Unexpected extracellular and intracellular sulfur species during growth of *Allochromatium vinosum* with reduced sulfur compounds

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Before its uptake and oxidation by purple sulfur bacteria, elemental sulfur probably first has to be mobilized. To obtain more insight into this mobilization process in the phototrophic purple sulfur bacterium *Allochromatium vinosum*, we used HPLC analysis and X-ray absorption near-edge structure (XANES) spectroscopy for the detection and identification of sulfur compounds in culture supernatants and bacterial cells. We intended to identify soluble sulfur compounds that specifically occur during growth on elemental sulfur, and therefore compared spectra of cultures grown on sulfur with those of cultures grown on sulfide or thiosulfate. While various unexpected oxidized organic sulfur species (sulfones, C–SO₂–C, and sulfonates, C–SO₃[–]) were observed via XANES spectroscopy in the supernatants, we obtained evidence for the presence of monosulfane sulfonic acids inside the bacterial cells by HPLC analysis. The concentrations of the latter compounds showed a tight correlation with the content of intracellular sulfur, reaching their maximum when sulfur began to be oxidized. None of the detected sulfur compounds appeared to be a specific soluble intermediate or product of elemental sulfur mobilization. It therefore seems unlikely that mobilization of elemental sulfur by purple sulfur bacteria involves excretion of soluble sulfur-containing substances that would be able to act on substrate distant from the cells.

Received 30 January 2009

Revised 28 April 2009

Accepted 1 May 2009

INTRODUCTION

It has been known for a long time that purple sulfur bacteria are able to take up and oxidize elemental sulfur (Thiele, 1968; Frigaard & Dahl, 2009). As elemental sulfur is poorly soluble in water (Steudel, 1996), it has been proposed that an initial mobilization step is necessary prior to its uptake. Different hypotheses and experimental findings concerning this step have been published. Originally, a reduction of elemental sulfur to sulfide was proposed for *Acidithiobacillus ferrooxidans* (Bacon & Ingledew, 1989), while later a mobilization of elemental sulfur by thiols of specific outer-membrane proteins and transport into the cytoplasm as persulfide sulfane sulfur was suggested for *Ath. ferrooxidans* and *Acidiphilum* spp.

(Rohwerder & Sand, 2003). Glutathione plays a catalytic role in this step. Furthermore, a conversion of insoluble elemental sulfur to thiosulfate occurs in *Streptomyces* strains (Yagi *et al.*, 1971). Very little information is available for uptake of solid elemental sulfur in phototrophic bacteria. For the purple sulfur bacterium *Allochromatium vinosum* it was found that the organism can only take up the polymeric fraction of elemental sulfur. Sulfur rings remain untouched. Furthermore, experimental evidence was obtained that an intimate physical cell–sulfur contact is a prerequisite for sulfur uptake in *Alc. vinosum* (Franz *et al.*, 2007). Despite this recent progress, nothing is known so far about the occurrence and possible role of soluble intermediates during the mobilization process in purple sulfur bacteria. In these organisms, the excretion of low-molecular-mass substances such as thiols for acquisition of insoluble sulfur could for example be envisioned. These would then react with exposed –SH or –S– groups

Abbreviations: mBBBr, monobromobimane; TCEP, tris(2-carboxyethyl)-phosphine; XANES, X-ray absorption near-edge structure.

on the surface of elemental sulfur to generate linear polysulfanes which could then be further metabolized.

To obtain more information on this process, we compared the presence of thiols in cells and culture supernatants of *Alc. vinosum* by an HPLC-based technique. These experiments were complemented by investigating culture supernatants of *Alc. vinosum* using X-ray absorption near-edge structure (XANES) spectroscopy. The latter method provides the possibility to investigate culture supernatant samples even with low concentrations of sulfur compounds (Franz *et al.*, 2009). Furthermore, XANES spectroscopy is non-destructive, and a 'quantitative analysis' of XANES spectra can be performed (e.g. Modrow *et al.*, 2001; Prange *et al.*, 2002, 2005). In addition to externally added elemental sulfur, *Alc. vinosum* can oxidize sulfide and thiosulfate to sulfate. Sulfur globules are formed inside the cells (in the periplasm) as an obligate intermediate during these processes. By comparing supernatants of cultures grown on elemental sulfur, sulfide or thiosulfate, we searched for soluble intermediates specifically occurring during growth on externally added elemental sulfur.

