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Investigation of S–H bonds in biologically important compounds by sulfur K-edge X-ray absorption spectroscopy

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Abstract. X-ray Absorption Near Edge Structure (XANES) spectroscopy, often provides a direct correlation between observed resonances in the spectrum and molecular bonds in the sample. This can be used as a fingerprint for the presence of a given molecular environment of the absorber atom in a sample. As the white line is found at similar energy positions for S–C and S–H bonds, this approach is impossible when both types of bond are present simultaneously, as often in biological systems. To develop a criterium for the presence of S–H bonds in such samples, reduced glutathione, reduced coenzyme A, cysteine and their corresponding oxidized forms were investigated using sulfur K-edge XANES, revealing a unique feature at 2 475.8 eV in the respective difference spectra. To correlate this structure to S–H bonds, H_2S and H_2S_2 were measured, whose difference spectrum also shows a structure at this energy position, whereas it is not present throughout a variety of C–S–C/C–S–S–C environments. Theoretical investigations suggest its correlation to a Rydberg transition occurring in the case of a S–H bond. Using this criterium, the presence of S–H bonds is in the purple sulfur bacterium *Allochromatium vinosum* during oxidation of intracellular accumulated sulfur, is proved, as expected from biological considerations.

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1 Introduction

Thiol-groups play an essential role in biological processes, *e.g.* in the prosthetic group like coenzyme A and in the amino acid L-cysteine. The S–H groups of cysteine residues often form S–S bridges by oxidation which are responsible for the stability of many proteins, *e.g.* the very stable keratines [1] and the flexibility *e.g.* of the gluten network [2]. Glutathione, γ-L-glutamyl-L-cysteinylglycine, is the most abundant thiol in animal cells [3,4] containing cysteinyl thiol with an important role for the redox state. Consequently, the *in situ* detection of S–H bonds is of crucial importance for the understanding of interaction mechanisms between the building blocks of life.

X-ray absorption near edge structure (XANES) spectroscopy at the sulfur K-edge provides an excellent tool to characterize the electronic and/or geometric structure of sulfur. Detailed information about the valency, the coordination geometry and the effective charge of the absorbing atom can be obtained [5,6]. This technique is well suited for the investigation of sulfur *e.g.* during the vulcanisation process of rubber [7–9] and in intact biological samples [4,10–12]. *In situ* measurements are possible in many cases because of the penetration strength of X-rays.

In general, the detection of S–H bonds in biologically relevant samples by S K-XANES measurements is a difficult task. Systems containing "pure" S–H-bonds have been subject to a number of studies [13,14], and the same is true for systems containing "pure" S–C bonds [8,15]. In most biological systems, however, both S–H and S–C bonds are found simultaneously. For this reason, a direct fingerprinting approach, relying on the shape and energy position of the white line, *i.e.* the most intense structure in the XANES spectrum, cannot be used, because the energy ranges typical for the presence of S–C bonds and S–H bonds coincide. In an attempt to find spectral features in the S K-XANES spectra that can be used for identifying S–H bonds in biological systems in which S–C bonds are also present, several biological model compounds (oxidized and reduced forms) and H_2S as well as H_2S_2 were

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investigated in this study. Furthermore, a variety of references for pure C–S–C bond environments were investigated as a "negative control". As an example for the applicability of the criterium, we investigated a complex biological system, the purple sulfur bacterium *Allochromatium vinosum*. It is known that *A. vinosum*, which belongs to the family Chromatiaceae, uses reduced sulfur compounds as electron donors for anoxygenic photosynthesis and CO_2 fixation. In a first step, sulfide (or thiosulfate) is oxidized to sulfur chains most probably carrying so far unidentified organic residues at one or both ends stored as intracellular sulfur globules as an intermediate [11,12]. When sulfide is depleted, the further oxidation to sulfate begins [16]. By taking "time resolved" S K-XANES spectra over 10 h during the formation and the following oxidation of the sulfur globules in *A. vinosum*, Prange *et al.* showed that the sulfur chains were gradually shortened [11]. During the oxidation from sulfur to sulfate a structure in the spectrum was observed, which could so far only vaguely be connected to the presence of S–H bonds as they occur in thiols. Independently, molecular genetic evidence led Pott and Dahl [17] to the proposal that thiols are involved in or occur as intermediates during the decomposition of sulfur globules.

