Synchrotron radiation based STXM analysis and micro-XRF mapping of differential expression of extracellular thiol groups by Acidithiobacillus ferrooxidans grown on Fe$^{2+}$ and S$^0$

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1. Introduction

Many studies have mentioned that acidophilic sulfur oxidizing bacteria (SOB), such as Acidithiobacillus ferrooxidans, play an important role in the bioleaching of metal sulfide minerals (Klauber, 2008; Rohwerder et al., 2003; Yang et al., 2013). In bioleaching process, elemental sulfur (S$^0$) and other reduced inorganic sulfur compounds (RISCs) could be produced (He et al., 2012; Kleinjan et al., 2003). S$^0$ is basically chemically inert and it could be exclusively eliminated by SOB (Tang et al., 2009). The understanding of sulfur oxidation mechanism of SOB is significant for bioleaching operations.

Sulfur produced by SOB can be stored in sulfur globules, located inside and/or outside the bacterial cell (He et al., 2010); (Kleinjan et al., 2003). Although the biochemical fundamentals of sulfur oxidation are very complex, several models for the oxidation of RISCs have been developed (Friedrich et al., 2005; Quatrini et al., 2009; Valdés et al., 2008). According to these models, S$^0$ is activated firstly by reacting with reactive thiol groups (−SH) of outer membrane proteins, forming −S$\times$H (n ≥ 2) complexes, and then transported into periplasmic space for further oxidation. The extracellular activation of S$^0$ is considered to be the rate-limiting stage for sulfur oxidation. However, it is still unclear about how the activation of S$^0$ is catalyzed for the lack of experimental evaluation of the required thiol groups during bacterial sulfur oxidation.

Several studies have been involved in thiol groups for sulfur activation. Buongiolio et al. (1993) and Ramírez et al. (2004) found that some out membrane proteins were highly expressed when A. ferrooxidans is grown on S$^0$. He et al. (2011) found that cyclo-octasulfur is necessarily converted to polymeric sulfur for the oxidation by A. ferrooxidans. Further studies by comparative proteomics identified six extracellular proteins which contain abundant thiol groups and four of the six proteins have CXXC functional motif when A. ferrooxidans is grown on S$^0$ (Zhang et al., 2008). RT-qPCR analysis demonstrated that these proteins were significantly up-regulated when A. ferrooxidans is cultured on S$^0$, contrary to that on Fe$^{2+}$ (Peng et al., 2012). Therefore, these proteins rich in thiol groups and/or CXXC functional motifs are probably directly involved in sulfur activation. However, so far there has been no literature that concerns the detection of the differential expression of thiol groups of A. ferrooxidans.

Thiol groups in a protein require modification if they are to be analyzed (Aitken and Learmonth, 1996). Iodoacetic acid is always used for the modification and alkylation of thiol groups of cysteine residues found in proteins and peptides, by forming Protein-SCH$_2$COOH (P-SCH$_2$COOH) (Zeng and Davies, 2005). If the carboxyl groups were
marked by calcium ions by forming P-SCH₂COOCa, the SCH₂COO-bonded Ca²⁺ could reflect the expression of the extracellular thiol groups.

Synchrotron radiation is an ideal X-ray source for probing trace elements in biological samples with high sensitivities, high spatial resolutions, and in situ analysis (Lobinski et al., 2006). The soft X-ray spectromicroscopy beamline (BL8B1U) at Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China, is useful for chemical distribution analysis in situ based on the dual-energy contrast analysis by two-dimensional scanning transmission X-ray microscopy (STXM), and the spatial resolution is better than 30 nm (Guo et al., 2011; Xue et al., 2010). By using STXM imaging, Wang et al. (2012) obtained the in situ iron distribution in *Helicobacter pylori*. The micro-beam X-ray absorption fine structure (micro-XAFS) beamline (BL15U1) at SSRF can be applied to detection of the relative contents and distribution of metals in materials and organisms by micro-X-ray fluorescence (μ-XRF) analysis. By using μ-XRF, Wang et al. (2010) detected the Ca, Fe, Cu and Zn and obtained the quantitative imaging of these trace elements in the section of bio-tissues. Combined with the reference materials, the relative changes of the spatial distributions of metals were clearly visible. Therefore μ-XRF could be used for analyzing the differential expression of extracellular thiol groups that are selectively labeled by specific metal ions.

