Nitrogen isotopic fractionation in the synthesis of photosynthetic pigments in *Rhodobacter capsulatus* and *Anabaena cylindrica*

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Abstract

Nitrogen isotopic discrimination was investigated for a purple nonsulfur bacterium, *Rhodobacter capsulatus*, during photoheterotrophic and photoautotrophic growth with *N₂* and ammonia, and compared with nitrogen assimilation in a cyanobacterium. In *R. capsulatus*, the $\Delta^{15}N$ values for biomass during growth with *N₂* fixation and ammonia assimilation, relative to the N-source, ranged from $-1.8$ to $-0.8\%$ and $-12.3$ to $-10.2\%$, respectively. In the cyanobacterium, *Anabaena cylindrica*, the $\Delta^{15}N$ value for biomass resulting from growth on molecular nitrogen was $-0.8$ to $-1.7\%$ relative to N-source. These values are similar to those observed for other microorganisms. Bacteriochlorophyll *a* in *R. capsulatus* was depleted in $^{15}N$ relative to biomass, whereas chlorophyll *a* in *A. cylindrica* was relatively enriched in $^{15}N$. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: N-isotopes; Purple nonsulfur bacteria; Cyanobacteria; Bacteriochlorophyll *a*; Chlorophyll *a*; Nitrogen fixation; Lipid biomarkers

1. Introduction

Anoxygenic photosynthetic bacteria are recognized as important contributors to microbial activity in various modern microenvironments such as those found in microbial mats (Castenholz et al., 1992; Paerl et al., 1996). These mats are important putative analogues to ancient stromatolites and as such are of particular significance in the history of the biosphere (Walter et al., 1992). The biological cycling of nitrogen is an important process in modern microbial mats, and was probably also important in ancient systems (Beaumont, 1994; Beaumont and Robert, 1998). Microbial mats exhibit diverse organisms at the taxonomic and metabolic level, and are most often dominated by some photosynthetic primary producer. High levels of nitrogen fixation are often recorded in such mats (Bautista and Paerl, 1985; Paerl et al., 1996), and the capacity to fix nitrogen enhances the ability of microbial mats to colonize nutrient-poor habitats (Bebout et al., 1994). However, the relative contribution of the different organisms in microbial mats to nitrogen fixation is less well assessed (Zehr et al., 1995). In seeking information to link knowledge of modern microbial mats to the ancient record, it is important to assess which organisms are able to fix nitrogen, what types of N-containing molecules they may synthesize and whether some vestige of information about ancient biochemistries may remain in the record of stable nitrogen isotopes.

Biological nitrogen fixation is a key process for supplying reduced nitrogen to natural environments. This process is carried out only by prokaryotes (archaeabacteria and bacteria including many cyanobacteria)
and involves the enzymatic reduction of N\textsubscript{2} to ammonium. The nitrogenase enzyme is composed of two proteins, dinitrogenase and dinitrogenase reductase. Dinitrogenase catalyzes the actual reduction of N\textsubscript{2} to NH\textsubscript{4}\textsuperscript{+} while dinitrogenase reductase is responsible only for reduction of the dinitrogenase enzyme. Although most N\textsubscript{2}-fixers possess a molybdenum-containing nitrogenase, it is becoming clear that a variety of genetically distinct alternative nitrogenases can be expressed in some organisms under certain environmental conditions (Bishop and Premakumar, 1992). Nitrogenase proteins are extremely O\textsubscript{2}-sensitive and N\textsubscript{2}-fixers exposed to highly aerobic conditions have devised various strategies to deal with this problem. In the cyanobacteria a common mechanism involves formation of a specialized cell called a heterocyst (Fay, 1992). Heterocysts lack photosystem II, the oxygen-evolving photosystem, and their thickened cell walls protect the nitrogenase enzymes by slowing the diffusion of oxygen into the cell. For microbes living in anoxic environments, the potential ill effects of oxygen are nil and no particular protective mechanisms to inhibit the diffusion of oxygen are necessary. It was of interest to understand the effects of these different strategies on nitrogen isotopic fractionation. We studied two N\textsubscript{2}-fixers, Rhodobacter capsulatus, an anoxygenic photosynthetic bacterium, and Anabaena cylindrica, an oxygenic photosynthetic cyanobacterium. Purple nonsulfur bacteria such as R. capsulatus are facultative (able to live in aerobic environments) and even fix nitrogen in the presence of low levels of oxygen (Madigan et al., 1984). R. capsulatus protects its nitrogenase through a high respiration rate which scavenges O\textsubscript{2}, thereby maintaining intracellular anaerobic conditions (Hochman and Burris, 1981). The cyanobacterium A. cylindrica, however, possesses heterocysts, and while nitrogen fixation is carried out in intracellular anaerobic conditions, the nitrogen must diffuse through a thick cell wall structure designed to limit gas diffusion.

