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Stable Sulfur Isotope Fractionation by the Green Bacterium *Chlorobaculum parvum* During Photolithoautotrophic Growth on Sulfide

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Abstract

Growing cultures of the green obligate photolithotroph, *Chlorobaculum parvum* DSM 263^T (formerly *Chlorobium vibrioforme* forma specialis *thiosulfatophilum* NCIB 8327), oxidized sulfide quantitatively to elemental sulfur, with no sulfate formation. In the early stages of growth and sulfide oxidation, the sulfur product became significantly enriched with ^{34}S, with a maximum $\delta^{34}S$ above +5‰, while the residual sulfide was progressively depleted in ^{34}S to $\delta^{34}S$ values greater than –4‰. As oxidation proceeded, the $\delta^{34}S$ of the sulfur declined to approach that of the initial sulfide when most of the substrate sulfide had been converted to sulfur in this closed culture system. No significant formation of sulfate occurred, and the substrate sulfide and elemental sulfur product accounted for all the sulfur provided throughout oxidation. The mean isotope fractionation factors (ε) for sulfide and sulfur were equivalent at ε values of –2.4‰ and +2.4‰ respectively. The significance of the experimentally-observed fractionation to the $^{34}S/^{32}S$ ratios seen in natural sulfur-containing minerals is considered.

K e y w o r d s: *Chlorobaculum parvum,* photolithotrophic sulfur bacteria, stable sulfur isotopes, sulfide oxidation

Introduction

There is a huge literature on sulfur isotope fractionation during bacterial sulfate reduction (*e.g*. McCready, 1975; Chambers and Trudinger, 1979; Canfield, 2001; Detmers *et al.*, 2001; Brunner and Bernasconi, 2005; Hoek and Canfield, 2008), but microbially-assisted fractionation occurring during the oxidation of inorganic sulfur compounds has received less and only intermittent study over the past six decades (*e.g*. Jones and Starkey, 1957; Kaplan and Rittenberg, 1964; Ivanov *et al.*, 1976; Fry *et al*., 1986; Kelly, 2008). Photolithotrophic sulfur bacteria including *Chromatium*, *Chlorobium* and *Ectothiorhodospira* have been shown to discriminate between ^{34}S and ^{32}S during the oxidation of sulfide (Kaplan *et al.*, 1960; Kaplan and Rittenberg, 1964; Mekhtieva and Kondratieva, 1966, Ivanov *et al.*, 1976; Chambers and Trudinger, 1979; Fry *et al.*, 1984, 1986, 1988). The general observations were that one or both of elemental sulfur or sulfate produced from sulfide became enriched with ^{34}S, while residual sulfide became enriched in ^{32}S (Kaplan *et al.*, 1960; Ivanov *et al.*, 1976; Chambers and Trudinger, 1979). There is some inconsistency in the literature in that some reports showed little or no enrichment of ^{34}S into elemental sulfur, while Kaplan *et al.* (1960) claimed enrichment of sulfur with ^{32}S rather than ^{34}S, and insignificant fractionation of sulfur isotopes in the sulfate produced from sulfide. A partial explanation for these inconsistencies could be the reported accumulation of polythionates ($S_nO_6^{2-}$) from sulfide oxidation, which become "sinks" for ^{34}S, in those cases where production of ^{32}S-enriched sulfur has been claimed (Kaplan and Rittenberg, 1964; Chambers and Trudinger, 1979). This was shown markedly to be the case during the accumulation of ^{34}S-enriched polythionate during thiosulfate oxidation by the aerobic chemolithotroph *Halothiobacillus neapolitanus* (Kelly, 2008). The most convincing study of isotope fractionation during sulfide oxidation by a phototroph is that of Fry *et al.* (1988), who showed increase of the ^{32}S content of sulfide during its oxidation to ^{34}S-enriched sulfur by *Chlorobium vibrioforme*. The end-product of sulfide oxidation by *Chlorobium vibrioforme* was, however, ^{34}S-depleted sulfate, for which elemental sulfur was a precursor (Fry *et al.*, 1988). Comparing different phototrophs, the magnitude of the preferential fractionation of ^{34}S into elemental sulfur observed by Fry *et al.* (1984, 1988) ranged from zero (for *Chromatium*) to +2.0 to +2.4‰ (for *Chlorobium*). In the

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case of *Chlorobium vibrioforme* any sulfate formation (with sulfate becoming depleted in ^{34}S) concurrent with the accumulation of ^{34}S-enriched elemental sulfur would have enhanced the observed ^{34}S content of the residual sulfur intermediate.

