of the form II RubisCO from the gammaproteobacterial endosymbiont of the hydrothermal vent tubeworm, *Riftia pachyptila*, and significantly smaller than the $\varepsilon$ value of spinach RubisCO (Table 3).

Table 3. $\varepsilon$ values from form I and II RubisCO enzymes

<table>
<thead>
<tr>
<th>Species</th>
<th>Form of RubisCO</th>
<th>$\varepsilon$ value (%)</th>
<th>{95% C.I.}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Solemya velum</em> symbiont</td>
<td>IA</td>
<td>24.5</td>
<td></td>
</tr>
<tr>
<td><em>Prochlorococcus marinus</em> MIT 9313</td>
<td>IA</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td><em>Spinacia oleracea</em></td>
<td>IB</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td><em>Anacystis nidulans</em></td>
<td>IB</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>IC</td>
<td>22.9</td>
<td>{21.4-24.7}</td>
</tr>
<tr>
<td><em>Riftia pachyptila</em> symbiont</td>
<td>II</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>II</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

The $\delta^{13}$C value of *R. sphaeroides* biomass, when it fixes carbon with form IC RubisCO, can be predicted from the RubisCO $\varepsilon$ value. Organisms that fix carbon with IC enzymes, should they all fractionate similarly to the *R. sphaeroides* enzyme, are likely to
have biomass $\delta^{13}C$ values similar to other CBB autotrophs whose RubisCO $\varepsilon$ values have been determined. When an organisms’ whole-cell isotopic discrimination ($\delta^{13}C_{\text{CO}_2} - \delta^{13}C_{\text{biomass}}$) is plotted against its RubisCO $\varepsilon$ value, it is apparent that organisms fixing carbon via RubisCOs with larger $\varepsilon$ values fractionate carbon to a greater extent (Table 4, Figure 6). Since the *R. sphaeroides* RubisCO has an $\varepsilon$ value similar to the enzyme from *R. rubrum*, it is likely that whole-cell discrimination by *R. sphaeroides* is comparable to that observed in *R. rubrum* when grown autotrophically. Indeed, biomass $\delta^{13}C$ values for *R. sphaeroides* can be predicted from the *R. rubrum* and *R. sphaeroides* $\varepsilon$ values, as well as *R. rubrum* whole-cell isotope discrimination:

$$\varepsilon_{R \text{rubrum}} = 22\%$$

$$\varepsilon_{R \text{sphaeroides}} = 22.9\%$$

For *R. rubrum*, $\delta^{13}C_{\text{CO}_2} - \delta^{13}C_{\text{biomass}} = 12.3\%$

Since $\varepsilon_{R \text{sphaeroides}} - \varepsilon_{R \text{rubrum}} = 0.9\%$,

For *R. sphaeroides*, predicted $\varepsilon_{\text{biomass}} = \delta^{13}C_{\text{CO}_2} - \delta^{13}C_{\text{biomass}} \approx 12.3 + 0.9$

$$= 13.2\%$$

If $\delta^{13}C_{\text{CO}_2} \approx -8\%$, then $\delta^{13}C_{R \text{sphaeroides}} \approx -8\% - 13.2\% = -21.2\%$ 

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Table 4. $\delta^{13}$C$_{\text{CO}_2}$ and $\delta^{13}$C$_{\text{biomass}}$ values from autotrophic organisms whose RubisCO $\varepsilon$ values have been determined

<table>
<thead>
<tr>
<th>Species</th>
<th>$\delta^{13}$C$_{\text{CO}_2}$ (‰)</th>
<th>$\delta^{13}$C$_{\text{biomass}}$ (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Riftia pachyptila</em> symbiont</td>
<td>-6.6 to -7.8</td>
<td>-9 to -16</td>
</tr>
<tr>
<td><em>Solemya velum</em> symbiont</td>
<td>-14 to -18</td>
<td>-30 to -35</td>
</tr>
<tr>
<td>C3 plants (Spinach)</td>
<td>-8</td>
<td>-22 to -30</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>-11.3</td>
<td>-23.6</td>
</tr>
</tbody>
</table>

Figure 6. Whole-organism isotopic discrimination ($\delta^{13}$C$_{\text{CO}_2}$ - $\delta^{13}$C$_{\text{biomass}}$) versus enzymatic isotopic discrimination (RubisCO $\varepsilon$ values). The predicted $\varepsilon_{\text{biomass}}$ value for *Rhodobacter sphaeroides* assumes whole-cell fractionation similar to *R. rubrum* (see Discussion for details), and the predicted range (whiskers on graph) assumes changes in whole-organism isotopic discrimination similar to what has been observed in other organisms in response to changes in CO$_2$ supply and/or demand.
A biomass $\delta^{13}C$ value of -21.2‰ would place *R. sphaeroides* and other form IC autotrophs within the range of $\delta^{13}C$ values observed for other CBB autotrophs, but would allow them to be distinguished from organisms using alternative carbon fixation pathways. Their $\delta^{13}C$ values should be more negative than what is expected for organisms using the rTCA or 3-hydroxypropionate cycles for carbon fixation, but not the acetyl-CoA pathway (17; Figure 7).

![Biomass $\delta^{13}C$ (‰)](image)

Figure 7. $\delta^{13}C$ values of atmospheric CO$_2$ and biomass from autotrophic organisms using the 3-hydroxypropionate pathway (3-HPP), the reverse citric acid cycle (rTCA), the acetyl-CoA pathway, and the C3-Calvin Benson Bassham cycle (C3-CBB).