It was the goal of this study to provide a spectral criterium enabling the detection of S–H bonds in biological systems in the presence of S–C bonds using S K-edge XANES and its application in order to find evidence for sulfhydryl groups during the sulfur oxidation in *A. vinosum*.

2 Material and methods

2.1 Bacterial strains, media, growth conditions

Allochromatium vinosum D DSM180^T was grown photolithoautotrophically in sulfide-containing medium (Pfennig's medium) [18] and prepared for the XANES spectroscopy measurements as described previously [11,12].

2.2 Reference compounds

For investigation of the S–H bonds reduced glutathione, coenzyme A (reduced form) and cysteine were chosen. The corresponding oxidized forms of these substances (oxidized glutathione, oxidized coenzyme A and cystine) with S–S bonds were used for comparison. These compounds were of reagent grade, purchased from Sigma (Deisenhofen) and used as received. All compounds were ground into fine powder and put on a self-adhesive kapton film. The solid reference compounds were ground into fine powder and put homogeneously on a self-adhesive kapton film. The thickness of the samples was optimized in order to avoid both possible thickness- and pinhole-effects.

H_2S was taken from a commercially obtained lecture bottle. Disulfane (H_2S_2) was prepared by the cracking distillation of a raw sulfane mixture [19]. In the presence of a rough or not acidified surfaces H_2S_2 reacts fast to H_2S and sulfur. However, with our experimental setup even after several measurements, no trace of sulfur was found inside the cell or on the windows, which strongly indicates the integrity of the measured substance. Another indicator for this fact is that inside the cooling traps H_2S_2 was accumulated quantitatively. Mono- and disulfanes R–S–S–R/R–S–R ($R = -CH(CH_3)_2$, $R = -C-(CH_3)_3$, $R = -(CH_2)_3CH_3$) were obtained commercially, whereas the series of bis (4-octene-3-yl)-*n*-sulfanes (n = 1–3) were synthesized as described previously [20]. 3-mercapto-4-octene, the key-compound in the synthesis of the bis (4-octene-3-yl)sulfanes was obtained from the reaction of the corresponding bromooctene with thiourea. The thiol was reacted with 3-bromo-4-octane, iodine and sulfurdichloride to yield the mono-, di- and trisulfane, respectively. In all cases, the *trans*-compounds showing small contamination by *cis*-configurated isomers were obtained.

2.3 Experimental

The XANES spectra were recorded using synchrotron radiation at the beamline BN3 of the Electron Stretcher Accelerator (ELSA) in Bonn [21] running in storage ring mode at 2.3 GeV with electron currents between 80 and 20 mA. A modified Lemonnier type double crystal monochromator [22] equipped with InSb (111) crystals was used. The monochromatic flux rate at the sample was approximately 10^9 photons per second and energy interval at 50 mA. The XANES spectra were obtained in transmission mode using standard techniques. Further details of the experimental procedure have been published previously [7,8,23]. For energy calibration of the XANES spectra, the spectrum of $ZnSO_4$ was used as a secondary standard near the sulfur K-edge. The energy of the white line of sulfate was defined to be at 2 481.4 eV. According to the step width of the monochromator, this value is reproducible to ±0.1 eV. The XANES spectra of H_2S/H_2S_2 were recorded in gas phase using a 15 cm long absorption cell made from Teflon. This cell was separated from the ionisation chambers by 12.5 μm thick windows made from kapton® (DuPont). To obtain the necessary vapour pressure inside the cell, the cell was first evacuated using a roughing pump in the exhaust line of the absorption cell. The pump was protected by a double cooling trap from traces of H_2S/H_2S_2. By variation of the ambient temperature of the cooled sample vial which was attached to the supply line of the cell, the vapour pressure of H_2S/H_2S_2 and thus the gas pressure in the measurement cell could be optimized to ≈0.5 mbar. It should be noted that due to the presence of the cooling traps during the measurement quasistatic conditions were present. Gas molecules were removed from the cell, being trapped inside the cooling cell; this loss was compensated by new molecules from the supply line. Spectra were scanned with step widths of 0.5 eV in the pre-edge region between 2 450–2 460 eV, 0.09 eV between 2 460–2 490 eV and 0.2 eV between 2 490–2 510 eV according to the spectral features and with an integration time of 1 s per data point. From the raw data

a linear background determined in the pre-edge region was substracted to correct for the absorption from higher shells and from supporting materials. The spectra were normalized at 2 490 eV where the variation of the absorption cross-section is already very small.