METHODS

Bacterial strains, medium and growth conditions. *Alc. vinosum* DSM 180^T was cultivated photolithoautotrophically in batch culture at 30 °C under anaerobic conditions in 100 ml culture bottles containing modified Pfennig's medium (0 medium without sulfide) (Hensen *et al.*, 2006). Solid elemental sulfur (S⁰; 50 mM), sulfide (2 mM) or thiosulfate (2 mM) were added as the sole sulfur sources. To maintain pH 7.0 ± 0.1, sterile HCl and Na₂CO₃ solutions were added. As an inoculum for the growth experiments, cells of *Alc. vinosum* grown photoorganoheterotrophically on malate were used and added to the 0 medium. Elemental sulfur was purchased and used as received from Riedel de Haen. For sulfide (Sigma-Aldrich), thiosulfate (Merck) and sulfite (Sigma-Aldrich) 1 M stock solutions were prepared.

Preparation of samples for HPLC. For HPLC measurements, 10 ml samples of culture suspension were centrifuged for 10 min at 11 000 g. An aliquot of the supernatant was immediately derivatized with monobromobimane (mBBr) as described by Rethmeier *et al.* (1997) and kept frozen until further analysis. The cell pellet was washed twice with 2 ml 20 mM HEPES buffer (pH 7.0) and resuspended in 125 µl 50 mM HEPES, pH 8.5, containing 5 mM EDTA. During washing and centrifugation steps the samples were kept under argon. Samples were stored for a maximum of 12 h at -18 °C before addition of 10 µl mBBr solution (48 mM in acetonitrile). After 3 min at room temperature, 125 µl acetonitrile was added followed by incubation at 60 °C for 15 min. The samples were then put on ice and 10 µl methanesulfonic acid (10 %, v/v) was added to stop the reaction. Samples were then centrifuged for 10 min at 11 000 g to remove cell debris. The supernatant was diluted 1:1 with methanesulfonic acid and again centrifuged for 10 min at 11 000 g. The resulting solution was either stored at -18 °C or immediately subjected to HPLC analysis.

Determination of sulfur compounds by HPLC. Sulfur compounds (sulfide, thiosulfate, sulfite, elemental sulfur and sulfate) were determined by HPLC (Thermo Separation Products) using the methods of Rethmeier *et al.* (1997).

Preparative HPLC and reduction with TCEP. Preparation of individual derivatized thiols and subsequent analysis by reduction were performed as follows. Samples (100 µl) of derivatized cell contents were injected onto a Lichrosphere RP18 (250-4, 5 µm; Merck) column and chromatographed according to Rethmeier *et al.* (1997). After chromatography, the fractions containing the desired compounds were neutralized with 1 M NaOH followed by addition of 50 µl 50 mM HEPES, pH 8.0, per ml solution collected. Subsequently, 20 µl 5 mM TCEP [tris(2-carboxyethyl)phosphine] in 50 mM HEPES, pH 8.0, was added followed by incubation at room temperature for 30 min. For detection of the HSMB group released by reductive cleavage with TCEP, 500 µl of the resulting solution was mixed with 40 µl methanesulfonic acid and an aliquot was subjected to HPLC analysis. A further 125 µl of the solution obtained after incubation with TCEP was again derivatized with mBBr and analysed as described above.

XANES spectroscopy: experimental. XANES spectra were recorded at the Double Crystal Monochromator beamline of the Center for Advanced Microstructures and Devices (CAMD), Baton Rouge, LA, USA (Hormes *et al.*, 2006). CAMD's storage ring was operated at an energy of 1.3 GeV with electron currents between 200 and 80 mA. The synchrotron radiation was monochromatized by a modified Lemonnier-type monochromator (Lemonnier *et al.*, 1978) equipped with InSb (111) crystals. The monochromatic flux rate per second on the sample was about 5×10^8 photons (at 200 mA). Measurements of supernatants (repeated twice) were performed in fluorescence mode using a Vortex silicon drift fluorescence detector (SII Nanotechnology) (experiment filled with helium in cases of 'anaerobic' samples). For energy calibration, the spectrum of zinc sulfate was used as a 'secondary standard', setting the maximum of the first resonance (white line) to an energy of 2481.44 eV (reproducibility ± 0.1 eV). Spectra were scanned with step widths of 0.5 eV between 2440 and 2468 eV, 0.1 eV between 2468 and 2485 eV, and 0.3 eV between 2485 and 2520 eV (integration time 1 s). Using the Origin program (OriginLab), a linear background determined in the pre-edge region was subtracted from the raw data and the spectra were normalized at 2510 eV.

Quantitative analysis of XANES spectra. The fitting and plotting package WinXAS (Ressler, 1998) was used. The program provides tools to find the minimum value of a multi-parameter function and to analyse the shape of the function around the minimum. A set of eight reference spectra was used for fitting the bacterial spectra (reduced and oxidized glutathione or cysteine and cystine, methionine sulfone, cysteic acid, thiosulfate and zinc sulfate). A chi-squared criterion was applied to find the linear combination of these spectra that reproduced the XANES spectrum of interest with the highest probability. Further details concerning the quantitative analysis, especially its verification, potential and restrictions, have been published previously (Prange *et al.*, 2002, 2003). The errors of the percentage contributions of sulfur species (Table 1) can be estimated to be <10 % (absolute value) (Prange *et al.*, 2002, 2003).