Future work with Form IC RubisCO will involve sampling the full phylogenetic breadth of Form IC RubisCOs to determine whether all Form ICs have $\varepsilon$ values that fall within the range observed for other RubisCO $\varepsilon$ values. In order to sample the full phylogenetic spectrum of this group, $\varepsilon$ values could be measured for RubisCOs from, for example, *Burkholderia xenovorans* LB400 and *Roseovarius sp.* HTCC 2601, as these two enzymes are present in IC clades distinct from the clade containing *R. sphaeroides*.
RubisCO (Figure 3). *B. xenovorans* LB400 is an aerobic soil microbe capable of degrading polychlorinated biphenyls (7). *Burkholderia sp.* have a great number of carbon metabolism genes that indicate the presence of different pathways for assimilating carbon, giving this strain an edge, ecologically (7). *Roseovarius sp.* HTCC 2601 is an aerobic, alphaproteobacterial isolate found in seawater collected from the Sargasso Sea, and is a member of the *Roseobacter* clade, which is important in the marine sulfur cycle (26, 51). This clade is responsible for degrading dimethylsulfoniopropionate (DMSP) to methanethiol (51).

It would also be of great interest to measure the ε value of RubisCO from the firmicute, *Sulfobacillus acidophilus*. *S. acidophilus* is most peculiar because it is an outlier from the Form IC and ID clades (Figure 3). *Sulfobacillus* species oxidize mineral sulfides and prefer environments with a high concentration of CO₂, and their use of this RubisCO substrate reveals much about the biogeochemical function of these acidophiles (3). Elucidating the degree of isotopic discrimination by form IC enzymes, broadly sampled, would make it possible to use stable carbon isotope analyses to learn more about the role form IC RubisCOs play in the environment and the global carbon cycle.
Chapter Three

Conclusion

*Rhodobacter sphaeroides* IC RubisCO has an \( \varepsilon \) value of 22.9‰, which is within the range measured for other RubisCOs. This implies that some CBB autotrophs using form IC enzymes can be predicted to have \( \delta^{13} \)C values similar to those previously measured in CBB autotrophs. Preliminary experiments have been conducted with form IC RubisCO from *Ralstonia eutropha*, and this enzyme appears to have an \( \varepsilon \) value of 26.6‰ (see Appendix table A-1), though replicate experiments are necessary to verify this measurement.

Experiments with Form ID RubisCO

\( \varepsilon \) values have also recently been collected for form ID RubisCOs from the phytoplankton species, *Skeletonema costatum* and *Emiliania huxleyi*. The ID RubisCOs have \( \varepsilon \) values that are lower than the \( \varepsilon \) value from *R. sphaeroides* RubisCO (\( \varepsilon = 11.1\% \) for *E. huxleyi*; \( \varepsilon = 18.6\% \) for *S. costatum*; see appendix). The diatom, *S. costatum*, and coccolithophore, *E. huxleyi*, are a major part of the phytoplankton community and contribute substantially to primary productivity in the oceans. The small \( \varepsilon \) values reveal that their ID RubisCOs are less isotopically selective for \(^{13}\)CO\(_2\) compared to other RubisCO enzymes. The \( \varepsilon \) values also provide a mechanism for the levels of isotopic enrichment observed in these organisms, and marine organic carbon in general.
It is apparent that IC/ID RubisCOs have a broad range of $\varepsilon$ values (11.1\% to 22.9\%). Currently, it is impossible to predict $\varepsilon$ values for RubisCO enzymes based on primary, secondary, tertiary, or quaternary structure. At this point, one must measure them, in the hope that at some point it will be possible to correlate $\varepsilon$ values with primary structure or other features of these enzymes.
References


Appendices
Appendix A: Table of Rubisco $\varepsilon$ values from *Ralstonia eutropha*

Table A-1. Rubisco $\varepsilon$ values determined from two independent experiments with the IC enzyme from *R. eutropha*

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>$\varepsilon$ value (%)</th>
<th># of Timepoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.1</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>26.2</td>
<td>8</td>
</tr>
</tbody>
</table>

Average $\varepsilon$ value = 26.6 ‰
Appendix B: Manuscript on Form ID RubisCO $\varepsilon$ value from *Emiliania huxleyi*

**A New Low for RubisCO**

(To be submitted to Science)

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**One-sentence summary**

The first high-precision measurement of stable carbon isotopic discrimination by form ID RubisCO, responsible for a substantial portion of marine carbon fixation, is reported here and indicates that this enzyme has a shockingly small fractionation factor, providing a mechanistic explanation for $^{13}$C-enrichment in marine organic carbon.
The $^{13}$C content of carbon is used to identify sources and sinks in the global carbon cycle. One enduring mystery is why the $^{13}$C content of marine organic carbon is relatively high. We tested the hypothesis that marine organic carbon $^{13}$C enrichment is due to reduced isotopic discrimination during carbon fixation by form ID RubisCOs (ribulose 1,5-bisphosphate carboxylase/oxygenase), found in a substantial portion of marine algae responsible for oceanic carbon fixation. Here, form ID RubisCO from coccolithophore *Emiliania huxleyi* discriminated substantially less against $^{13}$CO$_2$ than other RubisCO enzymes ($\varepsilon=11.1\%$). Reduced discrimination by form ID RubisCO may be a major factor dictating the high $^{13}$C content of marine organic carbon, necessitating re-evaluation of how biological $\delta^{13}$C values are integrated into global primary productivity models.
Stable carbon isotope analyses are critical to the identification and modeling of global carbon cycle sources and sinks. One key sink is marine carbon fixation (1), and phytoplankton δ\textsuperscript{13}C values (δ\textsuperscript{13}C = [(R_{sample}/R_{std})-1] x 1000, where R=\textsuperscript{13}C/\textsuperscript{12}C) have been used to untangle trophic links and to estimate \textit{in situ} growth rates (2). However, phytoplankton δ\textsuperscript{13}C values vary widely (-16 to -36‰; (2), and the factors responsible for this variation, as well as their relatively \textsuperscript{13}C-enriched δ\textsuperscript{13}C values compared to terrestrial C3 plants (3), are poorly understood. Given the substantial role that phytoplankton play in the global carbon cycle, this conceptual gap compromises not only the interpretation of phytoplankton δ\textsuperscript{13}C values, but also introduces uncertainty into carbon cycle modeling (4).