2.4 Computational

The Gaussian94 program [24] was employed for ground state calculations to optimize geometries and to compute LCAO coefficients and symmetries of the molecular orbitals of H_2S_2. The geometry of the molecules was preoptimized using a MNDO approach. The resulting geometry was then used as a starting point for another optimization using B3LYP [25,26] calculations on a 6-31G basis. The same method was then used for a full population analysis. As the sudden approximation is not valid for XANES measurements, one cannot expect to reproduce the experimental spectra by ground state calculations. Especially, neither transition probabilities nor correct energy positions of resonances can be obtained form this type of calculations. In spite of this fact, they provide useful information regarding local electron densities and virtual orbitals as well as the description of the bond in the LCAO scheme all of which is essential for the reliable interpretation of XANES spectra, as previously demonstrated in [27].

2.5 MINUIT fitting of spectra

For the quantitative analysis of the spectra of *Allochromatium vinosum*, *i.e.* the optimization of the coefficients of a linear combination of reference spectra in order to minimize the residual between the resulting sum spectrum and the spectrum obtained of *A. vinosum*, the interactive fitting and plotting package Mn-Fit 4.04/15 was used (available at: `http://www-zeus.physik.uni-bonn.de/brock/mn_fit.html`). Mn-Fit 4.04/15 uses the function minimization tool "MINUIT" which is part of the CERNlib available at CERN (`http://www.info.cern.ch/asdoc/minuit/node2.html`) to fit histograms or data. MINUIT is a tool to find the minimum value of a multi-parameter function. It analyzes the shape of the function around the minimum. In our case, a chi-square function was used to compute the best fit parameter values and to find a linear combination of spectra within the basis set of reference spectra, which reproduces the measured XANES-spectra of the bacteria with the highest probability. Non-statistical errors may occur from an incomplete set of reference spectra or from reference spectra which do not describe the local environment of the absorber atom "exactly". The error of the percentage contributions of sulfur (Tab. 2) can be estimated to be ±10% (absolute value).

3 Results and discussion

Due to the above mentioned problems of a direct fingerprinting approach using the white line, the most obvious approach to identify an indicator in XANES spectra for the presence of S–H bonds in a sample is to investigate reference compounds which do contain S–H bonds and their counterparts in which the hydrogen atom is replaced by another defined bonding partner whereas the rest of the molecular environment of the absorbing atom remains unchanged. There are a number of biologically relevant systems suitable for such investigations, such as coenzyme A and glutathione in oxidized and reduced form, respectively, or the cysteine/cystine system (Figs. 1a–1f). In accordance with earlier results [10,11] the S K-XANES spectra of the oxidized forms of these compounds (Figs. 1b, 1d, 1f) show a white line to which two (sets of) transitions contribute: One in the energy range between 2 471.6 and 2 471.8 eV, which is typical for transitions into molecular orbitals (MOs) forming a S–S bond, and a second peak at slightly higher energies (2 473 to 2 473.3 eV) which can be assigned to the MOs forming the S–C bond. While the energy position of the S $1s \rightarrow$ S–S transition is only slightly influenced by structural differences in higher coordination shells there is a clear influence of these higher shells on the position of the S $1s \rightarrow$ S–C transition. This effect has been observed and discussed in more detail in [8]. In the reduced forms (Figs. 1a, 1c, 1d), there is only one peak. This peak at an energy between 2 472.2 and 2 472.5 eV can be assigned to S $1s \rightarrow$ S–C as well as S $1s \rightarrow$ S–H transitions. Due to this ambiguity,