Preparation of samples for XANES spectroscopy. Although *Alc. vinosum* is an anaerobe, culture supernatant samples were prepared and measured under oxic conditions. This way of measuring yields the same results as measurements on samples prepared under anoxic conditions, with the advantage of less experimental effort (Franz *et al.*, 2009). For XANES measurements of culture supernatants 2 ml of culture suspension was centrifuged for 5 min at 9000 g and an aliquot of the supernatants was pipetted onto sulfur-free filter paper and directly measured.

Reference compounds for XANES spectroscopy. Glutathione (oxidized and reduced), cysteine and cystine, sodium thiosulfate, methionine sulfone, cysteic acid, methionine and zinc sulfate were

Table 1. Results of fitting the sulfur *K*-edge XANES spectra of *Alc. vinosum* culture supernatants to the sum of the reference spectra

Time (h)		Percentage contribution of sulfur species*						
Reference substance		Glutathione, oxidized	Glutathione, reduced	Cysteine	Cystine	Thiosulfate	Methionine sulfone	Cysteic acid
Sulfur species		C-S-S-C	C-S-H	C-S-H	C-S-S-C	S ₂ O ₃ ²⁻	C-SO ₂ -C	C-SO ₃ ⁻
Elemental sulfur	3	–	9	n.i.	n.i.	12	8	39
	6	11	7	n.i.	n.i.	16	14	30
	12	10	5	n.i.	n.i.	12	11	16
	48	6	–	n.i.	n.i.	6	–	24
Sulfide	0.25	4	10	n.i.	n.i.	28	3	46
	0.75	–	7	n.i.	n.i.	39	7	19
	3	–	8	n.i.	n.i.	32	9	22
	48	–	5	n.i.	n.i.	6	9	5
Thiosulfate	0.25	–	7	n.i.	n.i.	65	–	21
	0.75	–	5	n.i.	n.i.	79	–	10
	3	–	5	n.i.	n.i.	82	–	8
	48	n.i.	n.i.	–	14	3	–	24

*Different sulfur species and their percentage contribution to the sulfur speciation; error: $< \pm 10\%$; –, contribution $< 2\%$; n.i., not included.

used as reference compounds for fitting the spectra. These compounds were of reagent grade, purchased from Sigma and used as received. The reference compounds were ground into fine powder and placed homogeneously on a sulfur-free, self-adhesive Kapton film and/or sulfur-free filter paper (covered with polypropylene).

RESULTS

Growth on elemental sulfur, sulfide and thiosulfate followed by HPLC analysis: culture supernatants

Alc. vinosum was cultivated photolithoautotrophically in 100 ml bottles on medium containing 50 mM elemental sulfur, 2 mM sulfide or 2 mM thiosulfate as the sole sulfur source. Formation of sulfate during uptake and oxidation of elemental sulfur, and transformation of sulfide and thiosulfate into intracellularly stored sulfur and further oxidation to sulfate, respectively, were followed using HPLC (Fig. 1).

In *Alc. vinosum*, the polymeric fraction of elemental sulfur is first taken up by the cells and sulfur globules are formed in the periplasm as an obligate intermediate before the sulfur stored in the globules is oxidized to the final product sulfate (Franz *et al.*, 2007; Brune, 1995; Dahl, 2008). In the experiment shown in Fig. 1(a), *Alc. vinosum* was grown on 50 mM elemental sulfur. As described earlier, the cells first formed intracellular sulfur. The exact concentration of intracellularly stored sulfur could not be determined in this case, because it is not possible to quantitatively separate the cells containing sulfur globules from the solid elemental sulfur particles floating in the medium. The oxidation of

stored sulfur to sulfate started after 12 h. For *Ath. ferrooxidans* reduction of elemental sulfur to sulfide was observed (Bacon & Ingledew, 1989). In contrast, using HPLC analysis neither sulfide or polysulfides nor thiosulfate were detected at any point in time during growth of *Alc. vinosum* on elemental sulfur. According to Rethmeier *et al.* (1997) the detection limit for sulfide and thiosulfate with the HPLC protocol applied is 5 μ M and 1 μ M, respectively.

When 2 mM sulfide was added as the electron donor it was completely oxidized within the first 3 h and transformed into intracellular sulfur globules (Figs 1b and 2a). Polysulfides are formed as intermediates of this step (Fig. 2a) (Prange *et al.*, 2004). In Fig. 1, polysulfides are not shown for clarity. In addition, small amounts of thiosulfate (up to 0.15 mM, see Figs 1b and 2a) were detected during the phase of sulfide depletion. This has been reported before for *Alc. vinosum* wild-type (Steudel *et al.*, 1990) and also for several green sulfur bacteria (*Chlorobiaceae*) (Trüper & Fischer, 1982; Trüper, 1984). Oxidation of stored sulfur and concomitant formation of sulfate started when sulfide was completely used up.