While some variation in phytoplankton δ\textsuperscript{13}C values is clearly due to carbon concentrating mechanisms, C4 pathways, and diffusive limitation (5), the effect of isotopic discrimination by different forms of RubisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase), the CO\textsubscript{2} fixing enzyme of the Calvin-Benson-Bassham cycle, has not been widely considered. Instead, δ\textsuperscript{13}C analyses of oceanic primary productivity typically assume that isotopic discrimination by phytoplankton RubisCO is similar in extent to that of spinach RubisCO (e.g., (6-10). This assumption is untenable, however, as prior studies have shown that different forms of RubisCO discriminate to a greater or lesser extent against \textsuperscript{13}C (4, 11-16). There are three known forms of RubisCO (I, II, and III), which share as little as 25% in amino acid sequence identity, vary widely in $K_{CO_2}$
Appendix B (Continued)

and $V_{\text{max}}$ values, and display dramatic differences in tertiary and quaternary structure (17, 18). Form I enzymes are further subdivided into four subforms (IA – ID), whose amino acid sequences can differ by as much as 40% (17, 18). Marine algae fix carbon using at least four different RubisCO forms: IA in marine *Synechococcus* and *Prochlorococcus* spp., IB in algae with green plastids (and terrestrial plants), ID in ‘non-green’ algae (coccolithophores, diatoms, rhodophytes, and some dinoflagellates), and II in peridinin-containing dinoflagellates (19).

Isotopic discrimination, expressed as $\varepsilon$ values ($\varepsilon = (R_{\text{CO}_2}/R_{\text{fixed}} - 1) \times 1000$), have been measured for only three forms of RubisCO using high-precision methods (Table B-1). While values vary considerably, RubisCO forms IA and B ($\varepsilon = 22 - 29\%$) discriminate to a greater extent than form II ($\varepsilon = 18 - 22\%$) (4, 11-16). Given the prevalence of form ID RubisCO in dominant marine primary producers, many of which are used as model organisms for study in culture, determination of isotope discrimination by this enzyme is critical for the interpretation of environmental and culture $\delta^{13}C$ values, and in turn for global modeling efforts, paleo-oceanography, and other studies using $\delta^{13}C$ values. Here, we characterized and determined the $\varepsilon$ value of form ID RubisCO from a model marine alga, the coccolithophore *Emiliania huxleyi* CCMP 374.

Coccolithophores, whose massive blooms in the North Atlantic and Pacific oceans are visible from space (20, 21), fix CO$_2$ via form ID RubisCO (22) and play a major role as primary producers and in jettisoning carbon to the ocean floor (23). The
minute calcium carbonate plates (coccoliths) that cover coccolithophore cells enhance inorganic and organic carbon export from surface waters by ballasting fecal pellets into which they are packed (24, 25). In culture, the biomass δ^{13}C values of *Emiliania huxleyi* vary widely, from -9.71 to -38.6‰ (6, 7, 10, 26, 27). While some of this heterogeneity is likely due to variation in study strains and growth conditions, these δ^{13}C values cannot be rigorously interpreted without a RubisCO ε value.

Table B-1. ε values of different RubisCO forms, $\epsilon = \frac{R_{CO2}}{R_{fixed}} - 1$ x 1000

<table>
<thead>
<tr>
<th>Form</th>
<th>Organisms</th>
<th>ε value (‰)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Marine cyanobacteria and gammaproteobacteria</td>
<td>24 – 22.4</td>
<td>(4, 16)</td>
</tr>
<tr>
<td>IB</td>
<td>Terrestrial plants and freshwater cyanobacteria</td>
<td>21 – 30.3</td>
<td>(11, 13, 15)</td>
</tr>
<tr>
<td>ID</td>
<td>Marine coccolithophore <em>Emiliania huxleyi</em></td>
<td>11.1</td>
<td>This study</td>
</tr>
<tr>
<td>II</td>
<td>Alpha- and gammaproteobacteria</td>
<td>17.8 – 23.0</td>
<td>(12-15)</td>
</tr>
</tbody>
</table>

**RubisCO K_{CO2} and ε values**

In order to obtain sufficient *Emiliania huxleyi* Form ID RubisCO for determination of kinetic parameters and isotope fractionation (~20 mg per ε value experiment), 100 L of *E. huxleyi* CCMP 374 cultures were harvested and RubisCO was partially purified from sonicated cells via NH₄SO₄ precipitation (28). Carboxylase activity was verified to be
Appendix B (Continued)

due exclusively to RubisCO based on incubations in the presence and absence of the substrate ribulose 1,5-bisphosphate (RuBP; (28). To characterize *E. huxleyi* RubisCO activity, its Michaelis-Menten constants ($K_{CO2}$ and $V_{max}$) were measured radiometrically as in (29). The $\varepsilon$ value of *E. huxleyi* RubisCO was measured by the high-precision substrate depletion method, in which the concentration and isotopic composition of dissolved inorganic carbon are measured as they are consumed by RubisCO (4, 16, 28). The $\varepsilon$ value was calculated using a modified version of the Rayleigh distillation equation and Pitman estimators to calculate the least-biased average (28, 30).

*E. huxleyi* RubisCO Michaelis-Menten constants ($K_{CO2} = 111 \pm 40 \, \mu M; \ V_{max} = 146 \pm/\ 102 \, nmol/min \times mg$, calculated from five independent experiments) were quite different than those measured for other form ID enzymes from diatoms and rhodophytes ($K_{CO2} = 5 – 60 \, \mu M; \ V_{max} = 670 – 1670 \, nmol/min \times mg$; (31, 32). The larger $K_{CO2}$ value for the *Emiliania huxleyi* RubisCO may necessitate a carbon concentrating mechanism with active transport of dissolved inorganic carbon into the cells, since the concentration of CO$_2$ in seawater is only ~20 $\mu M$ (33). The lower $V_{max}$ value reflects the labile nature of this enzyme.