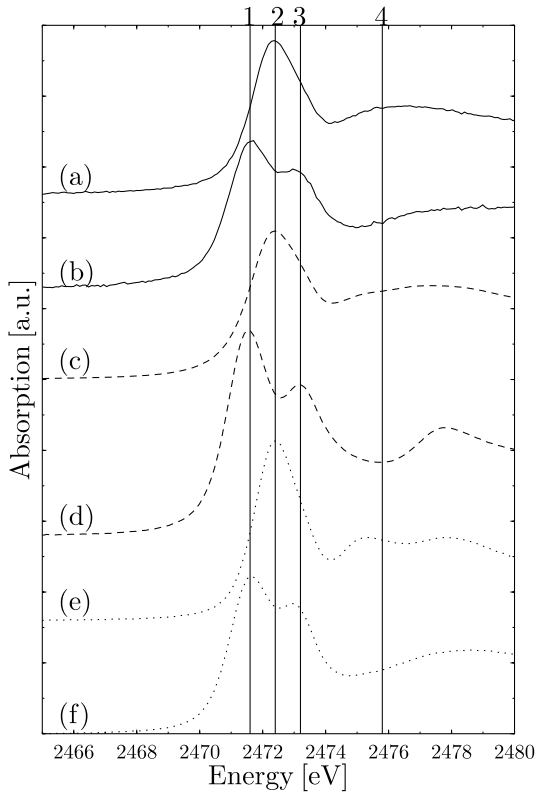

Fig. 1. Sulfur K-edge XANES spectra of coenzyme A (solid lines) in reduced (a) and oxidized (b) form, cysteine (broken lines) in reduced (c) and oxidized (= cystine) (d) form and glutathione (dotted lines) in reduced (e) and oxidized (f) form.

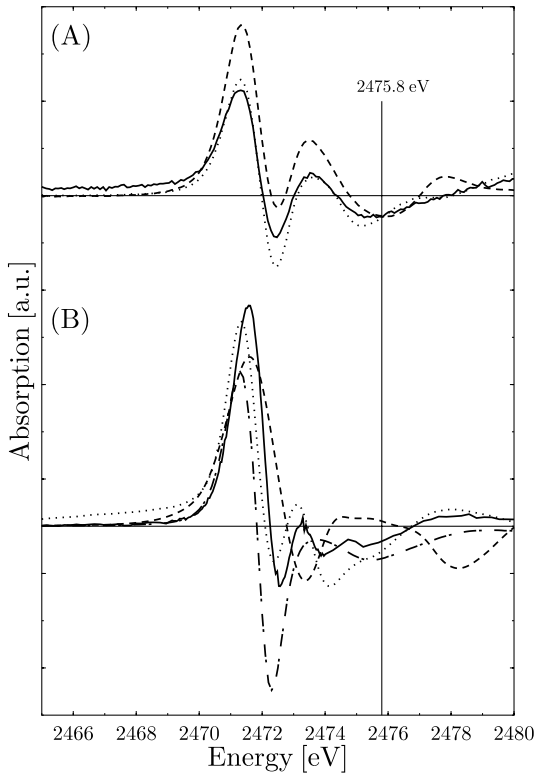

Fig. 2. (A) Subtraction of S K-edge XANES spectra of coenzyme A (solid line: coenzyme A oxidized form – reduced form), cysteine (broken line: cystine – cysteine) and glutathione (dotted line: glutathione oxidized form – reduced form). (B) Subtraction of S K-edge XANES spectra of compounds with the structures: R–S–S–R – R–S–R, R = –$CH(CH_3)_2$ (solid line), R = –$C_2H_2C_5H_{10}CH_3$ (dotted line), R = –C–$(CH_3)_3$ (broken line), R = –$(CH_2)_3CH_3$ (dotted/dashed line).