In the present study, synchrotron radiation based STXM imaging and μ-XRF mapping were applied for comparative analysis of the extracellular thiol groups of *A. ferrooxidans* cells. By comparing these two methods, it demonstrated the stability and accuracy of a new way for visible and in situ analysis of thiol groups distributed on the bacterial cells.

2. Materials and methods

2.1. Bacterial strain and culture conditions

*A. ferrooxidans* ATCC 23270 was purchased from the American Type Culture Collection (ATCC). The culture media used in this study were composed by 9K basal medium added with S⁰ (10 g/L) or FeSO₄ · 7 H₂O (44.7 g/L) as the energy substrate, and the initial pH was adjusted to 2.0. The 9K basal medium contained the following components: (NH₄)₂SO₄, 3.0 g/L; MgSO₄ · 7H₂O, 0.5 g/L; K₂HPO₄, 0.5 g/L; KCl, 0.1 g/L; Ca(NO₃)₂, 0.01 g/L. The S⁰ powder was pretreated with the method described by Konishi et al. (1995). The initial inoculated concentration was 1 × 10⁶ cells/mL. *A. ferrooxidans* ATCC 23270 was incubated in 250 mL Erlenmeyer flasks containing 100 mL culture medium on a rotary shaker at 30 °C and 170 r/min.

2.2. Labeling of thiol groups by Ca²⁺

In order to measure the quantities of extracellular thiol groups of *A. ferrooxidans* ATCC 23270 by STXM analysis and μ-XRF mapping, the thiol groups were selectively labeled with Ca²⁺. Before this experiment, *A. ferrooxidans* cells were collected by the method as previously described (Zhang et al., 2009), and then washed with 0.2 M phosphate buffer by triple-centrifugation at 10, 000 r/min. In order to determine the optimum amounts of iodoacetic acid to make sure that the extracellular thiol groups of *A. ferrooxidans* cells were totally alkylation, thiol groups of the samples were first detected based on 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) method reported by Ellman (1959). Briefly, the sample was diluted with 1 mL Tris–HCl buffer (0.25 M, pH 8.0) and then 5 mL DTNB solution (0.1 mM, prepared in 0.1 M phosphate buffer at pH 8.0) was added at 25 °C in the dark; after a 10 min incubation the absorbance of the solution was measured at 412 nm. The thiol concentration was determined referring to the calibration curve of standard cysteine. Results indicated that the total thiol concentrations of the bacterial samples grown on S⁰ and Fe²⁺ were 0.152 mM and 0.043 mM, respectively. The labeling of extracellular thiol groups with Ca²⁺ (Borman et al., 1982; Geoffroy et al., 1981; Lipari and Herscovics, 1996) could be divided into 3 steps: (1) extracellular carboxyl groups of *A. ferrooxidans* were firstly reacted with Ba²⁺ for about 2 h at room temperature by dispersing the cells in 45 mL 1.5 M Tris–HCl (pH 8.8) adding 5 mL 0.5 M BaCl₂, and then washed with 1.5 M Tris–HCl (pH 8.8) trpily at 10 min intervals. This step was to seal the extracellular carboxyl groups. (2) Extracellular thiol groups were selectively bound to iodoacetic acid for about 2 h at room temperature by dispersing the cells in 50 mL Tris–NaCl–EDTA buffer (pH 8.5) adding 0.5 g iodoacetic acid, and then washed with 1.5 M Tris–HCl (pH 8.8) trpily at 10 min intervals. The samples without labeling by Ca²⁺ were used as controls. All the samples were stored at 4 °C in nitrogen atmosphere until analysis.