Nitrogen fixation by cyanobacteria leads to a nitrogen isotopic composition averaging $\delta^{15}$N values of 0%o for cell biomass (Hoering and Ford, 1960; Delwiche and Steyn, 1970; Wada et al., 1975; Macko et al., 1987). Records of $\delta^{15}$N values around 0%o in aquatic sedimentary organic matter are usually interpreted to result from nitrogen fixation by cyanobacteria (Minagawa and Wada, 1986; Rau et al., 1987). However, few data are available for a variety of factors that might influence the nitrogen isotope record in ancient sedimentary material, such as the preservation of distinct N-containing biomarker molecules or the contributions to bulk $^{15}$N/$^{14}$N ratios of potentially more ancient metabolic groups of organisms like the anoxygenic photosynthetic bacteria. Geoporphyrins, the diagenetic products of bacteriochlorophylls and chlorophylls, are important geochemical fossils in more recent sediments. Even after loss of molecular structural information, these molecules have the potential to assist in the interpretation of the bulk N-isotope composition of even more ancient materials.

The isotopic consequences for production of biomass can occur as the result of the initial assimilation pathways or the subsequent allocation of elements during the biosynthesis of specific products (Hayes, 1993). This report presents a study of the nitrogen isotopic discrimination associated with nitrogen assimilation in an anoxygenic photosynthetic bacterium, R. capsulatus and compares one of its most significant findings, a major depletion in $^{15}$N associated with the biosynthesis of bacteriochlorophyll a, to biosynthesis of chlorophyll a in a cyanobacterium, Anabaena cylindrica. This is the first attempt to measure nitrogen isotopic fractionation associated with the growth of an anoxygenic photosynthetic bacterium. These findings indicate that $^{15}$N/$^{14}$N measurements may provide a valuable tool in assessing the activity of various metabolic groups in microbial ecosystems.

2. Materials and methods

2.1. Organisms and growth conditions

Bacteria were obtained from the American Type Culture Collection (ATCC), Manassas, VA. R. capsulatus (ATCC 23782) was grown in feed-batch cultures (open-system) anaerobically under phototrophic conditions either autotrophically with CO\textsubscript{2} and H\textsubscript{2} or heterotrophically with malate in the defined medium designated RCVBNP (Madigan et al., 1984). RCVBNP was supplemented with a slightly modified vitamin solution (0.4 ml l$^{-1}$) containing per 500 ml of deionized water: biotine, 50 mg; nicotinamide, 175 mg; thymine HCl, 150 mg; para-aminobenzoic acid, 100 mg; pyridoxine HCl, 50 mg; Ca pantothenate, 50 mg and cyanocobalamin, 25 mg. For growth with N\textsubscript{2}, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and EDTA were omitted from the medium, and Cu(NO\textsubscript{3})\textsubscript{2} was replaced by CuSO\textsubscript{4} in the trace element solution. Gas sparged cultures were grown in a 1-l water-jacketed flask maintained at 28°C by a circulating water bath and illuminated by a 30 W incandescent light source. Cultures were continuously sparged with a gas stream which for photoheterotrophic growth consisted of N\textsubscript{2} (25–30 ml/min) or for photoautotrophic growth consisted of a mixture (100 ml/min) of N\textsubscript{2}, H\textsubscript{2} and CO\textsubscript{2} (71:25:4), controlled by a rotometer. For growth with a fixed nitrogen source, cells were grown in filled and stoppered, 2-l flasks, stirred constantly and maintained between 28 and 30°C. A filter sterilized solution of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was supplied in large excess to the culture (≈900 ppm, i.e. 50 times the required quantity) to minimize closed-system isotope effects. Ammonium ion was quantitated in growth medium prior to inoculation and after harvesting cells using a DX-100 ion chromatograph.
calibrated for cation analysis. Flasks were fitted with a sampling port to allow intermittent removal of culture for turbidity measurements using a Klett-Summerson photometer fitted with either a blue (430), or a red (660) filter and cells harvested for isotopic analysis in the exponential phase.

*Anabaena cylindrica* (ATCC 29414) was grown as a feed-batch culture in BG-11 modified as a N-free medium (Castenholz, 1988). A 3-L water-jacketed flask maintained at 25°C was bubbled continuously with a gas stream of 0.5% CO₂ in nitrogen (100 ml min⁻¹) and illuminated by two 15-W daylight fluorescent tubes.

For all of the culture conditions described, starting inocula for the experimental growth flasks were grown under similar conditions (i.e. same N₂ or (NH₄)₂SO₄ source, media, temperaure, etc.) except inocula for sparged cultures were grown with 50-ml of medium, inoculated, then sealed in 250-ml flasks equipped with gassing ports and the headspace replaced with the appropriate gas mixture. Biomass of introduced inocula were minimal (< 5%) relative to recovered experimental biomass.