When *Alc. vinosum* was grown on 2 mM thiosulfate, the substrate was consumed within 12 h (Fig. 1c). Only the sulfane sulfur of thiosulfate is transferred to sulfur globules, while the sulfone sulfur is immediately oxidized to sulfate (Hensen *et al.*, 2006). In the experiment shown in Fig. 1(c), 2.2 mM sulfur accumulated inside the cells and 2.4 mM sulfate was formed simultaneously. Later the stored sulfur was also completely oxidized, leading to a final measured sulfate concentration of 4.11 mM.

Fig. 1. Turnover of sulfur compounds (◆, sulfide; ■, sulfate; ▲, sulfur; ●, thiosulfate) by *Alc. vinosum* during photolithoautotrophic growth on 50 mM elemental sulfur (a), 2 mM sulfide (b) and 2 mM thiosulfate (c). A representative of triplicate experiments is shown in each panel. Protein concentrations were between 80 and 120 $\mu\text{g ml}^{-1}$.

Inorganic thiols in *Alc. vinosum* cells

As a second step, we analysed the content of inorganic thiols in sulfide-grown *A. vinosum* cells that had been separated from culture supernatants by centrifugation. Thereby, we intended to identify compounds possibly involved in sulfur activation and/or oxidation that are not excreted into the medium in appreciable amounts. Fig. 3 shows an example of an HPLC chromatogram obtained for the biman-derivatized cellular thiols of *Alc. vinosum*. Peaks corresponding to thiosulfate, sulfide and two polysulfides of different chain length were readily identified. As described above, all of these compounds were also detected in the medium. It should be noted, however, that polysulfides and thiosulfate still occurred in the cells when

Fig. 2. Turnover of sulfur compounds (◆, sulfide; △ and ◇, polysulfides of different chain length; ▲, sulfur; ●, thiosulfate) and occurrence of monosulfane sulfonic acids (○, compound RT15; ■, compound RT30) by *Alc. vinosum* during photolithoautotrophic growth on 2 mM sulfide. A representative of triplicate experiments is shown. (a) Sulfur compounds in culture supernatants; (b) sulfur compounds detected in *Alc. vinosum* cells separated from the medium by centrifugation. Protein concentrations were between 80 and 120 $\mu\text{g ml}^{-1}$.

these compounds had long been depleted from the medium and the cells had clearly entered the phase of sulfur oxidation (compare Fig. 2a and b). In the HPLC chromatograms two peaks, assigned as RT15 and RT30 in Fig. 3, could not be attributed to any standards we applied. These substances were only present in the cells and not detected in the medium. Information about the chemical

Fig. 3. Typical HPLC chromatogram of cellular biman-derivatized thiols of *Alc. vinosum* grown photolithoautotrophically on sulfide. Peaks labelled 'H₂O' or 'c' indicate non-thiols or reagent peaks.

nature of these compounds was gained by isolating their bimeane derivatives as described in Methods. As expected, the pure compounds each yielded a single peak upon HPLC analysis (Fig. 4, trace 1). The isolated compounds were individually reduced with TCEP, which yielded monosubstituted sulfide originating from the terminal thiol group that had initially reacted with mBBR (Fig. 4, trace 2). Other parts of the original sulfur compounds were not detected in this run because they were not substituted with bimeane and thus invisible for the fluorescence detector. In a last step, the products of reduction with TCEP were again derivatized with mBBR, resulting in formation of disubstituted sulfide and derivatized thiosulfate (Fig. 4, trace 3). This finding allowed the identification of both RT15 and RT30 as sulfane monosulfonic acids, which are sulfur chain molecules with a thiol group at one end and a sulfone group at the other end. Reductive cleavage obviously did not occur between the terminal sulfone group and the directly neighbouring sulfur atom because this would have led to formation of sulfite. The bond between the last sulfur atom and the sulfone group appeared to be quite resistant to reductive cleavage. In accordance, reductive cleavage of thiosulfate by TCEP at room temperature also did not occur at appreciable rates. RT30 has a longer sulfur chain than RT15, as more sulfide was released from RT30 (not shown). It has not been possible to determine the exact chain lengths for the two molecules as the fluorescent factor for monosubstituted sulfide is not known. The intracellular concentrations of RT30 and especially RT15 showed a tight correlation with the content of intracellular sulfur, reaching their maximum when sulfur began to be oxidized. These compounds may therefore be relevant intermediates during stored sulfur oxidation inside the cells.