it is necessary to check for further changes in the spectra which can be connected to the presence of an S–H bond. This is done by analyzing the respective difference spectra ("reduced spectrum" subtracted from the "oxidized spectrum") as shown in Figure 2A. Evidently, from the first three extrema of these residuals, no unambiguous identification of an S–H bond is possible. However, it is a common feature of all three difference spectra of compounds containing S–H bonds that at a photon energy of about 2 475.8 eV a clear structure can be recognized. In principle, here, too, an ambiguity exists, as the observed changes could be due to the removal of the S–S bond or the addition of the S–H bond. To exclude the first possibility, Figure 2B displays the same situation for a number of reference spectra for the change from R–S–S–R to R–S–R-type compounds with widely varying organic restgroups R ("R–S–R spectrum" subtracted from the "R–S–S–R spectrum"). Here, the difference spectra do not feature a common structure. Instead, in these cases further structures vary significantly with respect to their energy positions. It is this variability which prevents using the presence of a structure at 2 475.8 eV as a necessary and sufficient proof for the presence of a S–H group, because it is impossible to exclude the existence of a specific organic restgroup which shifts a structure to this energy position. In fact, a structure in the energy region we assume to be characteristic for the presence of the S–H bond, is found in the difference spectra of the allyl-sulfane (R = –$(CH_2)_3CH_3$ (Fig. 2B dotted/dashed line)) (which is normally not likely to be encountered in a biological sample). However, in this difference spectrum the second maximum is by far less clearly outlined as compared to all the other analyzed difference spectra. This suggests that in this case the "true" third and fourth extremum in the subtracted spectra coincide partly. This reasoning is further supported by the observation that the second maximum in this case does not reach the zero-line any more which suggests interference between structures leading to positive and negative contributions to the spectrum.

All of the above systems containing the S–H bond bear in common that the organic restgroup which connects to the sulfur atom starts with a CH_2-group. Consequently, it is necessary to verify that the observed structure can in fact be related to a S–H bond and is not a typical feature connected to the presence of an identical nearest neighbour section of the terminating organic restgroup. To do so, the XANES spectra of H_2S and H_2S_2 were measured (Fig. 3). The S K-XANES spectrum of H_2S shows a white line at an energy position of about 2 472.4 eV which can be clearly assigned to S–H and also another well defined resonance at an energy of 2 475.8 eV, which can be assigned to a S Rydberg-state on the basis of previously published atomic calculations [13]. This assignment is supported by new ground state calculations using the B3LYP-approach [25,26] and 6-31g basis of Gaussian94 [24] presented in Table 1, which are described in detail in Section 2.4. Based on these calculations, it is possible to interpret the spectra in a very detailed way, although exact energy positions cannot be expected to be obtained due to the presence of the core hole in the measurement. One observes a clear separation between the first two virtual orbitals on the one hand and the next four virtual orbitals on the other hand. Based on the number of valence electrons present in the molecule, one may expect two virtual valence orbitals. The H_2S molecule is expected and found to belong to the c_{2v} point group. Consequently, two types of p-orbitals which transform differently under rotation contribute to the resulting electron configuration. In addition to that, to obtain the correct H–S–H angle, one may expect the calculated admixture of the S $3s$-orbital to the MO with a_1-symmetry, which explains why the corresponding antibonding orbital is located at slightly higher energies. Consequently, the following virtual MO's are to be related to Rydberg-like states. In fact, they are found to bear predominantly S $4p_y$, S $4p_z$, S $4s$ and S$4p_x$-character. It should be noted that due to the fact that the molecule is symmetric to the $x = 0$ plane molecule, the p_x orbital is present as a pure state as it is the only orbital of a_2 symmetry, which explains the strength of the observed second resonance.

The spectrum of H_2S_2 (Fig. 3) features a white line which includes the expected contributions of two (sets of) transitions, correlated to the S–S and S–H bond,