No previous study has been reported of the oxidation of sulfide exclusively to sulfur by a green phototrophic bacterium. Quantitative oxidation of sulfide to sulfur is a property of a number of species of *Chlorobiaceae* (van Niel, 1931; Kelly, 1974; Cork *et al.*, 1983; Imhoff, 2003). Data from such sulfur-producers are clearly needed to complement the observations on *Chlorobium vibrioforme*, for which sulfur is only an intermediate in sulfide oxidation. Chambers and Trudinger (1979) briefly alluded to a study in which a *Chlorobium* strain oxidized sulfide exclusively to produce sulfur enriched in ^{34}S by about 5‰ (citing unpublished results of Kelly, Chambers and Rafter). Those experiments with *Chlorobaculum parvum* were conducted in the late 1960s, but never published. The sulfur isotope fractionation data obtained for sulfide oxidation have now been reassessed for publication, as the only comparable work since then has been the study by Fry *et al.* (1988). This assessment enabled the progressive changes in the $\delta^{34}S$ of the sulfide and sulfur to be determined, and the sulfur isotope fractionation factors for the stoichiometric oxidation of sulfide to sulfur to be calculated (Mariotti *et al.*, 1981), and compared with results of Fry *et al.* (1988).

Experimental

Materials and Methods

Maintenance and growth of cultures. *Chlorobaculum parvum* DSM 263^T (formerly *Chlorobium vibrioforme* forma specialis *thiosulfatophilum* NCIB 8327; Kelly, 1974; Imhoff, 2003) was originally obtained from Dr June Lascelles in 1967 as "*Chlorobium thiosulfatophilum*" NCIB 8327, and maintained in continuously illuminated anaerobic culture on 4 mM thiosulfate with 0.5 mM sulfide as described previously (Kelly, 1974). For isotope discrimination experiments with sulfide as sole substrate, cultures were grown with 4 mM Na_2S in a nominally sulfate-free medium, containing (g/l in distilled water): $Na_2S \times 9H_2O$ (1.0), KH_2PO_4 (1.0), NH_4Cl (1.0), $MgCl_2 \times 6H_2O$ (0.5), $NaHCO_3$ (2.0), trace metal solution (Pfennig and Lippert, 1966; 10 ml), 0.1 M HCl (7.5 ml). Traces of sulfate were present in the initial medium as a result of sulfate-salts in the trace metal solution, and as a possible trace contaminant in other reagents. Sterilization was by autoclaving at 110°C for 10 min. Bicarbonate, sulfide and HCl were sterilized separately and added while hot to the rest of the medium. To provide as pure and accurate a supply of sulfide as possible, large sodium sulfide crystals were washed with distilled water and blotted dry before making up 10% (w/v) solutions for sterilization. Cultures were grown at 25°C in completely filled flat bottles (capacity 570 ml), with a 3–5% (v/v) inoculum of an actively growing culture. A layer of sterile paraffin oil was placed on top of the cultures before sealing the bottles. Illumination was by two banks of four 40 watt (40 J s^{-1}) fluorescent tubes placed 30 cm away from both sides of a double array of bottles. Dark control cultures were bottles prepared as above, but were wrapped in black paper and incubated together with the experimental bottles.

Sampling procedures and recovery of sulfide, sulfur and sulfate for analysis. At intervals from zero time until 270 h after inoculation, individual replicate bottles were opened, the paraffin layer removed by aspiration, and the bottle contents thoroughly mixed. To recover the residual sulfide, an aliquot (200 ml) was placed in a Quickfit Drechsel bottle, 10 ml of 1 M HCl injected through a sealed port, mixed, and sulfide expelled by bubbling with a flow of nitrogen for 2 h; the effluent gases were passed into a series of two traps containing 50 ml 0.2 M $AgNO_3$ to precipitate sulfide as Ag_2S. All the sulfide was recovered in the first trap. The Ag_2S precipitates were recovered by filtration through 0.45 μm pore-size Millipore filters (45 mm diameter).

A second aliquot (220 ml) was filtered through a 1.2 μm pore-size Millipore filter (45 mm diameter) to recover precipitated sulfur. The filtrate (200 ml) was assayed to determine any sulfate production, as described below.