XANES spectroscopy of culture supernatants

Suitability of the method. As described above, HPLC analysis of culture supernatants did not reveal the presence

of any soluble sulfur compounds that could potentially be involved in elemental sulfur mobilization. We therefore additionally applied the more sensitive XANES spectroscopy at the sulfur *K*-edge. This allows identification of most of the relevant sulfur compounds below the detection limit of HPLC. It should be pointed out, however, that this method is not suited for detection of sulfide in culture supernatants. Sulfide evaporates as hydrogen sulfide during the time necessary for sample preparation and XANES spectroscopy. Chemical transformation of sulfide into more oxidized forms by molecular oxygen can be excluded, as we have shown before that samples prepared under oxic and anoxic conditions do not significantly differ with regard to the percentage contributions of the various detected sulfur compounds (Franz *et al.*, 2009). HPLC remains the method of choice for determination of sulfide. With this minor limitation, XANES spectroscopy is an ideal complementary method to follow time-dependent changes of the contribution of individual sulfur species to all sulfur-containing compounds present.

At four time points, supernatants of *Alc. vinosum* cultures grown on elemental sulfur, thiosulfate or sulfide were investigated using XANES spectroscopy and the spectra were analysed quantitatively. Figs 5, 6 and 7 show the spectra for the different cultures and Table 1 summarizes the corresponding fitting results. Spectra of the reference substances used for fitting the supernatant spectra are presented in Fig. 8. Supernatant samples for XANES spectroscopy of sulfide- and thiosulfate-grown cultures were taken after 15 min, 45 min, 3 h and 48 h. For cultures grown on elemental sulfur, sampling points were at 15 min, 3 h, 12 h and 48 h, because uptake of elemental sulfur does not start until 3 h after inoculation (Franz *et al.*, 2007). For all culture supernatants fitting of the XANES spectra with WinXAS showed that the relative percentage contribution of sulfate was in good accordance with the HPLC results. After 48 h, sulfate was the dominant sulfur

Fig. 4. Identification of compound RT15 as monosulfane sulfonic acid by HPLC analysis of the isolated compound (trace 1), the products of reduction with TCEP (trace 2) and the compounds resulting from bimeane derivatization of the reduction products (trace 3). Equations for the underlying reactions are given next to the respective chromatograms.

Fig. 5. Sulfur *K*-edge XANES spectra (solid lines) of the supernatants of *Alc. vinosum* cultures during growth on elemental sulfur 15 min (a), 3 h (b), 12 h (c) and 48 h (d) after inoculation. Spectrum (e) shows the 0 medium before inoculation. Accompanying fits are shown as dashed lines (a.u., arbitrary units). Reduction factors used to scale all spectra to approximately the same size are given as asterisked numbers.

compound in all cultures according to both methods (Figs 1, 5, 6 and 7, Table 1). Furthermore, the depletion of thiosulfate during growth on sulfide or thiosulfate observed by HPLC analysis was mirrored by the XANES spectroscopic results. In contrast to HPLC analysis, XANES spectroscopic analysis detected small amounts of sulfate already in the samples taken after 15 min when sulfate formation by *Alc. vinosum* had not yet started. This sulfate originated from the culture medium, which contains micromolar amounts of FeSO_4 as part of the trace element solution (Fig. 5e, Table 1). At that concentration, sulfate is below the detection limit of the HPLC method applied. Traces of thiosulfate, C–S–H species, sulfones and sulfonates were also detected in the medium before inoculation.

Supernatants taken after 15 min from cultures grown on elemental sulfur showed a relatively high percentage contribution of sulfate. This might cause the misleading impression that these supernatants indeed contained high concentrations of sulfate. However, this is not the case. In fact, supernatants of cells grown on the insoluble substrate elemental sulfur have very low total concentrations of sulfur compounds, and the sulfate originating from the trace element solution is simply the major one of these

Fig. 6. Sulfur *K*-edge XANES spectra (solid lines) of the supernatants of *Alc. vinosum* cultures during growth on sulfide 15 min (a), 45 min (b), 3 h (c) and 48 h (d) after inoculation. Accompanying fits are shown as dashed lines (a.u., arbitrary units). Asterisked numbers: see Fig. 5.

minor players. In accordance with this interpretation, supernatants of sulfur-grown cells taken 15 min, 3 h and 12 h after substrate addition gave rise to comparatively noisy spectra due to the low total concentrations of soluble sulfur compounds (Figs 5, 6 and 7). In contrast, the spectrum for the sample taken after 48 h is essentially waveless, coinciding with a substantial increase in sulfate concentration. Interestingly, according to the XANES spectra, thiosulfate appears to be present in the *Alc. vinosum* culture grown on elemental sulfur although it was not detected by established HPLC methods with a detection limit of 1 μM thiosulfate (Rethmeier *et al.*, 1997).