*E. huxleyi* form ID RubisCO had an astonishingly low $\varepsilon$ value of 11.1‰ (95% CI: 9.8 - 12.6‰; Figure B-1), substantially smaller than any other RubisCO $\varepsilon$ measured to date (Table B-1). $\varepsilon$ values from replicate experiments were very consistent, falling within a 3.1‰ range (Figure B-1). Spinach form IB RubisCO, used as a control and
incubated under identical conditions to those used for the *E. huxleyi* enzyme, had an ε value of 27.5‰ (95% CI: 24.0 – 30.9‰; Figure B-1), similar to previously reported values (4, 11, 13). This is the first high-precision ε value to be measured for any eukaryotic algae of ecosystem-level importance; the peculiarity of its ε value highlights how little we know about the RubisCO enzymes responsible for marine primary productivity.

Figure B-1. Isotope fractionation by *E. huxleyi* RubisCO. R is the isotope ratio ($^{13}$C/$^{12}$C) of dissolved inorganic carbon (DIC) and [DIC] is its concentration. Solid symbols (▲, ♦, ■, and ●) correspond to independent incubations of *E. huxleyi* RubisCO, with ε = 10.8‰, 11.1‰, 11.8‰, and 13.9‰, respectively. Open symbols (□, ○, and Δ) correspond to independent incubations of spinach RubisCO, with ε = 28.3‰, 28.2‰, and 27.2‰, respectively.
Appendix B (Continued)

Implications for interpreting $\delta^{13}C$ values from environmental samples and phytoplankton cultures

This remarkably small RubisCO $\varepsilon$ value (11.1‰) suggests a novel explanation for the isotopically enriched $\delta^{13}C$ values typically observed for marine phytoplankton and the food webs they support. An enzymatic basis for these values must now be considered. Indeed, phytoplankton collected from environmental samples demonstrate whole-cell isotope discrimination values ($\varepsilon_p = \frac{R_{CO2}}{R_{biomass}} - 1) \times 1000$) that span this RubisCO $\varepsilon$ value. Samples collected from marine environments worldwide have values of $\varepsilon_p$ ranging from 7 to 19‰ (7, 34, 35). For environmental samples dominated by $E. huxleyi$, reported $\varepsilon_p$ values are 0-14‰ (calculated directly from biomass; (36) and 7-19‰ (calculated from $E. huxleyi$ alkanones, assuming a constant fractionation between alkanones and biomass; (7). Using the (incorrect) assumption of a RubisCO $\varepsilon$ value of 29‰, these $\varepsilon_p$ values suggest the isotope-enriching effects of carbon concentrating mechanisms or C4 pathways. However, given the small RubisCO $\varepsilon$ value measured here, it is not necessary to invoke these mechanisms to explain $^{13}C$-enriched biomass.

Similar to ocean samples, $\varepsilon_p$ values from $E. huxleyi$ culture studies are small and reasonably consistent with the measured RubisCO $\varepsilon$ value. In many of these studies, the $\delta^{13}C$ of dissolved inorganic carbon ($\delta^{13}C_{DIC}$), and not the $\delta^{13}C_{CO2}$, is reported. Therefore, to calculate $\varepsilon_p$ from these culture studies, the equilibrium isotope effect between dissolved CO$_2$ and HCO$_3^-$ ($=\varepsilon_{eq} = 1000 \times [\frac{R_{CO2}}{R_{HCO3^-}} - 1]$; = -10‰ at 15°C; (37) was
used to calculate the $\delta^{13}\text{C}_{\text{CO}_2}$ from $\delta^{13}\text{C}_{\text{DIC}}$. Based on these studies, $\varepsilon_p$ ranges from 2-18‰ for *Emiliania huxleyi* (6, 10, 38, 39). One potential factor that could be influencing the $\varepsilon_p$ value is the form of inorganic carbon transported, since there is an equilibrium isotope effect between HCO$_3^-$ and CO$_2$, which causes CO$_2$ to be isotopically depleted relative to HCO$_3^-$ (37). It is clear that *E. huxleyi* alters its pattern of inorganic carbon uptake in response to growth conditions (40). Perhaps smaller $\varepsilon_p$ values are the result of reliance on extracellular HCO$_3^-$, while larger $\varepsilon_p$ values may result from CO$_2$ utilization. Deciphering the form(s) of dissolved inorganic carbon taken up by this organism under different growth conditions is instrumental in developing a mechanistic understanding for the changes in dissolved inorganic carbon abundance and composition that accompany *E. huxleyi* blooms, which in turn determine whether these blooms are sources or sinks of atmospheric and dissolved CO$_2$.

The unexpectedly low $\varepsilon$ value of *E. huxleyi* RubisCO highlights the necessity of collecting RubisCO $\varepsilon$ values from other organisms, in order to uncover any phylogenetic patterns in isotope discrimination. At this point, given the small numbers of enzymes examined (2 form IA’s; 2 form IB’s; 1 form ID; 2 form II’s) it is irresponsible to suggest ‘typical’ $\varepsilon$ values for different forms of RubisCO, or even for RubisCO enzymes in general. Particularly with respect to the interpretation of marine $\delta^{13}\text{C}$ values, and to refine models of the global carbon cycle that rely on these $\delta^{13}\text{C}$ values, it is necessary to determine whether form ID RubisCO enzymes from other algae of ecosystem-level
importance (e.g., diatoms, rhodophytes) have low $\varepsilon$ values. Factors such as carbon concentrating mechanisms, inorganic carbon supply and demand, and $C_4$ pathways clearly exert an influence on the $\delta^{13}C$ values of algal biomass (38, 41-43), but more measurements of RubisCO $\varepsilon$ values are critical to discern their significance.
Appendix B (Continued)

References

Appendix B (Continued)

28. Materials and methods are available as supporting material on Science Online.
Appendix C: Manuscript on Form ID RubisCO $\varepsilon$ value from *Skeletonema costatum*