### 2.2. Nitrogenase assay

Nitrogenase activity was determined by measuring the reduction of the acetylene triple bond to ethylene (Hardy et al., 1968). Nitrogenase activity was determined in vivo by removing 5-ml culture samples intermittently throughout the exponential growth phase, sealing the cells in 26-ml serum vials equipped with two syringe needles for removing residual air. After 5 min of flushing with He, the vials were sealed, incubated at 30°C in a shaking water bath and the assay was initiated by injection of 1 ml of acetylene. The increase in ethylene content in the vial headspace was assayed periodically using a Perkin-Elmer Sigma 3B gas chromatograph equipped with a flame ionization detector and a J&W Scientific GS-Q column (30 m × 0.53 mm) operated isothermally at 35°C.

### 2.3. Lipid extraction and separation

Lyophilized cells were extracted twice by a modified Bligh and Dyer procedure which removes the solvent-extracted cell residue by centrifugation before initiating solvent partition (Kates, 1986). This method resulted in good recovery of the chlorophyll in the total lipid extract as judged by the lack of pigment color in the cell residue pellet. The total lipid extract was separated by solvent partition in cold acetone which precipitates phospholipids (Kates, 1986). Bacteriochlorophyll a (Bchl a) or chlorophyll a (Chl a) present in the neutral lipid (acetone supernatant) was separated on reverse phase thin-layer chromatography (RTLC) plates (Whatman KC₁₈) developed twice with methanol to 15 cm. The Bchl a (Rf 0.43) or Chl a (Rf 0.32) zones were visually selected, transferred to extraction tubes and eluted from the silica gel sequentially with ethyl acetate, then methanol. Eluates were filtered with fritted glass funnels using first medium and then fine frits (5 μm pore size) to remove residual silica gel. To recover all of the N-containing material from the RTLC plates, zones above and below the Bchl a band were also collected and eluted in a similar manner. All steps were carried out in dim light in order to minimize degradation of pigments. Spectral analysis of all pigmented zones was carried out using a Cary 3 UV-visible scanning spectrophotometer operated between 300 and 800 nm to monitor for the characteristic Bchl a peaks at 359 and 773 nm. Spectra for Bchl a in our samples were in good accord with those reported in other publications (Mauzerall, 1978), which attests to a good recovery of Bchl a through our methodology.

To determine the effects of this methodology on nitrogen isotopic compositions, commercial chlorophyll a (Sigma Chemical Co), isolated from *Anacystis nidulans*, was purified in a similar manner. The nitrogen isotopic composition of the retrieved chlorophyll and the original sample are reported in Table 1 and agreed to within 0.3%. Elution of a KC₁₈ silica gel blank of equivalent size showed no evidence for nitrogenous contaminants. Isotope values of experimental samples are reported without adjustment of measured values. The similarity in the molecular environment of nitrogen in the carbon skeletons of Bchl a and Chl a suggest that discrimination for both would be similar to the Chl a data.

Dried biomass, lipids and chlorophylls were submitted to a “Dumas combustion” (Minagawa et al., 1984; Rigby and Batts, 1985) to produce gases (N₂ and CO₂) which were purified cryogenically by standard line procedures for isotopic analysis (Des Marais et al., 1989). Isotopic measurements were made using a Nuclide 6-60RMS mass spectrometer modified for small sample analysis (Hayes et al., 1977). Nitrogen isotopic compositions are reported as δ¹⁵N in per mil (%o) units. Nitrogen isotopic discrimination between components and substrates are expressed using the Δ¹⁵N notation in per mil (%o) where:

\[ Δ^{15}N_{ps} = \delta^{15}N_{product} - \delta^{15}N_{source} \]  
\[ \delta^{15}N(%) = \left[ \left( R_{sample} - R_{standard} \right) / R_{standard} \right] \times 10^3 \]

### 3. Results

#### 3.1. Nitrogen fixation

*R. capsulatus* was grown photoheterotrophically with N₂ as sole nitrogen source. Samples were removed over...
the growth phase and monitored for both nitrogenase activity and nitrogen isotopic composition (Fig. 1). Nitrogenase activity increases throughout exponential growth, and then decreases as the culture enters the stationary phase. Values of $\Delta^{15}\text{N}$ for biomass relative to $\text{N}_2$ range from $-1.3\%$ to $-1.5\%$ and average $= -1.4\pm0.1\%$ (Fig. 1a, Table 2). They are constant throughout the growth cycle and appear to be independent of the nitrogenase activity.