Due to the thiol groups on the cell surface selectively bonded with Ca²⁺, the analysis for the bonded Ca²⁺ could reflect the distribution of thiol groups of *A. ferrooxidans*.

2.3. Synchrotron radiation based STXM imaging

The mapping of the spatial distribution for Ca²⁺ in a single cell was obtained by STXM based on the dual-energy contrast image analysis of the near-edge X-ray absorption fine structure (NEXAFS) of Ca²⁺ at BL8B1U at SSRF, Shanghai, China. CaCO₃ was chosen as the reference compound for NEXAFS analysis of SCH₂COOCa in the extracellular proteins. Through the test of the Ca L edge absorption spectra of CaCO₃, two energy values (on the absorption edge E₁ and away the absorption edge E₂) were obtained. The STXM transmission images of the samples were scanned in two dimensions with a step of 30 nm and a dwell time of 20 ms at E₁ and E₂ under ultrahigh vacuum (~10⁻⁵ Torr). At least three different regions of each sample were scanned. The distribution map of calcium element was obtained by digital division of two absorption-contrast images at dual photon energies of E₁ and E₂ using IDL 7.06 (Exelis Visual Information Solutions, Inc. Boulder, CO, USA) software (Xue et al., 2010; Zhang et al., 2010).

2.4. Synchrotron radiation based μ-XRF mapping

The two-dimensional elemental imaging for Ca²⁺ in masses of cells by using μ-XRF mapping was carried out at BL15U1 at SSRF in Shanghai, China. Prior to the experiment, a drop of the sample was put on the Quartz coverslip, smeared evenly, and dried at room temperature. Then the sample was positioned at a 45° angle to the incident X-ray beam, and the Kirkpatrick–Baez (KB) mirrors system was used to provide a focused beam with a spot size of 1 μm × 1 μm. The X-ray fluorescence was detected by a silicon detector oriented at a 90° angle to the incident beam. The μ-XRF mapping dimensions are 41 μm × 41 μm and it was recorded with a 1 μm step and a 4.038 KeV (which is the K-edge energy of Ca) excitation beam. The dwell time at each pixel was 2 s and the total scanning time of a sample was about 50 min. The intensities of the incident X-ray beam were monitored by the ionization chamber positioned upstream (I₀) of the sample. The fluorescence intensities of the mapping were normalized to the collecting time and the changes in I₀. Five different sections were selected for μ-XRF mappings analysis of each sample. The intensity map of calcium element was imaged using the software Igor Pro (WaveMetrics, USA). Statistical analysis for the data of normalized intensity was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was assessed by unpaired t-test, in which the probability value p < 0.05 is considered statistically significant.
3. Results and discussion

3.1. STXM imaging

The typical two-dimensional STXM imaging of Ca was scanned at two energies which were selected as being before and on the absorption edge of NEXAFS spectra of calcium. NEXAFS spectra provided important information about the electronic and structural properties (Chen, 1997). The spectra of Ca$^{2+}$ (CaCO$_3$) L$_2$, 3 edge absorption are shown in Fig. 1. The spectra were measured with a one-dimensional spectrum scan over an energy range of 344–354 eV and with a step of 0.1 eV, and the spectra were consistent with the literature (Couradeau et al., 2012; Fleet and Liu, 2009). The L$_2$ edge and L$_3$ edge of Ca were 347.6 eV and 350.8 eV, respectively. In this study, L$_2$ edge of Ca (350.8 eV) was chosen as the absorption edge (E1), and 348.6 eV was chosen as the energy away the absorption edge (E2).