Determination of sulfide, sulfur and sulfate, and sample preparation for isotope ratio mass spectrometry. Sulfide recovered as Ag_2S was washed on the filters with distilled water and transferred to weighed beakers and air-dried under an infrared lamp before weighing to estimate sulfide recovery. Standard sulfide solutions treated exactly as for the culture samples showed the procedure to give 100–103% recovery of the expected weights of Ag_2S. Recoveries of sulfide using traps with $CdCl_2$ or $ZnCl_2$ gave comparably high recoveries, but precipitation with silver was chosen as Ag_2S could be used directly for isotope ratio analyses.

Sulfur recovered on the filters was transferred into beakers and oxidized by heating on a sand bath for 1–2 h with 50 ml bromine-saturated concentrated nitric acid with 5 ml HCl and 5 ml 20% (w/v) NaCl. After standing overnight at 20°C, the liquid was evaporated to dryness and the residue taken up into 25 ml HCl and again evaporated. The residue was dissolved in 100 ml 0.4 M HCl and sulfate precipitated by boiling with 30 ml 0.05 M $BaCl_2$. The barium sulfate precipitate was recovered by filtration on to

a 1.2 μm pore-size filter, washed, dried at 120°C, and weighed. Replication of sulfur recovery among quadruplicate samples was ± 1.5% of the mean recovery.

Sulfate in the filtrates (200 ml) was precipitated by heating the samples on a boiling water bath with 2 ml HCl and dropwise addition of 0.05 M $BaCl_2$. After heating for 1 h, sulfate was recovered by filtration, dried and weighed as above.

Samples of Ag_2S and $BaSO_4$ recovered as described above were sent for sulfur isotope ratio analysis to the Rafter Stable Isotope Laboratory, Institute for Nuclear Studies, Lower Hutt, New Zealand. $\delta^{34}S$ values were determined from the $^{34}S/^{32}S$ ratios of sulfur in the various samples relative to the Cañon Diablo troilite standard (Krouse and Coplen, 1997), using the equation: $\delta^{34}S$ (‰) = [$^{34}S/^{32}S$(sample)/ $^{34}S/^{32}S$(standard) – 1] × 10^3. Methods for the analysis of the $^{34}S/^{32}S$ data are given in the legends to Fig. 1 and 2.

Results

^{34}S isotope discrimination during sulfide oxidation by growing cultures of *Chlorobaculum parvum*. Illuminated cultures grown with 4 mM sulfide consumed all the substrate in 50–70 h, with the production of sulfur equal to 96 ± 4% (four experiments) of the sulfide provided. There was no significant formation of sulfate from sulfide or sulfur (even in cultures incubated in the light for 200 h following sulfide exhaustion): the sulfate content of ten replicate illuminated cultures (24–270 h after inoculation) and of a 270 h dark control was constant at about 0.3 mM, all of which was due to sulfate introduced in the inoculum cultures. No evidence was obtained for the existence of any intermediate sulfur compound (*e.g.* polythionates) during sulfide oxidation, as the sulfide and sulfur recovered at each sampling time accounted for all the inorganic sulfur in the samples. This strain will also oxidize thiosulfate to sulfate as the photosynthetic electron donor, (Kelly, 1974), and thus has the capacity to convert the sulfane-sulfur (S–) of thiosulfate to sulfate, but under the conditions of the experiments described here, when sulfide rather than combined sulfane-sulfur was the substrate, it was converted quantitatively to elemental sulfur.

Marked enrichment of ^{34}S in the sulfur produced was paralleled by a decrease in the $\delta^{34}S$ of the residual sulfide. Fig. 1 shows the $\delta^{34}S$ values of the sulfur formed (relative to the $\delta^{34}S$ of the initial sulfide = 0), as a percentage of the amount expected for complete sulfide oxidation (4 mM), and the $\delta^{34}S$ of the residual sulfide, as a percentage of the initial 4 mM sulfide. Fractionation was greatest early in the growth, as expected when the sulfide concentration was highest. Thus, when less than 0.4 mM sulfur had been formed its $\delta^{34}S$ was +5.3‰, progressively declining to a $\delta^{34}S$ close to that of the initial substrate-sulfide when 80–90% of the expected sulfur had accumulated (Fig. 1). The residual sulfide became progressively enriched with ^{32}S, with its $\delta^{34}S$ declining from about –0.5‰ when only about 0.8 mM sulfide had been consumed to –4.3‰ when about more than 80% of the initial sulfide had been oxidized. The data show that proportional changes in the $\delta^{34}S$ of sulfide and sulfur during sulfide oxidation exclusively to elemental sulfur, with no evidence for the formation of transient intermediate sulfur compounds or sulfate: apparent fractionation declined to approach the original sulfide $\delta^{34}S$ value when sulfide conversion to sulfur neared 100%.