The biomass exhibited similar $^{15}\text{N}$ depletions for $R$. capsulatus grown photoheterotrophically and photoautotrophically ($\text{CO}_2+\text{H}_2$) with $\text{N}_2$ as sole N-source (Table 2). Although photoautotrophic growth was much slower than photoheterotrophic growth (65 h per doubling versus 22 h, respectively), the exponential phase of the photoautotrophs continued for a longer time, resulting in higher biomass yields. The $\Delta^{15}\text{N}$ values for biomass relative to $\text{N}_2$ range from $-0.8\%$ to $-1.8\%$ (average $= -1.4\pm0.4$). These values were constant within 1% over the growth period, and their average value was similar to the value obtained under heterotrophic growth conditions.

Table 1
Comparison of nitrogen isotopic composition for a commercial chlorophyll $a$ before and after isolation from reverse phase thin layer chromatography (RTLC) plates as described in Section 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\delta^{15}\text{N}_{\text{air}}$ (%)</th>
<th>$\text{N}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl $a$ pure</td>
<td>5.5</td>
<td>9.8</td>
</tr>
<tr>
<td>Chl $a$ pure</td>
<td>5.3</td>
<td>9.2</td>
</tr>
<tr>
<td>Chl $a$ RTLC</td>
<td>5.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Chl $a$ RTLC</td>
<td>5.6</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Table 2
Nitrogen isotopic discrimination during photoheterotrophic and photoautotrophic growth of $Rhodobacter$ capsulatus with $\text{N}_2$ as nitrogen source

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>$\delta^{15}\text{N}_{\text{source}}$ (%)</th>
<th>Growth stage</th>
<th>O.D. 660 nm</th>
<th>$\Delta^{15}\text{N}_{\text{AIR}}$ (biomass-source) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{N}_2+\text{Malate}$</td>
<td>$-1.3^a$</td>
<td>Starting inoculum</td>
<td>$-1.3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.7$</td>
<td>0.1</td>
<td>$-1.5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.3$</td>
<td>0.3</td>
<td>$-1.3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.4$</td>
<td>0.4</td>
<td>$-1.5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.5$</td>
<td>0.5</td>
<td>$-1.4$</td>
<td></td>
</tr>
<tr>
<td>$\text{N}_2+\text{CO}_2$</td>
<td>$-2.4^a$</td>
<td>Starting inoculum</td>
<td>$-1.7^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-2.4$</td>
<td>0.3</td>
<td>$-0.8$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.5$</td>
<td>0.5</td>
<td>$-1.8$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.8$</td>
<td>0.8</td>
<td>$-1.8$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$-0.8$</td>
<td>0.8</td>
<td>$-1.3$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The starting culture used as the inoculum was grown under similar conditions to approximate experimental growth condition, so that $\delta^{15}\text{N}$ value of inoculum $\approx \delta^{15}\text{N}$ of experimental biomass, and isotopic shift due to the $\delta^{15}\text{N}$ of inoculum would be minimal.

Fig. 1. Isotopic discrimination by $Rhodobacter$ capsulatus during photoheterotrophic growth with $\text{N}_2$ as N-source. (a) Growth curve and nitrogen isotopic composition of biomass relative to N-source. Labels on growth curve indicate sample numbers removed during growth. Samples 3, 5, 7 and 9 were analyzed for nitrogen isotopic composition. Samples 1, 4, 5, 6 and 7 were measured for nitrogenase activity. (b) Nitrogenase activity relative to growth phase.
3.2. Ammonia assimilation

To compare the N-isotope fractionation associated with N2 fixation versus ammonia assimilation, *R. capsulatus* was grown photoheterotrophically with a source of reduced nitrogen (Table 3). In the first of two experiments, 7.4% of the ammonia was consumed, and in the second experiment, 2.0% of ammonia was consumed. Values for the fractionation factor ($\epsilon$) of $-10.6\%$ and $-12.4\%$ for *R. capsulatus* (1) and (2), respectively, were calculated according to Eq. (11) of Mariotti et al. (1981) after rearrangement to solve for $\epsilon$ where $\delta_{\text{sub}0}$ is the value of substrate (NH$_4^+$) at time zero, $\tilde{\delta}_{\text{product}}$ is the value of accumulated product, and $f$ is the fraction of reactant remaining:

$$\epsilon_{\text{p/s}} = [\delta_{\text{sub}0} - \tilde{\delta}_{\text{product}}] \cdot [1 - f/(\ln f)]$$

3.3. Lipids and chlorophylls

The $\Delta^{15}\text{N}$ values of all isolated compounds, relative to their N source (Fig. 2) and relative to biomass, are reported in Table 3 for *R. capsulatus* grown photoheterotrophically and photoautotrophically with both N$_2$ and ammonia. Total lipids were relatively depleted in $^{15}\text{N}$ compared to the cell residues. N-depletions for total lipids relative to biomass were as low as $-5.5\%$. For the neutral lipid fractions, $\Delta^{15}\text{N}$ values ranged from $-9.2\%$ to $-8.7\%$ under photoheterotrophic and photoautotrophic growth, respectively. Similar $^{15}\text{N}$ depletions were obtained for the isolated Bchl $a$ from these same N$_2$ grown samples; the $\Delta^{15}\text{N}$ values relative to biomass were