**Isotopically less selective RubisCO from the diatom *Skeletonema costatum***

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**Acknowledgments**

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Form ID ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is present in several major oceanic primary producers that grow in large blooms. These large blooms may be one of the causes of $^{13}$C enriched marine carbon because these organisms use an isotopically less selective form of RubisCO for carbon fixation. The purpose of this study is to determine the isotopic discrimination and $K_{CO2}$ of *Skeletonema costatum* RubisCO, and to compare its large subunit gene (*rbcL*) with form ID *rbcL* genes from other organisms. We found the isotopic discrimination of *S. costatum* RubisCO is 18.5‰ and its $K_{CO2}$ is 90 (+/- 20) μM. Comparatively, this form ID RubisCO is isotopically less selective than forms IA and IB RubisCOs (22-29‰), which are also present in some oceanic primary producers. However, the ID RubisCO from the coccolithophore, *Emiliana huxleyi* (11.1‰) is the most non-selective RubisCO measured. Since ID RubisCOs are less selective against $^{13}$C, the isotopic discrimination by form ID RubisCO must be considered when making carbon cycle models or biological food chain models using $^{13}$C values. Consequently, current carbon models could miscalculate global carbon fixation.
Appendix C (Continued)

Diatoms are major primary producers in the ocean and contribute at least a quarter of inorganic carbon fixed each year (10). Most diatoms live in pelagic marine and freshwater ecosystems, but can also be found at the water-sediment interface. In areas of nutrient upwelling, large blooms of diatoms quickly dominate the phytoplankton communities (18) because they contain high proportions of growth machinery that allows for exponential growth (1). Because of their larger cell size and abundance, diatoms form the base of bloom associated food webs. *Skeletonema costatum* is a common, global member of the plankton community in temperate areas, and therefore, is a good candidate for further investigation into diatom carbon fixation kinetics.

At least five forms of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) catalyze carbon fixation in the marine organisms. Form IB RubisCO is the most extensively studied because it is present in eukaryotic green chloroplasts from algae to land plants. Other forms of RubisCO, prevalent in marine organisms, include form ID RubisCO, which is present in eukaryotic organisms with non-green chloroplasts, such as coccolithophores, rhodophytes, and diatoms (31), and form IA, IC, and II RubisCO, which are present in diverse marine prokaryotes (4, 21, 30). Different forms of RubisCO are found in diverse intracellular environments, and hence have evolved varying structural and kinetic properties to fit their individual conditions (29). However, most carbon cycle models are based on properties associated with IB RubisCO, even though other forms of RubisCO are major contributors to carbon fixation, particularly in the marine environment.
Stable carbon isotope compositions ($\delta^{13}$C values) of phytoplankton biomass have been used to infer the physical and physiological factors influencing carbon fixation in the ocean (15). $\delta^{13}$C values are determined by measuring amounts of $^{13}$C and $^{12}$C in biomass and comparing the ratio to a limestone standard (19). The main influence of $\delta^{13}$C values measured in phytoplankton biomass is the selectivity of $^{13}$C by the RubisCO. However, physical factors, such as dissolved inorganic carbon (DIC) pool composition and nutrient availability, as well as physiological factors, such as growth rate and type of carbon concentrating mechanism (CCM) utilized, can also influence the $\delta^{13}$C value of marine phytoplankton (15). However, the effects of different factors on the $\delta^{13}$C biomass values are difficult to interpret without the initial baseline isotopic selectivity from RubisCO. The isotopic selectivity of very few RubisCOs have been measured to date; therefore, other forms of RubisCO need to be examined to be able to correctly identify factors which play a role in biomass composition.

Most biological models assume RubisCOs isotopic discrimination ($\varepsilon$ value; $= \frac{R_{CO2}}{R_{fixed}} - 1 \times 1000$) is $\approx 25\%$ (12). This model value is reasonable when using $\varepsilon$ values from terrestrial plants, cyanobacteria, and bacterial chemolithoautotrophs, with $\varepsilon$ values ranging from 18-29\% (14, 21, 22, 23, 26). However, the very isotopically non-selective ID RubisCO from the coccolithophore, *Emiliana huxleyi*, differs greatly from the model value ($\varepsilon = 11.8\%$) (2).
Appendix C (Continued)

If all ID RubisCO are isotopically non-selective, carbon models based on an isotopic discrimination of 25‰ will underestimate the amount of $^{13}$C cycled, therefore miscalculating total carbon fixation. Therefore, to determine if form ID RubisCOs have similar carbon fixation kinetic parameters, the kinetic isotopic effect (KIE) and the $K_{CO2}$ of *S. costatum* RubisCO was measured. *Skeletonema costatum* RubisCO large subunit gene ($rbcL$) was also sequenced and compared to other ID RubisCOs from various organisms.

**Methods**

**Cell Culture Methods**

*Skeletonema costatum* cultures were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP1332; West Boothbay Harbor, Maine). The cells were grown in f/2 medium with silica (13) at 18-22°C, under constant illumination. One L of f/2 media was inoculated with 5 mL of cell culture. After one week of growth, the 1 L culture was added to 4 L of fresh media for another week of growth with aeration.

**RubisCO purification from *S. costatum***

RubisCO was extracted and partially purified from frozen cell cultures using ammonium sulfate precipitation. For all purification steps, samples were kept at 4°C. Cells were harvested from the 5L cultures by centrifugation (5000g, 15min), flash-frozen in liquid nitrogen, and stored at -80°C. Cell pellets were resuspended in lysis buffer [20mmol L$^{-1}$ Tris pH 7.5, 10mmol L$^{-1}$ MgCl$_2$, 5mmol L$^{-1}$ NaHCO$_3$, 1mmol L$^{-1}$ EDTA,
and 1 mmol L$^{-1}$ dithiothreitol (DTT)] per gram of pellet, sonicated twice for 30 sec with glass beads, and centrifuged (5000g, 15 min). (NH$_4$)$_2$SO$_4$ was added to the supernatant to a final concentration of 30% saturation, and proteins were allowed to precipitate for 15 min. After centrifugation (5000g, 15 min), the pellet was discarded and the supernatant was brought to 50% (NH$_4$)$_2$SO$_4$ saturation. Proteins that precipitated at 30-50% (NH$_4$)$_2$SO$_4$ saturation were collected by centrifugation and dissolved in BBMD buffer (50 mmol L$^{-1}$ Bicine pH 7.5, 25 mmol L$^{-1}$ MgCl$_2$, 5 mmol L$^{-1}$ NaHCO$_3$, and 1 mmol L$^{-1}$ DTT). The partially purified proteins were desalted with an anion exchange column (HiPrep 26/10 desalting column; Amersham Biosciences, New Jersey, USA) equilibrated and eluted with BBMD buffer. The eluant was monitored at 280 nm and peaks that had an absorbance of 0.5 or higher were collected (<15 ml).

