Utilization of ‘elemental’ sulfur by different phototrophic sulfur bacteria (Chromatiaceae, Ectothiorhodospiraceae): A sulfur K-edge XANES spectroscopy study

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Abstract. Phototrophic sulfur bacteria are generally able to use elemental sulfur as an electron donor for anoxygenic photosynthesis. Elemental sulfur is mainly a mixture of cyclo-octasulfur and polymeric sulfur. The purple sulfur bacterium Allochromatium vinosum strongly prefers the polymeric sulfur fraction showing that sulfur speciation has a strong influence on availability of elemental sulfur. X-ray absorption near edge structure (XANES) spectroscopy was used to investigate whether polymeric sulfur is also the preferred sulfur species in other purple sulfur bacteria belonging to the families Chromatiaceae and Ectothiorhodospiraceae. The cultures were fed with 50 mM of elemental sulfur consisting of 68% polymeric sulfur and 30% cyclo-octasulfur. In all cultures, elemental sulfur was converted into intra- or extracellular sulfur globules, respectively, and further oxidized to sulfate. Sulfate concentrations were determined by HPLC and turbidometric assays, respectively. However, the added elemental sulfur was only partly used by the bacteria, one part of the ‘elemental sulfur’ remained in the cultures and was not taken up. XANES spectroscopy revealed that only the polymeric sulfur fraction was taken up by all cultures investigated. This strongly indicates that polymeric ‘chain-like’ sulfur is the form preferably used by phototrophic sulfur bacteria.

Introduction
The utilization of reduced sulfur compounds as electron donors for anoxygenic photosynthesis is common among the purple sulfur bacteria [1]. The purple sulfur bacteria belong to the $\gamma$-proteobacteria and cover the two families Chromatiaceae and Ectothiorhodospiraceae. Both form deposits of sulfur as an intermediate during the oxidation of reduced sulfur compounds. The most important difference between the two families is the formation of sulfur globules inside the cell in Chromatiaceae while Ectothiorhodospiraceae accumulate sulfur globules outside the cell.

Next to sulfide and in some cases also thiosulfate, many members of the Chromatiaceae and Ectothiorhodospiraceae can use elemental sulfur as an electron donor. All sulfur allotropes are

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hydrophobic, not wetted by water and they hardly dissolve in water [2]. Cyclic, orthorhombic α-sulfur ($\alpha-S_8$) (cyclo-octasulfur or $S_8$ rings) is the thermodynamically most stable form of elemental sulfur at ambient temperature and pressure [e.g. 3, 4]. However, the customary in trade typical elemental sulfur (“flowers of sulfur”) mainly consists of $S_8$ rings and some polymeric sulfur which consists of chain-like macromolecules [5, 6]. The bonding energy between S-S bonds in polymeric sulfur is 2.4 kJ mol$^{-1}$ weaker than in cyclo-octasulfur [7, 8], therefore, polymeric sulfur, i.e. chain-like sulfur, might be easier accessible for sulfur oxidizing bacteria and elemental sulfur utilization.

And in fact, when *Allochromatium vinosum*, a member of the *Chromatiaceae*, was cultivated photolithoautotrophically with elemental sulfur as single sulfur source, we found evidence that only the polymeric sulfur fraction was used [9]. The goal of this study was to investigate whether polymeric sulfur also is the preferred sulfur species for three other phototrophic sulfur bacteria.

**Material and Methods**

*Bacterial strains, medium, growth conditions.* Thiocapsa roseopersicina BBS was cultivated in Pfennig’s medium [10] under anaerobic conditions at 30 °C in the light. Halorhodospira halophila SL1 DSMZ 244 and Halorhodospira abdelmalekii DSMZ 2110 were cultivated in medium for extremely halophiles [11] under anaerobic conditions at 40 °C in the light. Growth experiments with 50 mM elemental sulfur were performed like described in [9] with the respective medium without sulfide and thiosulfate. As sulfur source commercially available ‘elemental’ sulfur was purchased and used as received from Riedel de Haen (Seelze, Germany) in the growth experiments. All experiments were carried out in duplicate, in the results section one of the two experiments is shown as a representative.

**Determination of sulfur compounds by HPLC.** Sulfur compounds (sulfide, thiosulfate, sulfite, sulfate) were determined by High Pressure Liquid Chromatography (HPLC) using the methods described in [12]. Sulfate was measured in culture supernatants.