![Fig. 2: The relative nitrogen isotopic fractionation ($\Delta^{15}\text{N}_{\text{component-N2}}$) of isolated components from *Rhodobacter capsulatus* in an open system with N$_2$ as sole nitrogen source, grown either photoheterotrophically (■) or photoautotrophically (□).](image)

Table 3
The effect of growth conditions on the nitrogen isotopic compositions of isolated lipid components from *Rhodobacter capsulatus* and *Anabaena cylindrica*, relative to nitrogen source and biomass

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth condition</th>
<th>Nitrogen source $\delta^{15}\text{N}_{\text{air}}$ (%)</th>
<th>Analyzed component</th>
<th>$\Delta^{15}\text{N}$ (component-source) ($%$)</th>
<th>$\Delta^{15}\text{N}$ (component-biomass) ($%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. capsulatus</em></td>
<td>N$_2$ + Malate</td>
<td>-0.5</td>
<td>Biomass</td>
<td>-1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extracted residue</td>
<td>-0.9</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total lipid</td>
<td>-6.7</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polar lipid</td>
<td>-1.8</td>
<td>-0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutral lipid</td>
<td>-10.4</td>
<td>-9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bchl $a$</td>
<td>-8.7</td>
<td>-7.4</td>
</tr>
<tr>
<td><em>R. capsulatus</em></td>
<td>N$_2$ + CO$_2$</td>
<td>0.6</td>
<td>Biomass</td>
<td>-1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extracted residue</td>
<td>-0.9</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total lipid</td>
<td>-6.9</td>
<td>-5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polar lipid</td>
<td>-2.3</td>
<td>-1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutral lipid</td>
<td>-9.9</td>
<td>-8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bchl $a$</td>
<td>-8.7</td>
<td>-7.5</td>
</tr>
<tr>
<td><em>R. capsulatus</em> (1)</td>
<td>NH$_4^+$ + Malate</td>
<td>0.6</td>
<td>Biomass</td>
<td>-10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bchl $a$</td>
<td>-21.0</td>
<td>-10.9</td>
</tr>
<tr>
<td><em>R. capsulatus</em> (2)</td>
<td>NH$_4^+$ + Malate</td>
<td>0.6</td>
<td>Biomass</td>
<td>-12.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bchl $a$</td>
<td>-20.9</td>
<td>-8.6</td>
</tr>
<tr>
<td><em>A. cylindrica</em></td>
<td>N$_2$ + CO$_2$</td>
<td>-0.9</td>
<td>Biomass</td>
<td>-0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chl $a$</td>
<td>7.2</td>
<td>8.0</td>
</tr>
<tr>
<td><em>A. cylindrica</em></td>
<td>N$_2$ + CO$_2$</td>
<td>-0.9</td>
<td>Biomass</td>
<td>-1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chl $a$</td>
<td>7.3</td>
<td>9.0</td>
</tr>
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</table>
measured at −7.4%o for photoheterotrophic and −7.5%o for photoautotrophic growth. Growth with ammonium yielded a similar discrimination (Table 3). Although the amount of biomass was not sufficient for N-isotope analyses to be done on all fractions, a $\Delta^{15}N$ relative to biomass of −10.9%o for one growth experiment and −8.6%o for the other are in good agreement with the results obtained from the N$_2$-grown cells. Little nitrogen was recovered in analysis of the other RTLC silica gel eluates (some of these contained large amounts of carotenoids), indicating that almost all nitrogen in neutral lipids was contained in recovered Bchl a.

In order to evaluate the effect of tetrapyrrole biosynthesis on the potential for N-depletion in other bacteria, several growth experiments were carried out with a cyanobacterium, Anabaena cylindrica. In both cases the $\Delta^{15}N$ value for A. cylindrica Chl a relative to biomass was substantially enriched (Table 3) as evidenced by values of +8.0%o and +9.0%o for the two independent growth experiments.