STXM images obtained by dual-energy contrast analysis for *A. ferrooxidans* grown on S$^0$ and Fe$^{2+}$ are shown in Fig. 2A and B and C and D, respectively, where Fig. 2A and C was referring to no labeling of Ca$^{2+}$ and was taken as the controls for the two cultures, respectively. It shows that Ca$^{2+}$ distribution of S$^0$ grown cells was increased apparently after the selective labeling of SH with Ca$^{2+}$ by comparison with that grown on Fe$^{2+}$. The color distribution bar of each image in Fig. 2 showed the content range of Ca$^{2+}$ distributed in each sample. According to the color bar of each image, the surface densities of Ca$^{2+}$ spread on the cells grown on S$^0$ before and after Ca$^{2+}$-labeling were $0.99 \times 10^{-5}$–$2.51 \times 10^{-5}$ g/cm$^2$ and $1.23 \times 10^{-5}$–$3.54 \times 10^{-5}$ g/cm$^2$, respectively; the surface densities of Ca$^{2+}$ spread on the cells grown on Fe$^{2+}$ before and after Ca$^{2+}$-labeling were $0.89 \times 10^{-5}$–$2.29 \times 10^{-5}$ g/cm$^2$ and $0.92 \times 10^{-5}$–$2.57 \times 10^{-5}$ g/cm$^2$, respectively. The results showed that the increased value of surface densities of Ca$^{2+}$ for the cells grown on S$^0$ were larger than that on Fe$^{2+}$ after labeling by Ca$^{2+}$. It indicated that the thiol group content of *A. ferrooxidans* grown on S$^0$ is higher than that on Fe$^{2+}$.

Rohwerder and Sand (2003) had suggested that thiol group-containing protein(s) of mesophilic leaching bacteria could activate the oxidation of S$^0$, which is probably by a mechanism described as Eq. (1). Chi et al. (2007) and Zhang et al. (2008) had demonstrated that cysteine-containing outer membrane proteins were highly expressed by S$^0$ grown *A. ferrooxidans*. In this study, the higher expression of extracellular thiol groups of *A. ferrooxidans* grown on S$^0$ observed by the STXM imaging further indicates that the reduced –SH groups probably played an important role in sulfur activation during sulfur oxidation.

$$\text{S}_8 + P - \text{SH} \rightarrow (P - \text{SS}_8\text{H}) \rightarrow P - \text{SS}_n\text{H} \quad (n \geq 1).$$  \hspace{1cm} (1)

It is worthy to note that although the mechanism of sulfur activation had been postulated to be related with thiol groups of outer
membrane proteins, in situ observation of the thiol groups expression has not yet been detected before. In this study the STXM imaging of Ca2+ ions that were selectively labeled to thiol groups clearly provides an in situ analysis method for investigating thiol group expression.

3.2. μ-XRF mapping

In synchrotron μ-XRF analysis, the intensity and clarity of the fluorescence depend on the varied beam current intensity of X-ray with scanning time and the differences in size of the beam and the distance between detector and sample (Lai et al., 1995). The relative quantitative changes of metals distributed on the samples could be determined if the scan conditions were the same. By using μ-XRF analysis of Ca content in each sample, it was valuable for further evaluating the method for selectively labeling of SH with Ca2+.

The μ-XRF mappings for the Ca content in the samples of A. ferrooxidans grown on Fe2+ or S0 are shown in Fig. 3A and B and C and D, respectively, where Fig. 3A and C was referring to the sample before labeling of Ca2+ and was taken as the controls for cells grown on S0 and Fe2+, respectively. The fluorescence was recorded with a 1 μm step using Ca Kα-edge energy (4.038 KeV) and normalized by the collecting time and the changes in the beam current intensity. The normalized intensity of each sample could directly reflect the content of Ca2+ in A. ferrooxidans. Statistical analysis of the data of normalized intensity of each sample in Fig. 3 is shown in Table 1. The results showed that, compared with A. ferrooxidans cells before they were labeled of Ca2+ (Fig. 3A and C), the content of Ca2+ for the samples after they were labeled of Ca2+ (Fig. 3B and D) had an apparent increase. By subtracting the fluorescence intensities in Fig. 3A and C (before labeling of Ca2+) from that in Fig. 3B and D (after labeling of Ca2+), respectively, it could derive the relatively increased fluorescence intensities, i.e., 0.0101 a.u. and 0.0026 a.u. for the cells grown on S0 and Fe2+, respectively, as shown in Fig. 4.