Detection of organic thiols and disulfides. In addition to thiosulfate and sulfate, the XANES spectra of samples taken at the first three time points from cultures with the three different electron donors showed clear structures at energy positions of 2471–2475 eV (Figs 5, 6 and 7). These are due to sulfur compounds with the structure C–S–S–C and/or C–S–H. None of these compounds appeared to be specific for growth on elemental sulfur and they are therefore unlikely candidates for mobilization of this insoluble substrate. However, the development of percentage contributions of these compounds over time exhibited significant differences when growth on sulfide, thiosulfate

Fig. 7. Sulfur *K*-edge XANES spectra (solid lines) of the supernatants of *Alc. vinosum* cultures during growth on thiosulfate 15 min (a), 45 min (b), 3 h (c) and 48 h (d) after inoculation. Accompanying fits are shown as dashed lines (a.u., arbitrary units). Asterisked numbers: see Fig. 5.

and elemental sulfur was compared (Table 1). In sulfide-grown cultures, exclusively species of the structure C–S–H were present after 45 min, 3 h and 48 h. When thiosulfate was used as electron donor for anoxygenic photosynthesis, C–S–H sulfur species were present after 15 min, 45 min and 3 h, but completely disappeared after 48 h. At that time point, C–S–S–C species were found. It is noteworthy that the spectrum of culture supernatant after 48 h growth on thiosulfate could not be fitted with oxidized and reduced glutathione as reference compounds for C–S–S–C and C–S–H species. Instead, fitting with cystine and cysteine yielded useful results, showing that glutathione itself is not present in culture supernatants of *Alc. vinosum*. During uptake and oxidation of elemental sulfur, both C–S–S–C and C–S–H species were present in varying amounts.

Detection of sulfones and sulfonic acids. In addition to the features described above, XANES spectra of samples taken at the first three time points from cultures with the three different electron donors showed clear structures at energy positions of 2478.5–2480.0 eV (Figs 5, 6 and 7a, b, c, respectively). These are characteristic for higher oxidized sulfur species (Vairavamurthy *et al.*, 1994). Fitting of the spectra taken after 48 h (Figs 5, 6 and 7d, respectively) still indicated minor contribution of spectroscopic features

Fig. 8. Sulfur *K*-edge XANES spectra of the reference compounds that were used for fitting spectra of supernatants: oxidized glutathione (a), reduced glutathione (b), cysteine (c), cystine (d), sodium thiosulfate (e), methionine sulfone (f), cysteic acid (g) and zinc sulfate (h) (a.u., arbitrary units).

caused by sulfone and sulfonates (Table 1); however, at that time point, sulfate with a structure at 2481.4 eV was clearly the dominant sulfur compound in the supernatants. Methionine sulfone and cysteic acid were identified as adequate reference compounds for fitting the conspicuous spectral structures described. This finding showed that the culture supernatants contained sulfur compounds belonging to the organic sulfones (C–SO₂–C) and sulfonates (C–SO₃[–]). Detailed quantitative analysis of the XANES spectroscopic data revealed that organic sulfones were present during growth on sulfide and elemental sulfur (Table 1). Their percentage contribution increased slightly over time during growth on these substrates. On thiosulfate as a substrate, sulfones were not detected. In contrast, organic sulfonates were present in all culture supernatants (Table 1). Their relative percentage contributions decreased dramatically within 48 h in the culture grown on sulfide; they decreased less in cultures grown on thiosulfate, and first decreased and then increased again in cultures grown on elemental sulfur.

DISCUSSION

One of the most interesting findings of our study is the presence of polysulfides and monosulfane sulfonic acids in cells of *Alc. vinosum* grown on sulfide (Fig. 2). Of the latter,

the concentration of compound RT15 showed an especially tight correlation with the amount of intracellularly stored sulfur. RT15 reached its maximum when the cells had just started to oxidize sulfur. To understand the probable origin and relevance of the detected sulfur compounds, we have to look at the current models for stored sulfur oxidation in *Alc. vinosum* (Dahl *et al.*, 2008; Cort *et al.*, 2008). In this organism several proteins encoded in the *dsr* operon have been shown to be essential for the degradation of periplasmic sulfur globules to the end product sulfate. The Dsr proteins are either cytoplasmic or membrane-bound. It is proposed that sulfur is transported into the cytoplasm in a persulfidic form, possibly as glutathione amide persulfide. Once in the cytoplasm, the sulfane sulfur is made available to sulfite reductase, probably via multiple sulfur transfer reactions involving the proteins DsrEFH and DsrC (Dahl *et al.*, 2008; Cort *et al.*, 2008), and oxidized to sulfite. We have previously indicated the possibility that DsrC serves as a substrate-donating molecule for sulfite reductase (Cort *et al.*, 2008). This would imply oxidation of DsrC-bound sulfur to a sulfone group. Possibly, more than one sulfur atom can be transferred to the conserved active-site cysteine of DsrEFH and DsrC, which could explain release of short-chain polysulfides or sulfane monosulfonic acids. On this background, we can envision intracellular polysulfides and also monosulfane sulfonic acids as intermediates or side products of sulfur trafficking between persulfidic sulfur imported into the cytoplasm and sulfite reductase.