**Turbidometric determination of sulfate.** Due to the high salt content in the medium of the *Halorhodospira* species which overlaid the desired peak sulfate could not be determined via anion-exchange chromatography like described above. Therefore, we used the turbidometric method of Sörbo [13].

**Determination of protein concentration.** The protein concentration of batch culture samples of the various bacteria was determined using Bradford reagent (Sigma, Taufkirchen, Germany) as specified by the manufacturer.

**X-ray absorption near edge structure (XANES) spectroscopy – Experimental and Sample preparation.** XANES spectroscopy and sample preparation was performed like described in [9,14].

**Reference compounds.** Pure cyclo-octasulfur ($S_8$ rings) and polymeric sulfur were used as reference compounds, both kindly provided by Prof. Dr. R. Steudel, TU Berlin. The reference compounds were ground into fine powder and put homogeneously on a Kapton® film.

**Results and Discussion**

The aim of this investigation was to clarify whether other phototrophic sulfur bacteria prefer -like *Alc. vinosum*- sulfur chains for utilization of water-insoluble elemental sulfur. Therefore, *Tca. roseopersicina*, *Hlr. halophila* and *Hlr. abdelmalekii* were cultivated with 50 mM of ‘elemental’ (zero-valent) sulfur as single sulfur source. The formation and subsequent degradation of sulfur globules of externally added elemental sulfur was observed microscopically and quantified indirectly by determining the final oxidation product sulfate. Also, the concentration of protein in the cultures was determined as an indicator of growth of the cells.

Firstly, the exact chemical speciation of ‘elemental’ sulfur that was used for the growth experiments was analyzed by XANES spectroscopy to determine the ratio of $S_8$ rings and polymeric sulfur. Figure 1a, b shows the sulfur K-edge XANES spectra of the reference compounds pure cyclo-octasulfur and pure polymeric sulfur, Figure 1c the sulfur K-edge XANES spectrum of the elemental
sulfur used for the growth experiments and its corresponding fit with the reference compounds. The quantitative analysis of the spectrum showed a percentage contribution of 68% for polymeric sulfur and 30% for cyclo-octasulfur (Table 1).

Figure 1. Sulfur K-edge XANES spectra of the reference compounds polymeric sulfur (a), cyclo-octasulfur (S₈ rings) (b) which were used for fitting sulfur spectra, elemental sulfur used in the growth experiments (c), remaining sulfur platelets in the cultures and accompanying fits (dashed lines) of Tca. roseopersicina (d), Hlr. halophila (e) and Hlr. abdelmalekii (f) (a.u. = arbitrary units).

Table 1. Results of fitting the sulfur K-edge XANES spectra of the added sulfur and the remaining sulfur platelets in the various cultures to the sum of the reference spectra.

| sample                      | percentage contribution of sulfur species* |
|-----------------------------|--------------------------------------------|
| sulfur added                | polymeric sulfur                           |
|                             | sulfur remaining                           |
| Thiocapsa roseopersicina    | 30                                          |
| sulfur remaining            | 68                                          |
| Halorhodospira halophila    | 84                                          |
| sulfur remaining            | 16                                          |
| Halorhodospira abdelmalekii | 59                                          |
| sulfur remaining            | 41                                          |
|                             | 58                                          |
|                             | 42                                          |

*different sulfur species and their percentage contribution to the sulfur speciation; error: < ± 10%

When Tca. roseopersicina, Hlr. halophila and Hlr. abdelmalekii were fed with elemental sulfur, all organisms started to accumulate sulfur globules - intracellular ones by Tca. roseopersicina and extracellular ones by the Halorhodospira species - of the externally added elemental sulfur, but, however, they showed some differences in velocity of elemental sulfur conversion (Table 2). Hlr. halophila took up elemental sulfur during the first 3 h after addition of elemental sulfur, Hlr. abdelmalekii started formation of sulfur globules after 6 h and Tca. roseopersicina after 12 h. Furthermore, for all strains tested the protein concentration of the cultures increased (data not shown) during the course of the experiments indicating growth of all cultures with elemental sulfur.
Further oxidation to sulfate and therefore degradation of the sulfur globules was detected for *Thiocapsa roseopersicina* and *Halorhodospira halophila*. Sulfate formation did not occur for *Halorhodospira abdelmalekii* during 240 h. This is in accordance with the observations of Then and Trüper [15]. According to their experiments *Halorhodospira abdelmalekii* does not appear to be able to oxidize stored sulfur to sulfate. In all cultures the elemental sulfur formed small sulfur platelets at the beginning of sulfate formation or 48 h after addition of elemental sulfur in case of *Halorhodospira abdelmalekii*. They were separated from the cells by centrifugation and their speciation was determined by XANES spectroscopy (Fig. 1d, e, f). Spectra were fitted with cyclo-octasulfur and polymeric sulfur as reference substances (Table 1). For *Thiocapsa roseopersicina* as well as for the *Halorhodospira* strains the cyclo-octafraction of the remaining elemental sulfur increased significantly while the polymeric fraction decreased. This strongly indicates that all strains at least prefer the polymeric fraction of elemental sulfur.