Table 1

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Energy source</th>
<th>Ca2+-labeling</th>
<th>Mean value ± SD (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>S0</td>
<td>−</td>
<td>0.0970 ± 0.0021</td>
</tr>
<tr>
<td>B</td>
<td>S0</td>
<td>+</td>
<td>0.1071 ± 0.0023</td>
</tr>
<tr>
<td>C</td>
<td>Fe2+</td>
<td>−</td>
<td>0.1056 ± 0.0022</td>
</tr>
<tr>
<td>D</td>
<td>Fe2+</td>
<td>+</td>
<td>0.1081 ± 0.0023</td>
</tr>
</tbody>
</table>

* The letters (A, B, C, D) represent the samples of Fig. 3A-D.
** Mean value is the mean of intensity of all the points in each map, SD means standard deviation (n = 5).
*** p < 0.05 compared with control group.

Fig. 3. The μ-XRF mappings of the content of calcium in A. ferrooxidans grown on S0 without (A) and with (B) labeling Ca2+, and on Fe2+ without (C) and with (D) labeling Ca2+, respectively. The map dimensions are 41 x 41 μm and they were recorded with a 1 μm step and 4.038 KeV excitation beam. The color bar means the normalized intensity of fluorescence yields (unit: a.u.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. The μ-XRF intensities referring to the –SOHCOO-bonded Ca2+ for A. ferrooxidans cells grown on S0 and Fe2+, respectively; they were obtained by subtracting the a.u. values in Fig. 3A and C (before labeling of Ca2+) from the a.u. values in Fig. 3B and D (after labeling of Ca2+), respectively. Error bar was the standard deviation based on the statistical analysis of the a.u. values (n = 5).
Since the extracellular thiol groups of A. ferrooxidans were selectively labeled with Ca++, the increased fluorescence intensity of the sample after Ca++-labeling resulted from the emission of --S-CH2COO-bonded Ca++, and it could reflect the expression of extracellular thiol groups. The results shown in Fig. 4 indicate that the cells express 3.88 times more SH in culture S0 to that in culture Fe2+. It basically confirmed the result of STXM imaging analysis (Fig. 2), further indicating that the extracellular thiol groups play an important role during sulfur oxidation. In addition, results of thiol groups of the samples based on DTNB method showed that the total thiol concentrations of the bacterial samples grown on S0 and Fe2+ were 0.152 mmol and 0.043 mmol, respectively, indicating that the cells expressed 3.62 times more SH in culture S0 than that in culture Fe2+. It indicated that the method for selectively labeling of SH with Ca++ is valuable for SH determination and in situ analysis. In this study, the thiol groups were selectively labeled with Ca++, and the S-CH2COO-bonded Ca++ was detected with STXM imaging and μ-XRF mapping. It should be pointed out that though STXM imaging achieved an in situ analysis of single bacterial cell its operation was complicated and took a long time to focus and scan the sample of interest, whereas μ-XRF mapping was relatively simple and rapid though it could not be used for analysis of single A. ferrooxidans cell (typically 0.3–0.5 μm in width and 1–2 μm in length), because the minimum spot size of μ-XAFS beamline was only 1–2 μm. It suggested that combining with STXM imaging, which gives an in situ evaluation of extracellular SH groups of single cell, μ-XRF mapping could be of value in rapid analysis of differential expression of thiol groups for a large number of samples.

4. Conclusions

Synchrotron radiation based STXM analysis and μ-XRF mapping provide powerful tools for analysis of trace elements in biological samples. By selectively bonding to Ca++, the extracellular thiol groups of A. ferrooxidans grown on different energy substrates Fe2+ and S0 were analyzed in an in situ and rapid way, respectively, with STXM imaging and μ-XRF mapping of S2-CH2COO-bonded Ca++. The results showed that the thiol group content of A. ferrooxidans grown on S0 is about 3.88 times more that on Fe2+, providing direct evidence that the extracellular SH groups play an important role in sulfur activation during sulfur oxidation.

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