RubisCO activity assays

RubisCO activity in partially purified protein extracts was monitored by the incorporation of $^{14}$CO$_2$ into 3-phosphoglycerate (PGA). Purified RubisCO extracts were added to the assay buffer [50 mmol L$^{-1}$ Bicine, pH 8.0, 10 mmol L$^{-1}$ MgCl$_2$, 1 mmol L$^{-1}$ EDTA, 5 mmol L$^{-1}$ DTT, and 25 mmol L$^{-1}$ NaH$^{14}$CO$_3$ 55 mCi/mmol bicarbonate (MP Biomedicals, Irvine CA)] in a 1:2 ratio and pre-incubated for 15 min. Reactions were started by the addition of 1 mmol L$^{-1}$ RuBP (Sigma R0878, USA). At 3-30 sec intervals, one third of the sample was removed from the reaction mix and added to acetic acid to stop the reaction. After the samples were dried by sparging with air, scintillation cocktail (Fisher Scientific, USA) was added to the samples. Assays lacking RuBP were performed.
Appendix C (Continued)

as a control for background CO₂ fixation.

**Rubisco isotope discrimination by kinetic isotope effect (KIE)**

Using the partially purified protein from *S. costatum*, the kinetic isotope effect (KIE) of Rubisco was measured at pH 7.5 using the high-precision substrate depletion method (7, 26). Previous KIE were performed at pH 8.5 (14, 22), however, the optimal pH for partially purified *S. costatum* Rubisco is pH 7.5. To test the effects of the altered parameter on KIE’s, control experiments using spinach Rubisco were also conducted.

The reaction was prepared by sparging BBMD buffer with N₂ to minimize CO₂ and O₂ concentration, and 1mg/25mL of bovine erythrocyte carbonic anhydrase (CA; Sigma 3934, USA) was added to maintain DIC at chemical and isotopic equilibrium. Approximately 5mL of partially purified Rubisco was added to the BBMD buffer, filter sterilized (0.45µm), and loaded into a heat-sterilized, septum-sealed 25 mL glass gastight syringe with a stir bar. Ribulose 1,5-bisphosphate (RuBP), substrate for Rubisco, was enzymatically synthesized from ribose 5-phosphate using spinach phosphoriboisomerase (PRI; Sigma P9752-5KU) and purified phosphoribulokinase (PRK)(26). After allowing the Rubisco to pre-incubate for 15 min at 25°C, filter-sterilized (0.22 µm) RuBP (~100 mmoles) was injected into the gastight syringe to begin the reaction.

The concentration and δ¹³C value were measured as the CO₂ was consumed by the Rubisco reaction. Samples were removed at 8 time intervals from the reaction syringe based on the decrease of the DIC concentration. Triplicate samples were acidified in a gas tight syringe with 43% phosphoric acid (1:4 ratio) to terminate the reaction and
convert the DIC to CO₂. The [DIC] of the triplicate samples was measured using a gas chromatograph (HP/Agilent 5890A, USA) (7). The remaining sample was injected into gas-tight syringes with 43% phosphoric acid (1:1 ratio). Using a vacuum line, the CO₂ was cryodistilled from the sample and sent to Boston University Stable Carbon Isotope Laboratory (Robert Michener) to determine their δ¹³C values using a gas inlet mass spectrometer (7).

Five independent KIE’s were performed using partially purified *S. costatum* RubisCO and three independent reactions were completed using spinach RubisCO (10mg; Sigma R8000), for a positive control. The average ε values for the KIE’s were calculated using the Pitman estimator with a 95% confidence interval (25). To ensure alternate carboxylases were not fixing CO₂, KIE experiments with *S. costatum* RubisCO were also run without RuBP and without RuBP + 5 mmol L⁻¹ 3-PGA for negative controls (Sigma P8877, USA).

**KCO₂ measurements**

The KCO₂ and Vmax of *S. costatum* RubisCO were measured radiometrically (17, 24). The assay buffer (50 mM Bicine, pH 7.5, 30 mM MgCl₂, 0.4 mM RuBP, 1 mM DTT) was prepared with low CO₂ and O₂ concentrations as in Schwedock et al., (2004). Eight different incubations were conducted with DIC ranging from 0.2 to 14 mM in glass vials primed with stir bars and sealed under a N₂ headspace with gas-tight septa. Before the reaction was started, 25 mmol NaH¹⁴CO₃ (SA = 55 mCi/mmol bicarbonate) along with bovine erythrocyte carbonic anhydrase (40 μg/mL; Sigma C3934), to maintain CO₂
and HCO$_3$ in chemical equilibrium, was added to each vial. Partially purified *S. costatum* RubisCO in BBMD buffer that lacked RuBP was added to assay buffer, immediately before the reaction was started. To begin a reaction, RuBP was injected into a vial, and samples were removed with a gastight syringe at 1 min intervals over a 4 min time course. These samples were immediately injected into scintillation vials containing glacial acetic acid to remove all $^{14}$CO$_2$ and collect acid stable $^{14}$C. The samples were sparged with air and left for approximately 6 hours to ensure samples were completely dry before scintillation cocktail was added. The initial activity of each incubation was measured by injecting 10μl samples into scintillation cocktail containing phenylethylamine (sx10-1000 Fisher Scientific, USA) to trap the NaH$^{14}$CO$_3$. Both acid stable $^{14}$C samples and the initial activities were measured via scintillation counting. Five independent experiments were conducted, and carbon fixation followed the Michaelis-Menten response curve. $K_{CO2}$ and $V_{max}$ values for the five experiments were estimated from the carbon fixation rates using direct linear plots (9).