4. Discussion

4.1. Nitrogen assimilation

Although the potential pathways of nitrogen to the site of nitrogenase activity are considerably different in R. capsulatus and A. cylindrica, N-isotope discrimination was quite similar for the purple nonsulfur bacterium and the cyanobacterium with average $\Delta^{15}N$ values of −1.2%o for both organisms. No significant effect on the isotopic composition seemed to be linked to the O$_2$-protective mechanism. Nitrogen fixation by this anoxygenic photosynthetic bacterium did not result in any apparent isotopic fractionation that would distinguish the $\Delta^{15}N$ of R. capsulatus from that of other freeliving or symbiotically associated nitrogen-fixers (Hoering, 1955; Delwiche and Steyn, 1970; Wada et al., 1975; Wada and Hattori, 1976; Minagawa and Wada, 1986; Macko et al., 1987). These measurements provide evidence supporting the universality of the isotopic fractionation associated with the nitrogenase reaction, and clearly show that the small N-isotope discrimination is not the consequence of any O$_2$ protective mechanism such as the heterocyst.

The photoheterotrophic growth of R. capsulatus with ammonia resulted in N-depletions of up to −12.4%o. The magnitude of N-isotope discrimination in R. capsulatus is similar to the $^{15}N$-depletion reported for a cyanobacterium, Anabaena sp., grown under similar conditions (Macko et al., 1987). This finding supports the hypothesis that the mechanisms involved in ammonia assimilation in these two metabolically diverse bacteria are similar and that the bacterial processes involved in this aspect of the cycling of ammonia in anaerobic and aerobic environments will result in similar N-discrimination.

4.2. Lipids and chlorophylls

In R. capsulatus, Bchl a is $^{15}$N-depleted relative to total biomass under all growth conditions. Average values of $\Delta^{15}N_{Bchl-biom}$ are −7.5%o for growth using nitrogen fixation and −9.7%o using ammonia assimilation. Growth condition had little effect on $\Delta^{15}N_{Bchl-biom}$, even though the isotopic composition of the biomass was highly dependent on the N-assimilation process. Values of $\Delta^{15}N_{Bchl-biom}$ ranged from −7.4 to −10.9%o while the $\Delta^{15}N_{biom-source}$ varied from −1.2 to −12.3%o. The N-fractionation steps associated with the biosynthesis of Bchl a appeared to be largely independent from the fractionations associated with nitrogen assimilation.

The isotopic composition of individual metabolites is the result of both potential kinetic isotope effects associated with specific reactions and the branching ratios of metabolic precursors along synthetic pathways (Hayes, 1993). In R. capsulatus, an illustration of the consequences of differences in branch point ratios is readily apparent in the nitrogen isotopic contrast between bacteriochlorophyll and polar lipids (Fig. 2). Serine is the metabolic precursor for the synthesis of both phosphotidylethanoamine (PE) and bacteriochlorophyll (Fig. 3). PE is the major membrane lipid in many bacteria including R. capsulatus (Imhoff et al., 1982), and is produced by the decarboxylation of phosphatidylserine (Harwood and Russell, 1984). Indeed, it is probable that most of the nitrogen in the relatively enriched polar lipid fraction ($\Delta^{15}N_{PL-biom}$ of −0.5 to −1.2%o) can be accounted for as N-associated PE. The relatively depleted bacteriochlorophyll ($\Delta^{15}N_{Bchl-biom}$ of −7.5%o), however, is also derived from serine, since in this organism, glycine is the sole N-source for tetrapyrrole synthesis (discussion below) and the major source of glycine is the serine hydroxymethyltransferase (Datta, 1978).

The $\Delta^{15}N_{Bchl-biom}$ values measured here for R. capsulatus and one value reported for a cyanobacterium, Synechococcus, (Sachs et al., 1999) are considerably more depleted than those previously reported for chlorophylls from higher plants and marine algae (Kennicutt et al., 1992; Sachs et al., 1999), or the enriched values measured here for A. cylindrica Chl a (Fig. 4). In this respect, it is important to note the existing differences in metabolic end-products, metabolic processes and potential branch points in these evolutionarily diverse organisms.

Unfortunately, little is known about specific isotope effects involved in synthesis of nitrogenous compounds. An isotope effect has been measured for transamination of oxaloacetate by glutamate which results in N-depleted aspartate (Macko et al., 1986). Glutamate plays a central role in amino acid synthesis because the amination
of α-ketoglutarate to form glutamate is the central source of amino groups for synthesis of all of the major classes of nitrogenous compounds (Fig. 3). Certain amino acids that are the products of a series of transamination steps, such as serine, are generally more depleted than the average pool (Macko et al., 1987). Proteins and nucleic acids account for most of cell biomass, making up 55 and 23% of dry weight, respectively of an Escherichia coli cell (Neidhardt et al., 1990). Amino acids are also important precursors for the synthesis of other N-compounds (Fig. 3), and, depending on composition of the initial pool, as well as pathway branch points and/or specific isotope effects associated with subsequent biosynthetic steps (see Fig. 3), the N-isotopic composition of metabolites will most likely vary.