In *Thiocapsa roseopersicina* cultures the concentration of sulfate reached 240 h after the addition of elemental sulfur 34.6 mM and remained constant for further 23 h. When *Thiocapsa roseopersicina* completely oxidizes 50 mM elemental sulfur, one would expect the formation of 50 mM sulfate. A concentration of 34.5 mM corresponds to 69 % of the expected 50 mM which fits quite well to the 68 % of polymeric sulfur in the added elemental sulfur. This yields further evidence that *Thiocapsa roseopersicina* uses only the polymeric fraction of elemental sulfur. However, *Halorhodospira halophila* forms only 12.7 mM sulfate which corresponds to 25 % of the expected 50mM of sulfate which is far from the 68 % polymeric fraction in the added elemental sulfur. This is probably caused by an incomplete further oxidation of extracellularly stored sulfur which also explains the high polymeric contribution in the XANES spectra. Sulfur globules in *Halorhodospira halophila* consist of sulfur chains [16] which would simulate higher polymeric sulfur content of the remaining sulfur. This is also the case for *Halorhodospira abdelmalekii*.

**Table 2.** Sulfate determination and observation by light microscopy of sulfur globule formation in cultures fed with 50 mM elemental sulfur. The % values in parentheses show the relative amount of sulfur recovered as sulfate, compared to a total of 50 mM in the culture.

| Time [h] | Thiocapsa roseopersicina | Halorhodospira halophila | Halorhodospira abdelmalekii |
|---------|---------------------------|--------------------------|-----------------------------|
| 0       | 0.0                       | 0.0                      | 0.0                         |
| 3       | 0.0                       | 0.0                      | 0.0                         |
| 12      | 1.7                       | 2.6                      | 0.0                         |
| 33      | 6.9                       | 3.4                      | 0.0                         |
| 72      | 29.2                      | 4.2                      | 0.0                         |
| 124     | 25.3                      | 5.2                      | 0.0                         |
| 192     | 34.6                      | 10.9                     | 0.0                         |
| 240     | 34.5                      | 12.7                     | 0.0                         |
| 263     |                           |                           |                             |

* sulfate globules were not observed

In summary, our results show that next to *Alc. vinosum* other members of the *Chromatiaceae* as well as members of the *Ectothiorhodospiraceae* appear to use only or at least strongly prefer the polymeric fraction of elemental sulfur as an electron donor for anoxygenic photosynthesis. All strains tested - the one belonging to the *Chromatiaceae* that form intracellular sulfur globules as well as the members of *Ectothiorhodospiraceae* that accumulate extracellular sulfur deposits -
showed as an similarity that they prefer the polymeric fraction of elemental sulfur. Our results point towards ‘sulfur chains’ as “microbiologically preferred form of elemental, zero valent sulfur” in purple sulfur bacteria. Next to preferred utilization of externally added polymeric sulfur the sulfur globules of all tested organisms consist exclusively of sulfur chains [16]. Therefore, it appears that the organisms are completely unable to deal with sulfur rings, the chemically more stable cyclo-octasulfur, neither outside nor inside the cell. Other sulfur oxidizing bacteria might also prefer ‘sulfur chains’. This hypothesis gains some support by the general agreement that rather linear sulfur forms than the octameric sulfur rings are the actual substrates for the sulfur oxidizing enzymes in acidophilic sulfur oxidizing prokaryotes [17].

Our results show that the molecular composition of elemental sulfur is an important factor for its utilization in purple sulfur bacteria. They can only use or at least strongly prefer the polymeric sulfur fraction (sulfur chains) when taking up ‘elemental’ sulfur. Probably, purple sulfur bacteria cannot deal with cyclo-octasulfur at all - neither outside nor inside the cell.

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