One major goal of our study was to identify soluble sulfur compounds possibly involved in the mobilization of externally added elemental sulfur. XANES spectroscopy proved to be a valuable tool to detect sulfur compounds present in culture supernatants in very low concentrations. Using this method, we succeeded in detecting thiolic and disulfidic compounds as well as sulfones and sulfonic acids in varying concentrations during growth on three different sulfur substrates (elemental sulfur, thiosulfate and sulfide). The occurrence of these compounds in the medium is in itself interesting and cannot be easily explained by the current models for sulfur compound oxidation in phototrophic sulfur bacteria (Frigaard & Dahl, 2009). It will be an interesting and demanding task for the future to clarify the origin and role of these substances.

In conclusion, we have to note that none of the newly detected compounds occurred specifically during growth on elemental sulfur, which renders unlikely the idea that any of these compounds are specifically involved in mobilization of this insoluble substrate. One probable reason for the lack of specific soluble intermediates in the culture supernatants of *Alc. vinosum* during growth on elemental sulfur is the close cell–sulfur contact necessary for uptake of elemental sulfur (Franz *et al.*, 2007), during which exchange of soluble substances may not be required. Cell–sulfur contact may be provided by extracellular polymeric substances (EPS), as has been described for *Ath. ferrooxidans* (Gehrke *et al.*, 1998). Intermediates

formed during elemental sulfur mobilization could stay embedded in the EPS film and therefore be removed together with sulfur and cells from culture supernatants by centrifugation. Future experiments will try to answer this question by investigating EPS extracted from *Alc. vinosum* via HPLC and XANES spectroscopy. Another useful experimental strategy is ‘blocking’ of the sulfur cell contact by the ‘layer-by-layer’ technique (e.g. Lvov & Möhwald, 2000).

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG grants no. PR708/4-1, DA351/3-4 and DA351/5-1). The authors would like to thank the State of Louisiana for providing the operating budget of CAMD and CAMD’s staff, especially John Scott, Amitava Roy, Gregory Merchan and Russell ‘Rusty’ L. Louis for their support.

REFERENCES

- Bacon, M. & Ingledew, W. J. (1989). The reductive reactions of *Thiobacillus ferrooxidans* on sulphur and selenium. *FEMS Microbiol Lett* **58**, 189–194.
- Brune, D. C. (1995). Sulfur compounds as photosynthetic electron donors. In *Anoxygenic Photosynthetic Bacteria*, pp. 847–870. Edited by R. E. Blankenship, M. T. Madigan & C. E. Bauer. Dordrecht: Kluwer.
- Cort, J. R., Selan, U. M., Schulte, A., Grimm, F., Kennedy, M. A. & Dahl, C. (2008). *Allochromatium vinosum* DsrC: solution-state NMR structure, redox properties and interaction with DsrEFH, a protein essential for purple sulfur bacterial sulfur oxidation. *J Mol Biol* **382**, 692–707.
- Dahl, C. (2008). Inorganic sulfur compounds as electron donors in purple sulfur bacteria. In *Sulfur in Phototrophic Organisms*, pp. 289–317. Edited by R. Hell, C. Dahl, D. B. Knaff & T. Leustek. Dordrecht: Springer.
- Dahl, C., Schulte, A., Stockdreher, Y., Hong, C., Grimm, F., Sander, J., Kim, R., Kim, S.-H. & Shin, D. H. (2008). Structural and molecular genetic insight into a wide-spread bacterial sulfur oxidation pathway. *J Mol Biol* **384**, 1287–1300.
- Franz, B., Lichtenberg, H., Hormes, J., Modrow, H., Dahl, C. & Prange, A. (2007). Utilization of solid “elemental” sulfur by the phototrophic purple bacterium *Allochromatium vinosum*: A sulfur K-edge XANES spectroscopy study. *Microbiology* **153**, 1268–1274.
- Franz, B., Lichtenberg, H., Hormes, J., Dahl, C. & Prange, A. (2009). The speciation of soluble sulfur compounds in bacterial culture fluids by X-ray absorption near edge structure spectroscopy. *Environ Technol* (in press).
- Frigaard, N.-U. & Dahl, C. (2009). Sulfur metabolism in phototrophic sulfur bacteria. *Adv Microb Physiol* **54**, 103–200.
- Gehrke, T., Telegdi, J., Thierry, D. & Sand, W. (1998). Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. *Appl Environ Microbiol* **64**, 2743–2747.
- Hensen, D., Sperling, D., Trüper, H. G., Brune, D. C. & Dahl, C. (2006). Thiosulfate oxidation in the phototrophic sulfur bacterium *Allochromatium vinosum*. *Mol Microbiol* **62**, 794–810.
- Hormes, J., Scott, J. D. & Suller, V. P. (2006). Facility update: the Center for Advanced Microstructures and Devices: a status report. *Synchr Rad News* **19**, 27–30.