**Cloning and sequencing of the RubisCO large subunit gene (rbcL)**

DNA was purified from *S. costatum* cells using the CTAB method (11). Primers for the *S. costatum* *rbcL* gene were designed from a partial *rbcL* sequence (6) and purchased from Invitrogen (USA). Primers used to replicate the first half of the gene (1bp - 840 bp) include the forward primer (GGGTTACTGGGATGCTTCATACAC) and reverse primer (CCAACAGCTTTAGCATACTCAC). The primers used to replicate the second half of the gene (560bp - 1428bp) include forward primer

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Appendix C (Continued)

(GGAAGGTATTAACCGTGCATCAGC) and reverse primer
(TCTGTTTGCAGTTGGTGTTTCAGC). The two RubisCO gene sequences were
replicated with PCR, using the above primers, and the PCR product was ligated into the
pCR2.1 vector using TA Cloning Kit (Invitrogen, 45-0046; California, USA). The
plasmids with RubisCO gene inserts were transformed into One Shot TOP10 cells and
grown on plates with Luria broth agar with 100µg/ml ampicillin, 50 µg/ml kanamycin,
and spread with X-gal. Colonies which had the plasmid + insert were grown in liquid LB
overnight and the plasmids were purified using the QIAniprep Spin Miniprep Kit (Qiagen,
12125; California, USA). Three different purified plasmids for each of the two RubisCO
gene inserts were sent for sequencing (Macrogen, Maryland, USA). A consensus
sequence was made using repetitive nucleotides from the three sequenced plasmid inserts.

Phylogenetic Analysis

A phylogenetic analysis of the large subunit RubisCO gene (rbcL) from S.
costatum and other form ID rbcL genes from diverse organisms was accomplished using
a maximum parsimony tree with rbcL sequences obtained from NCBI- BLAST
nucleotide searches. Sequences were aligned and a maximum parsimony tree, with 1500
replicates, was constructed using MEGA 4.0 software (28).

Results

The five independent incubations of S. costatum RubisCO had ε values of 19.0‰,
18.6‰, 19.7‰, 15.9‰ and 20.2‰, respectively (Figure C-1). The average ε value
calculated using the Pitman estimator was 18.5‰ (95% CI: 17.0-19.9‰). Three spinach
Appendix C (Continued)

RubisCO incubations had \( \varepsilon \) values of 28.3\%, 28.2\%, and 27.2\%, with a Pitman average \( \varepsilon \) value of 27.5\% (95% CI: 24.0 – 30.9\%; Figure C-1) that was within range of previously published values. In order to further characterize \( S. \ costatum \) RubisCO activity, its \( K_{\text{CO}_2} \) was measured radiometrically in oxygen-free incubations. The \( K_{\text{CO}_2} \) and \( V_{\text{max}} \) were calculated from five independent experiments and found to be 90 (+/- 20) \( \mu \text{M} \) and 15.6 (+/- 5.8) nmol/min\(^*\) mg, respectively.

Figure C-1. Isotopic fractionation of dissolved inorganic carbon (DIC) as CO\(_2\) is consumed by \( S. \ costatum \) RubisCO and spinach RubisCO. The slope of the line is used to estimate the \( \varepsilon \) value for each organism.

Since partially purified extracts of \( S. \ costatum \) RubisCO were used in the KIE, controls for alternative carboxylase activity were run. KIE incubations with RuBP showed a decrease in [DIC] over time, which is consistent with \( S. \ costatum \) RubisCO catalyzing the carbon fixation in these assays (Figure C-2). Conversely, control experiments, which did not contain RuBP, showed no change of [DIC] in the reaction.
syringe. DIC also was constant in the RuBP-free incubations that contained 3-PGA. A more sensitive bicarbonate $^{14}$C activity assay performed on partially purified enzyme also showed carboxylation occurring only in the presence of RuBP. Therefore, RubisCO was the only active carboxylase in the partially purified extracts (Figure C-3).

Figure C-2. The consumption of DIC over time by *S. costatum* RubisCO. Incubations that included RuBP (solid symbols) were used to calculate the $\varepsilon$ value of *S. costatum* RubisCO. In absence of RuBP, (open symbols) no DIC was consumed even with 3- PGA included in the incubation.
Figure C-3. Radiometric assay of partially purified *S. costatum* RubisCO. Assays were conducted in the presence and absence of RuBP to confirm that all detectable carboxylase activity was due to RubisCO.

Since the isotopic discrimination of form ID RubisCOs differ, a phylogenetic analysis was completed to identify if the ID RubisCO *rbcL* genes are divergent. In the maximum parsimony tree, the *rbcL* genes formed clades, which included diatoms, coccolithophores, and rhodophytes (Figure C-4). Higher bootstrap values at the base of the clades differentiate the *rbcL* genes based on their phylogenetic groups. Furthermore, the rhodophyte *rbcL* genes group with the diatoms genes, while the coccolithophore *rbcL* genes formed a separate branch. The form IC *rbcL* genes formed a separate group away from the ID RubisCO genes.
Figure C-4. Phylogenetic analysis of selected RubisCO large subunit genes (rbcL). Sequences were aligned using the maximum parsimony method (MEGA software). The numbers next to the branches represents the number of times, of the 1500 bootstrap trials, that the associated taxa clustered together as shown.

Discussion

*Skeletonema costatum* RubisCO is less selective against $^{13}$C; consequently, providing a basis for enriched $^{13}$C marine carbon biomass. The carbon isotope compositions of *S. costatum* biomass correlate with the selectivity of its RubisCO, having $\delta^{13}$C values ranging from -16.8 to -27.6‰. (3, 16). The higher (-16.8‰) $\delta^{13}$C biomass values can be attributed primarily to carbon fixation of bicarbonate carbon by the less selective RubisCO. At marine pH values, bicarbonate (HCO$_3^-$) is the main form of DIC
in the ocean; however, RubisCO fixes DIC in the form of CO₂ (5). To cope with the paucity of CO₂ in the environment, some marine autotrophs will convert HCO₃⁻ into CO₂ using carbonic anhydrase (CA). Organisms, that use HCO₃⁻ as a main carbon source, have higher δ¹³C biomass values because HCO₃⁻ is isotopically enriched compared CO₂.