Estimation of sulfur isotope fractionation factors (ε) for sulfide oxidation and sulfur formation. The $\delta^{34}S$ values of Fig. 1 were plotted against the natural logarithms of the fractions (f) of sulfide consumed and sulfur produced (where f = 1 and ln f = 0 for the

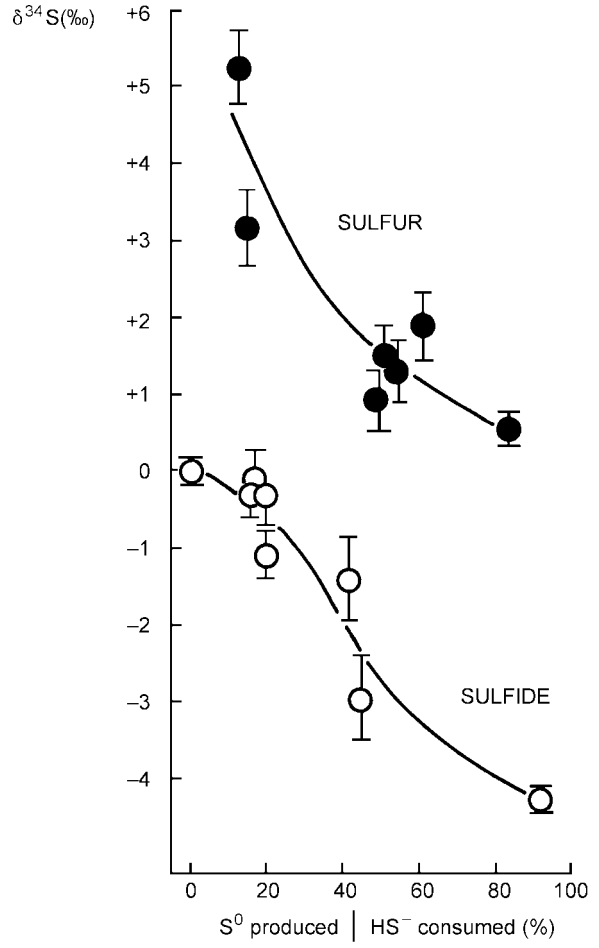

Fig. 1. Isotopic changes in sulfide (○) and sulfur (●) during sulfide oxidation to sulfur by *Chlorobaculum parvum*.

Changes in $\delta^{34}S$ of sulfide (initially 100% of the total sulfur present) and sulfur (initially 0% of total sulfur) are shown relative to the fraction (%) of sulfur present as sulfide or sulfur. The $\delta^{34}S$ of the sulfide used in all the experiments was +8.80 ± 0.26‰: in order to relate the experimental $\delta^{34}S$ values to that of the substrate-sulfide, the data were recalculated with respect to the $^{34}S/^{32}S$ of the initial sulfide to normalize the substrate-sulfide value to $\delta^{34}S$ = 0‰.