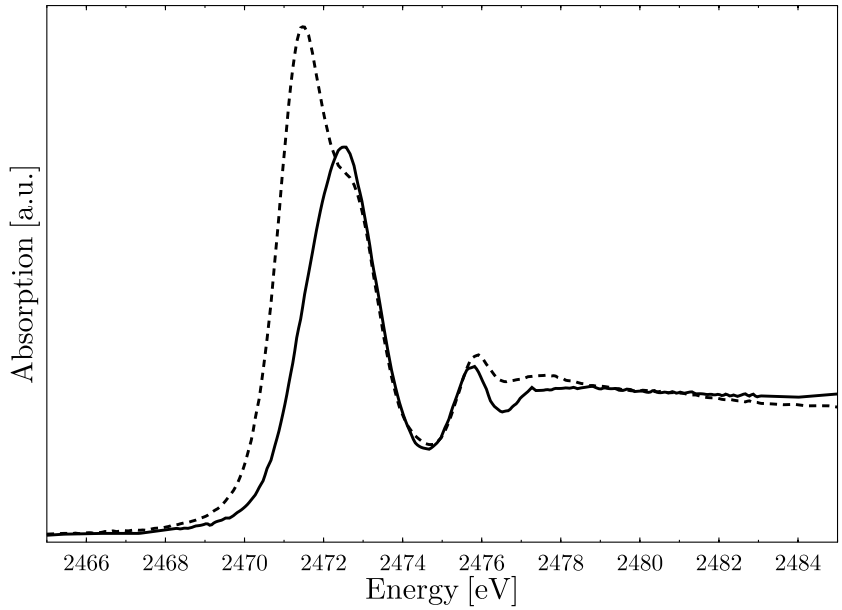

Fig. 3. Sulfur K-edge XANES spectra of H_2S (solid line) and H_2S_2 (broken line).

Table 1. Calculated energy positions, symmetry and main constituents in terms of atomic orbitals for the lowest unoccupied states of H_2S and H_2S_2, respectively.

H_2S			H_2S_2		
Energy*	Symmetry	Interpretation/main constituents	Energy*	Symmetry	Interpretation/main constituents
			−1.9 eV	B	S–S (S 3s, S 3 p_y)
0.6 eV	B_2	S–H (H 1s, S 3p_y)	0.6 eV	B	S–H (H 1s, S 3p_y, some S 3p_x)
0.9 eV	A_1	S–H (H 1s, S 3s, S 3p_z)	0.6 eV	A	S–H (H 1s, S 3s, S 3p_z)
9.0 eV	B_2	S 4p_y, some H	9.1 eV	A	S 4s, some S 4s, some H
9.4 eV	A_1	S 4p_z, some S 4s, some H	9.6 eV	B	S 4p_y, some S 4p_x, some H
9.8 eV	A_1	S 4s, some S 4p_z, some H	9.9 eV	B	S 4p_x, some S 4p_y, some H
9.8 eV	A_2	Pure S 4p_x	9.9 eV	A	S 4p_z, some S 4s, some H

* Relative to E_{vac} of H_2S, *i.e.* correcting the different core level shift of the S 1s orbital.

respectively. This results in a white line, with a maximum at 2 471.3 eV and a high energy shoulder at an energy of 2 472.4 eV. In addition to that, there is also a well defined resonance visible at an energy of 2 475.8 eV, like in the spectrum of H_2S. Motivated by the spectrum of H_2S, the straight forward assignment of the resonances at 2 472.4 eV and at 2 475.8 eV as characteristics of the S–H bond is possible. As a consequence, the first resonance can be assigned to a S–S orbital. Here, too, an agreement exists with the typical energy range in which the S–S bound is found. Its slight shift towards lower energies in comparison to the biological samples mentioned above is easily understood based on the fact that the hydrogen atom is a better electron donor than the organic moieties which are present in the biological samples. Again, these assignments are qualitatively supported by the results of ground state calculations (see Tab. 1) using the B3LYP-approach [25,26] and 6-31g basis of Gaussian94 [24]. As before, a clear separation in energy between the three expected virtual valence- and the following Rydberg-type orbitals is visible. The most stable configuration bears c_2-symmetry. As before, a similar splitting of the 2nd and 3rd virtual MO, which form the S–H bond, as before is observed and can be understood on the basis of the above arguments. The expected S–S bond is identified as the first virtual MO by the fact that only S p_y and S s orbitals contribute significantly. These calculations also indicate the reason why in this case the structure which was previously assigned to the Rydberg states is no longer as intense as before, as now other orbitals couple into the p_x-dominated Rydberg-orbital, so that a pure Rydberg state no longer exists.

In summary, it can be concluded from these investigations that the feature at an energy of 2 475.8 eV in the S K-XANES spectra provides indirect evidence for the presence of S–H bonds in a sample. This allows to detect S–H bonds in a "C–S–H environment" which is typical for biological samples.

Next, we show the usefulness of this criterium for the investigation of a complex biological example: in batch

Table 2. Results of fitting the sulfur K-edge XANES spectra of *Allochromatium vinosum* recorded 1, 6 and 10 h (*cf.* Figs. 4a–4c) after the cells had been transferred to sulfide-containing medium.