The N-isotope composition for the amino acids measured in two bacteria (Vibrio sp. and Anabaena sp.) grown with different N-sources has a wide range of values but displays a somewhat consistent relative pattern that appears independent of organism and growth conditions, with glutamate generally enriched relative to other amino acids such as glycine (Macko et al., 1987). Depending on organism, either glutamate or glycine may serve as the precursor amino acid in tetrapyrrole synthesis, and thus contribute to isotopic differences between these products. As mentioned above, in the bacteria of the α-subdivision of the Proteobacteria (which includes the genera Rhodobacter, other purple nonsulfur bacteria, and novel aerobic, anoxygenic phototrophs such as Erythrobacter), the condensation of glycine and succinyl-CoA results in synthesis of δ-aminolevulinic acid (ALA), the metabolic N-intermediate required for tetrapyrrole biosynthesis in all organisms. In plants, algae, cyanobacteria and other photosynthetic bacteria the tetrapyrrole is synthesized by the so called C5-pathway, and involves conversion of glutamate via a transamination of glutamate-1-semialdehyde to ALA (Avissar et al., 1989; Senge and Smith, 1995). The steps

Fig. 3. Overview of precursor amino acids and pathways leading to major N-containing cell constituents in (a) Rhodobacter capsulatus and (b) Anabaena cylindrica with (c) representing synthesis of N-products common to both cell types. Steps with potential and/or measured kinetic isotope effects are indicated for N–C bonds (see Handley and Raven, 1992 for review) at (1) amination of carbon skeletons such as α-ketoglutarate by glutamate dehydrogenase; (2) transfer of amino group by transamination within amino acid network; (3) GSA aminotransferase; (4) PBG synthetase; and (5) PBG deamination. Additionally, potential inverse isotope effects for N-bonds in association with C–C bond cleavage indicated for (6) synthesis of PE by phosphatidylserine decarboxylase or (7) serine hydroxymethyltransferase. Abbreviations: ALA, δ-aminolevulinic acid; GSA, glutamate-1-semialdehyde; PBG, porphobilinogen; PE, phosphatidylethanolamine.
subsequent to ALA in porphyrin synthesis involve condensation of two ALA molecules to form porphobilinogen, and condensation and deamination of four porphobilinogen molecules to form the tetrapyrrole ring. These steps appear to be universally conserved in organisms (Beale, 1995).

Total protein within a cell tends to be slightly enriched in $^{15}$N by about $3\%$ relative to total biomass, with glycine most closely representing the average amino acid composition and with glutamate even further enriched (Macko et al., 1987). Indeed, Macko et al. (1987) have reported glutamate values enriched by approximately $6\%$ relative to biomass for an *Anabaena* sp. also grown utilizing $N_2$ fixation. Although it is tempting to view the relative enrichment of *A. cylindrica* Chl $a$ measured here as a reflection of a possible enrichment in the initial precursor, glutamate, this would assume that no other isotope effects and/or branch points affected isotopic composition of this pigment, which is unlikely. Clearly, this would not explain the $^{15}$N depletion in Chl $a$ reported for other marine phytoplankton and plants (Kennicutt et al., 1992; Sachs et al., 1999) or the depletion associated with another cyanobacterium, *Synechococcus WH7803* (Sachs et al., 1999). A more likely explanation is the variety of metabolic mechanisms and the need to assess environmentally important groups on an individual basis.

Metabolic end-products may vary among organisms, even among closely related cyanobacteria. In most groups of photosynthetic organisms (plants, green algae and photosynthetic bacteria), the Mg branch in tetrapyrrole synthesis is the major sink for N-precursors (Fig. 3). However, in cyanobacteria the relative distribution of N-precursors into the Fe and Mg branches is equal because of the high cellular content of phycobiliproteins which are synthesized from heme precursors (Beale, 1995). Additionally, cyanobacteria are known to synthesize large amounts of N-storage compounds. Cyanophycin is a particularly important N-storage compound in $N_2$-fixing cyanobacteria such as *A. cylindrica* (Wolk et al., 1994), while the accumulation of considerable quantities of a phycobilin, phycoerythrin, is thought to serve a similar function in picoplanktonic cyanobacteria such as *Synechococcus WH7803* (Sachs et al., 1999; Wyman et al., 1985). Variations such as these may lead to differences in metabolic flow in reaction networks and the observed broad range in $\delta^{15}$N values for the end-products of tetrapyrrole synthesis in photosynthetic organisms.