- Lemonnier, M., Collet, O., Depautex, C., Esteva, J.-M. & Raoux, D. (1978). High vacuum two crystal soft X-ray monochromator. *Nucl Instrum Methods* **152**, 109–111.
- Lvov, Y. & Möhwald, H. (editors) (2000). *Protein Architecture: Interfacing Molecular Assemblies and Immobilization Biotechnology*. New York: Marcel Dekker Publications.
- Modrow, H., Visel, F., Zimmer, R. & Hormes, J. (2001). Monitoring thermal oxidation of sulfur crosslinks in SRB-elastomers by quantitative analysis of sulfur K-edge XANES-spectra. *Rubber Chem Tech* **74**, 281–294.
- Prange, A., Chauvistré, R., Modrow, H., Hormes, J., Trüper, H. G. & Dahl, C. (2002). Quantitative speciation of sulfur in bacterial sulfur globules: X-ray absorption spectroscopy reveals at least three different speciations of sulfur. *Microbiology* **148**, 267–276.
- Prange, A., Birzele, B., Krämer, J., Chauvistré, R., Modrow, H., Hormes, J. & Köhler, P. (2003). Characterization of sulfur speciation in low molecular weight subunits of glutenin after reoxidation with potassium iodate and potassium bromate at different pH values using X-ray absorption near edge structure (XANES) spectroscopy. *J Agric Food Chem* **51**, 7431–7438.
- Prange, A., Engelhardt, H., Trüper, H. G. & Dahl, C. (2004). The role of the sulfur globule proteins of *Allochrocatium vinosum*: mutagenesis of the sulfur globule protein genes and expression studies by real-time RT PCR. *Arch Microbiol* **182**, 165–174.
- Prange, A., Modrow, H., Hormes, J., Krämer, J. & Köhler, P. (2005). Influence of mycotoxin producing fungi (*Fusarium*, *Aspergillus*, *Penicillium*) on gluten proteins during suboptimal storage of wheat after harvest and competitive interactions between field and storage fungi. *J Agric Food Chem* **53**, 6930–6938.
- Ressler, T. (1998). WinXAS: a program for X-ray absorption spectroscopy data analysis under MS-Windows. *J Synchrotron Radiat* **5**, 118–122.
- Rethmeier, J., Rabenstein, A., Langer, M. & Fischer, U. (1997). Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods. *J Chromatogr A* **760**, 295–302.
- Rohwerder, T. & Sand, W. (2003). The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp. *Microbiology* **149**, 1699–1709.
- Steudel, R. (1996). Das gelbe Element und seine erstaunliche Vielseitigkeit. *Chem Unserer Zeit* **30**, 226–234.
- Steudel, R., Holdt, G., Visscher, P. T. & van Gernerden, H. (1990). Search for polythionates in cultures of *Chromatium vinosum* after sulfide incubation. *Arch Microbiol* **153**, 432–437.
- Thiele, H. H. (1968). Sulfur metabolism in Thiorhodaceae. V. Enzymes of sulfur metabolism in *Thiocapsa floridana* and *Chromatium* species. *Antonie Van Leeuwenhoek* **34**, 350–356.
- Trüper, H. G. (1984). Phototrophic bacteria and their sulfur metabolism. In *Sulfur, its Significance for Chemistry, for the Geo-, Bio-, and Cosmo-sphere and Technology*, pp. 367–382. Edited by A. Müller & B. Krebs. Amsterdam: Elsevier Science Publishers B.V.
- Trüper, H. G. & Fischer, U. (1982). Anaerobic oxidation of sulphur compounds as electron donors for bacterial photosynthesis. *Philos Trans R Soc Lond B Biol Sci* **298**, 529–542.
- Vairavamurthy, A., Zhou, W., Eglinton, T. & Manowitz, B. (1994). Sulfonates: a novel class of organic sulfur compounds in marine sediments. *Geochim Cosmochim Acta* **58**, 4681–4687.
- Yagi, S., Kitai, S. & Kimura, T. (1971). Oxidation of elemental sulfur to thiosulfate by *Streptomyces*. *Appl Microbiol* **22**, 157–159.

Edited by: G. Muyzer