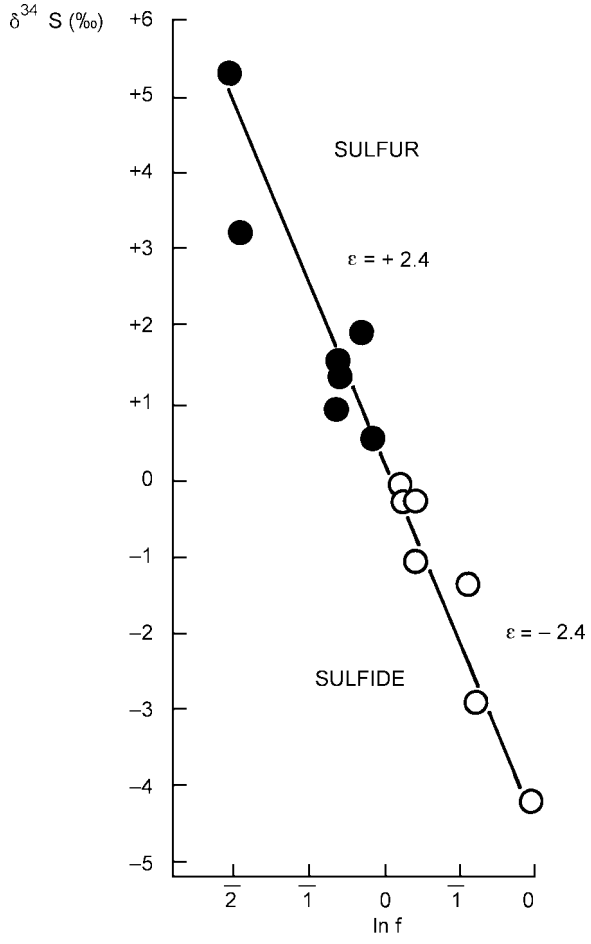

Fig. 2. Isotopic values ($\delta^{34}S$) of substrate-sulfide and product-sulfur as functions of the natural logarithms of the fractions of the initial sulfide remaining or of sulfur formed (ln f) during sulfide oxidation by *Chlorobaculum parvum* (data of Fig. 1).

Isotope fractionation factors (ε, ‰) were calculated from the slopes of these plots. For irreversible reactions, the ε values indicate the expected difference between the product and its substrate (Mariotti *et al.*, 1981). A negative ε value (for residual sulfide) indicated depletion of ^{34}S in the substrate, matched by a positive ε value in the product (sulfur), in which the ^{34}S content was increased.

initial 4 mM sulfide and for the 4 mM sulfur expected for complete oxidation of the added sulfide). The best-fit lines of plots of $\delta^{34}S$ values against ln f gave matching slopes for sulfur and sulfide. Simply combining the two plots showed the best fit line to run through all the data (Fig. 2). This showed that the mean decline in the ^{34}S content of the sulfide was paralleled, with 95% confidence, by the increase in the ^{34}S of the sulfur. The isotope discrimination factors (e) given by the slope of this line were –2.4 for sulfide and +2.4 for sulfur. The factors are the same as those calculated for sulfide oxidation to sulfur by *Chlorobium vibrioforme* (Fry *et al.*, 1988). This treatment of the results effectively smoothes the data to reveal the mean fractionation over the whole time period, and obscures the initial $\delta^{34}S$ increase to +5–6‰ in the sulfur formed.

Discussion

The results obtained allow a clear interpretation of what appears to be a relatively simple physiological system, in which sulfide and its oxidation product, elemental sulfur, are the only detectable sulfur species at all stages of the complete oxidation of the sulfide provided. The culture method used, a batch culture with a limited initial sulfide concentration, is a closed system, so it was expected, and shown, that the greatest change in $\delta^{34}S$ would occur early in the oxidation, with progressive decrease as oxidation neared completion. Thus, initially there was high discrimination in favour of the formation of ^{34}S-elemental sulfur, decreasing as less sulfide remained, but a progressive increase in the ^{32}S-content of the sulfide showed that positive discrimination in favour of ^{34}S-sulfide as a substrate continued throughout oxidation (Fig. 1). In an open system, such as a continuous flow system with passage of some excess sulfide, the decreased $\delta^{34}S$ of the residual sulfide would be sustained as the sulfide flowed out of the system. The environmental relevance of these results is that in habitats permanently rich in sulfide (*e.g.* from a continuous sulfide input from bacterial sulfate reduction in an anoxic sediment), with only a minor part of this being oxidized to sulfur by photolithotrophs, a significant accumulation of ^{34}S-enriched elemental sulfur would result, with $\delta^{34}S$ values approaching +6‰ (*cf.* Fig. 1). In contrast, in habitats in which most of the input sulfide was oxidized to sulfur, the small residual "steady-state" pool of sulfide would show a large decrease in $\delta^{34}S$. The enrichment observed experimentally was to a $\delta^{34}S$-sulfide value of –4 to –5‰ (Fig. 1), which is consistent with the value of about –5.3‰ estimated from the data of Fry *et al.* (1988) for *Chlorobium vibrioforme*. Thus, in natural environments, where both closed and open systems occur, oxidation of sulfide to sulfur by phototrophs such as *Chlorobaculum* could in part explain the large negative $\delta^{34}S$ values observed in some mineral sulfides, deposited in habitats where sulfide was in relatively abundant supply from sulfate reduction, but where oxidation to sulfur would result in ^{34}S-depleted sulfide-minerals (Mekhtieva and Kondratieva, 1966; Nissenbaum and Rafter, 1967; Chambers and Trudinger, 1979; Detmers *et al.*, 2001; Habicht and Canfield, 1996, 2001; Hoek and Canfield, 2008).

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