Compound	Glutathione (red.)	Glutathione (ox.)	Dimethyl-sulfoxide	Trisulfane	Polymeric sulfur	Sulfate
sulfur species	*C–S–H	*H–S–S–H	*C–SO–C$^-$	*C–S–S–S–C	*S_μ	*SO_4^{2-}
A. vinosum, 1 h after sulfide addition	---	---	---	32%	67%	---
A. vinosum, 6 h after sulfide addition	37%	28%	---	---	19%	15%
A. vinosum, 10 h after sulfide addition	51%	---	(4%)	(2%)	---	45%

* Different sulfur species and percentage contribution, error: ±10%; --- = contribution <0.1%.

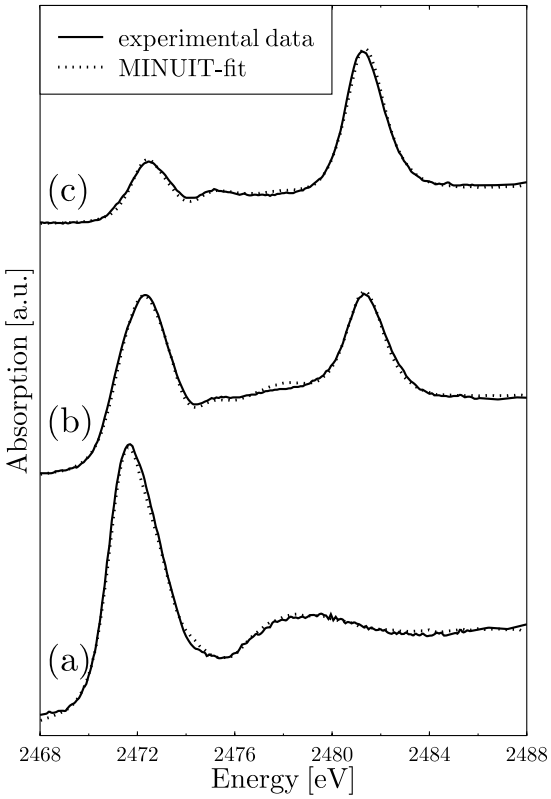

Fig. 4. Sulfur K-edge XANES spectra *Allochromatium vinosum* recorded 1 h (a), 6 h (b) and 10 h (c) after the cells had been transferred to sulfide-containing medium and linear combinations of the reference spectra optimized using MINUIT (dotted lines).

culture, sulfide oxidation to sulfate by *Allochromatium vinosum* and other purple sulfur bacteria proceeds in a biphasic manner: as long as sulfide is present, sulfur is accumulated as intracellular sulfur globules in the periplasm [28]. When sulfide is depleted, further oxidation of sulfur starts [16,29]. Figure 4 shows the spectra and MINUIT-fits of *A. vinosum* 1, 6 and 10 hours after the cells had been transferred to sulfide-containing medium. A detailed description of the growth experiment and spectra (white line positions) has been published previously [11]. It has also been proposed that the sulfur in *A. vinosum* occurs as longs chains, terminated by organic residues at one or both ends [11,12]. The existence of glutathione in its amidated form and its perthiol and its function as sulfur carrier was also proposed [17,30] for sulfur globules of *A. vinosum*. Consequently, the occurrence of S–H bonds should be visible using the structure at 2 475.8 eV as "fingerprint" in the spectra (Figs. 4b and 4c). In fact, the minimum in the energy range of 2 474.5–2 477 eV on the high energy side of the white line is filled up in the spectrum 6 h (Fig. 4b) and a small peak can be seen at about 2 575.8 eV especially in the sample taken after 10 h (Fig. 4c). This new feature can be correlated to S–H bonds by the above described criterium to detect these bonds. This represents the first *in situ*-experimental evidence for S–H during the decomposition of the sulfur globules of *A. vinosum*. To corroborate the fingerprint results and to analyze the spectra quantitatively in a next step, MINUIT (*cf.* Sect. 2.5) was used. Now, different sets of reference compounds were tested to fit the three spectra in order to obtain a quantitative description of the spectra. Good fits for all three spectra are obtained by optimizing a linear combination of the set of spectra of the reference compounds listed in Table 2 using MINUIT. It should be emphasized that this degree of quality of the fit is only obtainable when including reduced glutathione in the basis set and cannot be obtained using cysteine or reduced coenzyme A instead. The need to include reduced glutathione in the basis set can be related to a previous study of Bartsch *et al.* [30] showing the presence of glutathione modified with an amidated glycine carboxylic group and its perthiol in *A. vinosum*. As this modification occurs only in the 7th coordination shell of the sulfur atom, it should not influence on the S K-edge XANES spectrum significantly. It should be noted that sulfoxide, which was included in the set of references, does not contribute significantly to the fit, although the maxima of the "white lines" of sulfoxides have an energy position at about 2 475.5 eV. This corroborates the suitability of the feature at 2 475.8 eV to provide indirect evidence for S–H bonds in a complex biological system. By fitting the spectra, a quantitative description of the sulfur speciation during sulfide oxidation