Even though *S. costatum* RubisCO is less isotopically selective, its biomass has less ¹³C than expected, with δ¹³C values as low as -27.6‰ (3). Low δ¹³C values could be attributed to *S. costatum* RubisCO using a carbon source depleted in ¹³C, such as isotopically depleted CO₂ (-8‰). Furthermore, carbon-concentrating mechanisms (CCM) could also play a role in low biomass δ¹³C values. In diatoms, the internal DIC concentration can reach 3.5 times higher than external DIC concentrations (20). This high internal concentration is not achievable strictly though CO₂ diffusion, which is indicative of a CCM; however, the exact CCM used by *S. costatum* is unknown. The CCM may consist of active transport of CO₂ or HCO₃⁻ into the cell as well as CA to carry out the interconversion of the two substrates (20). The CCM could actively transport less ¹³C into the cell for RubisCO to fix, also causing low δ¹³C biomass values in *S. costatum*. Once the exact mechanism of *S. costatum*’s CCM is elucidated, the isotopic effect associated with the CCM can be investigated.

Because of their bloom life style, organisms, such as diatoms and coccolithophores that have a less selective form ID RubisCO, can greatly alter the δ¹³C of oceanic biomass. In areas of nutrient upwelling, large blooms of phytoplankton can trigger the larval spawning of grazing zooplankton (27). Consuming organisms with ID
RubisCO causes the zooplankton biomass to become rich in $^{13}$C. Furthermore, diatoms are very efficient at exporting carbon to the sea floor because the cells are larger and heavier, which enhance sinking rates; therefore, increasing the amount of $^{13}$C in deep waters (8). An increase in oceanic biomass $\delta^{13}$C values can be caused by zooplankton grazing and increased vertical carbon flux of diatoms.

With rising atmospheric CO$_2$ values, it is essential to have accurate carbon cycle modeling. Although isotopic discrimination by different forms of RubisCO vary between organisms, most carbon models assume that all RubisCOs discriminate against $^{13}$CO$_2$ to the same degree (12). Form ID RubisCOs, including $S.\ costatum$ and $E.\ huxleyi$, are isotopically less selective than other forms of RubisCO (Table C-1), calling for changes in current modeling equations. However, rhodophytes, another major phylogenetic group with ID RubisCO, also need to be investigated to understand the full range of isotopic fractionation by ID RubisCO. Collecting isotopic fractionation data from RubisCO of diverse organisms will allow for more inclusive carbon models that will generate better estimations of global carbon fixation.
Table C-1. Epsilon values of different forms of RubisCO that have been measured with kinetic isotope experiments.

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<th>Form of RubisCO</th>
<th>ε value (‰)</th>
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<td><em>Solemya velum</em> symbiont IA</td>
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Appendix C (Continued)

References


Appendix D: Abstract from Publication of *Thiomicrospira crunogena* XCL-2 genome

**The Genome of Deep-Sea Vent Chemolithoautotroph *Thiomicrospira crunogena***

**XCL-2**

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Appendix D (Continued)

ABSTRACT

Presented here is the complete genome sequence of *Thiamicospira crunogena* XCL-2, representative of ubiquitous chemolithoautotrophic sulfur-oxidizing bacteria isolated from deep-sea hydrothermal vents. This gammaproteobacterium has a single chromosome (2,427,734 bp), and its genome illustrates many of the adaptations that have enabled it to thrive at vents globally. It has 14 methyl-accepting chemotaxis protein genes, including four that may assist in positioning it in the redoxcline. A relative abundance of CDSs encoding regulatory proteins likely control the expression of genes encoding carboxysomes, multiple dissolved inorganic nitrogen and phosphate transporters, as well as a phosphonate operon, which provide this species with a variety of options for acquiring these substrates from the environment. *T. crunogena* XCL-2 is unusual among obligate sulfur oxidizing bacteria in relying on the Sox system for the oxidation of reduced sulfur compounds. The genome has characteristics consistent with an obligately chemolithoautotrophic lifestyle, including few transporters predicted to have organic allocrits, and Calvin-Benson-Bassham cycle CDSs scattered throughout the genome.
Appendix D (Continued)

Figure D-1. PloS Biology Open Access License for use of the above abstract.
Appendix E: Abstract from Publication of *Sulfurimonas denitrificans* genome

**Genome of the Epsilonproteobacterial Chemolithoautotroph *Sulfurimonas denitrificans***

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ABSTRACT

Sulfur-oxidizing epsilonproteobacteria are common in a variety of sulfidogenic environments. These autotrophic and mixotrophic sulfur-oxidizing bacteria are believed to contribute substantially to the oxidative portion of the global sulfur cycle. In order to better understand the ecology and roles of sulfur-oxidizing epsilonproteobacteria, in particular the widespread genus *Sulfurimonas*, in biogeochemical cycles, the genome of *Sulfurimonas denitrificans* DSM1251 was sequenced. This genome has many features, including a larger size (2.2 Mbp), that suggest a greater degree of metabolic versatility or responsiveness to the environment than most of the other sequenced epsilonproteobacteria. A branched electron transport chain is apparent, with genes encoding complexes for the oxidation of hydrogen, reduced sulfur compounds, and formate, and the reduction of nitrate and oxygen. Genes are present for a complete, autotrophic reductive citric acid cycle. Many genes are present that could facilitate growth in the spatially and temporally heterogeneous sediment habitat from where *Sulfurimonas denitrificans* was originally isolated. Many resistance-nodulation-development-family transporter genes (11 total) are present, several of which are predicted to encode heavy metal efflux transporters. An elaborate arsenal of sensory and regulatory protein-encoding genes is in place, as well as genes necessary to prevent and respond to oxidative stress.
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