4.3. Geological implications

Chlorophylls have a high potential as geochemical markers because their porphyrin rings can be preserved through the diagenetic processes that create geoporphyrins, thus leaving their isotopic compositions intact (Macko et al., 1993). Indeed, the isotopic composition of geoporphorphyin biomarkers have been shown to allow identification of specific sources and aid in reconstructing element cycling in paleoenvironments (Hayes et al., 1987). Chicarelli et al. (1993) reported a $\delta^{15}$N value for sedimentary alkyl porphyrin nitrogen of $-3.3\%$ vs. air for the Triassic Serpiano shale, and suggested that $N_2$ fixing cyanobacteria were a likely source. The associated biomarker assemblage of this shale, particularly the high abundance of hopanoids and the low levels of steranes, suggests that this paleoenvironment was bacterially-dominated (McEvoy and Giger, 1986), with the preponderance of 2-methylhopanoids supporting a cyanobacterial input (Summons and Jahnke, 1990; Summons et al., 1999). The conditions in restricted anoxic basins such as the one involved in formation of the Serpiano oil shale are thought to encourage cyanobacterial $N_2$ fixation in surface waters and contribute to relatively light ($\delta^{15}$N $\sim 2\%$) organic matter (Haug et al., 1998). Only a few genera of cyanobacteria in the open ocean are known to fix nitrogen (Carr and Mann, 1994). The dominant picoplanktonic cyanobacteria, *Synechococcus* and *Prochlorococcus*, are limited to obtaining nitrogen from ammonia and in some cases nitrate (Carr and Mann, 1994; Partensky et al., 1999). Because nitrogen
assimilated by these cyanobacteria is expected to be $^{15}$N-depleted, *Synechococcus* WH7803 (Sachs et al., 1999) is not expected to be a major source of nitrogen for such anoxic sediments. The relatively enriched chlorophyll of a $N_2$-fixer such as the *Anabaena* studied here ($\delta^{15}N + 8.1\%$ vs. air) might provide a more likely source of the geoporphyrins of the Serpiano shale. At least one marine $N_2$-fixing cyanobacterium, *Cyanothece*, has been shown to accumulate the N-storage compound, cyanophycin, found in *Anabaena* (Reddy et al., 1993). If cyanophycin and its synthetic pathway are widely shared among $N_2$-fixers, the $^{15}N$-enrichment of chlorophyll might be widespread within this group.

Photosynthetic microorganisms such as the purple nonsulfur bacteria must also be considered in assessing potential sources of the relatively depleted geoporphyrins. Purple nonsulfur bacteria are able to fix $N_2$ and contain high levels of hopanoids (Rohmer et al., 1984) similar to those reported for this shale (McEvoy and Giger, 1986). The relative depletion of $^{15}N$-porphyrins suggests a possible contribution by this photosynthetic group to the residual organic matter in this shale.

Purple nonsulfur bacteria are widely distributed in nature and are found in freshwater, marine and hypersaline aquatic habitats, where they are well-adapted to utilizing sulfide. Purple nonsulfur bacteria often accompany purple sulfur bacteria in stratified environments, and are found in great abundance in the muds of eutrophic water bodies (Imhoff and Trüper, 1992; Lopez-Cortes, 1990; Imhoff, 1992). These organisms have been demonstrated to use ferrous iron to reduce $CO_2$ (Widdel et al., 1993) and, therefore, may have been important contributors to the early biosphere (Gest and Schopf, 1983). The $\delta^{15}N$ values reported by Beaumont (Beaumont, 1994; Beaumont and Robert, 1998) for Archean sedimentary organic matter (average $\delta^{15}N = 0\%$, but including values as low as $-6\%$) are somewhat depleted relative to modern values. It is tempting to suggest that purple nonsulfur bacteria, or some other metabolic group of anoxicogenic photosynthetic bacteria yet to be studied, may have contributed substantially to these observed values as a consequence of the selective preservation of $N$-depleted porphyrins.

These results emphasize the importance of performing molecular isotopic fractionation studies in living organisms in order to achieve a better understanding of fossil microenvironments and life on early Earth.

5. Conclusions

1. The nitrogen isotope fractionation associated with $N_2$ fixation by bacteria appears independent of bacterial group, metabolic growth mechanism and/or fixation strategy. Isotopic depletions of $\sim 1.4\%$ were observed for an anoxicogenic, photo-synthetic bacterium grown photoautotrophically or photoheterotrophically, while a depletion of $1.3\%$ was measured for an oxygenic, photosynthetic cyanobacterium using a specialized cell (heterocyst) for $N_2$ fixation.

2. The isotopic discrimination associated with porphyrin synthesis by an organism appear to be independent of the mode of nitrogen assimilation by the organism (e.g. for *Rhodobacter capsulatus*, the $\Delta^{15}N$ values Bchl $a$ relative to biomass were similar for growth utilizing $NH_4^+$ or $N_2$).

3. The variety of enzymes and reaction networks exhibited among diverse bacterial groups can create a variety of nitrogen isotopic patterns within metabolic end-products.

4. In order to assess properly the isotopic significance of organic biomarkers in natural environments, both modern and ancient, environmentally-significant populations of bacteria must be related to specific kinetic isotope effects of individual reactions and/or fractionations associated with reaction networks.

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