to sulfate is obtained (Tab. 2). In the sulfur globules of *A. vinosum* 1 h after exposition to sulfide, polymeric sulfur is the dominant sulfur species (≈67%). Furthermore, the presence of the "initial, short chains" at the beginning of sulfide oxidation is indicated by the occurrence (≈32%) of their model compound dioctenyltrisulfane (R–S_3–R, R = –$C_2H_2C_5H_{10}CH_3$). After an exposition time of one hour, sulfide oxidation and formation of the sulfur globules are still in progress [11,31]. Thus, the presence of a considerable amount of the "initial chains" is to be expected. Definite statements regarding the terminal groups on the end of the chains are not possible, but it seems plausible that they are at least partly formed by glutathione groups based on the argument that some of these are expected to be present in the sample. After 6 h, the degradation of the globules is in full progress. This fact is clearly indicated by the decreasing amount of polymeric sulfur (≈19%), the appearance (≈15%) of the sulfate species and the increasing amount of reduced glutathione (≈37%) and oxidized glutathione (28%). The presence of oxidized glutathione might either be related to small sulfur chains and to the complete shortening of chains in case glutathione is the terminating group of the chains. Alternatively, a combination of both species (reduced and oxidized glutathione) might be use as an indicator for perthiol-groups.

In order to start the oxidation process, sulfur in the globules must first undergo reductive cleavage [32], which can occur *via* nucleophilic attack of glutathione (here, in the amidated form known to be present in *A. vinosum*). Assuming this mechanism for the degradation of the globules, the massive occurrence of reduced glutathione in the sample (≈51%), which has also been observed in comparable growth experiments by Gehrke [33] and Bartsch *et al.* [30], can be explained. In the final state of degradation, which is reached after 10 hours, both polymeric sulfur and the initial chains have been removed completely and only the oxidized species and reduced glutathione contribute to the fit of the spectrum. Based on the combination of these results and previous studies [12,17,30,33] the assumption that glutathione amide can be the sulfur carrier shuttleling one sulfur atom of the sulfur (after nucleophilic attack resulting in glutathioneamide perthiol) from the sulfur globules which are located in the periplasm [28] (pH "acidic" [32]) across the sulfur globule protein envelope and the cytoplasmic membrane into the cytoplasm (pH "neutral" [32]) seems plausible. There, further oxidation of the sulfur takes place and glutathione amide can function again as carrier. Furthermore, it seems very probable that glutathione in its amidated form is the organic residue of the organyl sulfanes in the sulfur globules of *A. vinosum*.

4 Conclusions

Using the S K-XANES spectra of a set of biologically significant reference compounds in their respective oxidized and reduced forms, a common structure at 2 475.8 eV was found. This structure is not consistently found in a corresponding set of R–S–R and R–S–S–R compounds. By comparison with the S K-XANES spectra and ground state calculations of H_2S and H_2S_2, it was established that the structure can be explained by a Rydberg-like transition which is typical for S–H bonds, even though it is not possible to prove that it is unique for this bond. As this criterium does not rely on the energy position of the white line, it can also be applied to samples where S–C and S–H might be present simultaneously. Applying the criterium, a new level of understanding of the sulfur metabolism during the decomposition of the sulfur globules of *Allochromatium vinosum* can be reached, giving detailed insight into this process defining the very basics of life.

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