Sulfur-Binding Protein of Flagella of Thiobacillus ferrooxidans

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The sulfur-binding protein of Thiobacillus ferrooxidans ATCC 23270 was investigated. The protein composition of the bacterium’s cell surface changed according to the culture substrate. Sulfur-grown cells showed greater adhesion to sulfur than iron-grown cells. The sulfur-grown cells synthesized a 40-kDa surface protein which was not synthesized by iron-grown cells. The 40-kDa protein had thiol groups and strongly adhered to elemental sulfur powder. This adhesion was not disturbed by Triton X-100, which can quench hydrophobic interactions. However, adhesion was disturbed by 2-mercaptoethanol, which broke the disulfide bond. The thiol groups of the 40-kDa protein formed a disulfide bond with elemental sulfur and mediated the strong adhesion between T. ferrooxidans cells and elemental sulfur. The 40-kDa protein was located on the flagella. The location of the protein would make it possible for cells to be in closer contact with the surface of elemental sulfur powder.

It is widely accepted that the chemolithotroph Thiobacillus ferrooxidans oxidizes elemental sulfur and/or ferrous ions in the periplasm of the bacterium (13, 16). Soluble ferrous ion can easily pass across the outer membrane and be incorporated in the periplasm, while bulky insoluble elemental sulfur encounters steric hindrance. Therefore, its incorporation has been of interest for a long time (24, 27). It has been assumed that the bacterium may release reduced glutathione to form a soluble polysulfide chain by nucleophilic attack of the glutathione and that the polysulfide products are incorporated into cells (25). If the incorporation occurs by the assumed mechanism, the bacterial cells would continuously release the reduced glutathione to produce the polysulfide. The continuous release would make the bacteria consume substantial amounts of high-energy materials. To minimize consumption of the materials, the bacteria must approach the surface of elemental sulfur as closely as possible. The cells would be in contact with elemental sulfur before release of the glutathione. In fact, it was observed that T. ferrooxidans cells adhered to the surface of sulfur particles (4).

Components on the bacterial cell envelope play an important role in adhesion (9, 17), and the cell envelope is subject to change according to the growth conditions (1, 6, 8a, 10, 11). We considered that if T. ferrooxidans were grown on an insoluble substrate such as elemental sulfur, the bacterium might synthesize specific components for adhesion to utilize the insoluble substrate in their envelope. The purpose of this study is to clarify the specific component for adhesion to elemental sulfur particles.

MATERIALS AND METHODS

Bacterium, medium, and culture conditions. The iron-oxidizing bacterium T. ferrooxidans ATCC 23270 was used throughout this study. The strain was cultured in 9K basal medium (23) containing 44.2 g of FeSO4·7H2O per liter to prepare iron-grown cells and in medium containing 10.0 g of elemental sulfur per liter to prepare sulfur-grown cells. The initial pH of these media was adjusted to 2.5 with H2SO4. The culture was transferred more than 10 times on the iron or sulfur medium for adaptation to each substrate. Large-scale cultivation on iron or sulfur medium was carried out in a 20-liter carboy (working volume, 15 liters) under aeration at 30°C for 4 days. The pH of the sulfur medium was maintained at 2.5 with the addition of 4 M NaOH during cultivation.

Preparations of cell suspension and cell surface proteins. The culture broth was filtered through a filter paper (no. 2; Advantec Co., Ltd., Tokyo, Japan) to remove the precipitated ferric compounds or elemental sulfur. The filtrate was centrifuged at 15,000 × g for 20 min to collect the sulfur-grown or iron-grown cells. The collected cells were gently washed three times with sulfuric acid (pH 2.0). Cell surface proteins were obtained from the cells by the method described below (1). The iron- or sulfur-grown cells were further washed with sulfuric acid solution (pH 2.0) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM EDTA and 150 μg of lysozyme per ml (2). The suspension was incubated for 1 h at room temperature and then centrifuged at 15,000 × g for 20 min to remove cells and large debris. The supernatant thus obtained was ultracentrifuged at 100,000 × g for 2 h to collect the insoluble sediment. From the sediment, the substances soluble in N-lauroylsarcosine were eliminated through incubation in 50 mM Tris-HCl buffer (pH 6.8) containing 0.5% sodium N-lauroylsarcosine for 1 h at 37°C and ultracentrifugation at 100,000 × g for 2 h. The remaining detergent-insoluble sediment was solubilized and denatured in Laemmli buffer (15) for 5 min at 100°C and was then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Adhesion of cells to elemental sulfur. The method described in a previous report (26) was modified for the adhesion experiments in this study. Each cell suspension of iron- and sulfur-grown cells was diluted with sulfuric acid solution (pH 2.0) to an optical density of 0.1 at 600 nm. Five milliliters of the suspension was preincubated for 10 min at 30°C and mixed with 5 ml of sulfur suspension (pH 2.0, 30°C) containing 2.0 g of elemental sulfur powder (Wako Co., Ltd., Tokyo, Japan) of particle size below 0.14 mm in diameter. After standing for 10 min, the mixture was filtered through filter paper (no. 5B; Toyo Co., Ltd., Tokyo, Japan) to remove elemental sulfur, and then the optical density at 600 nm was measured to determine the amount of free cells. (When the optical density at 600 nm of a cell suspension is 0.1, the cell density of the suspension is 1.765 × 108 cells/ml.) The amount of adhered cells was determined by subtracting free cells from the added cells. For the experiment to examine thiol masking on the cell surface, iodoacetamide solution (pH 2.0) was added to the cell suspension to a concentration of 25 mM before contact with elemental sulfur (26). The optical density at 600 nm was measured with a model DU-650 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Fluorescent labeling of thiol groups. To label thiol groups on the cell surface, 20 μl of sulfuric acid solution (pH 2.0) containing 0.5 mg N-(7-dimethylamino-4-methylcoumarin)maleimide (DAMC) was added to each suspension of iron- and sulfur-grown cells (29). The suspension was allowed to stand for 10 min at room temperature and was collected by centrifugation at 15,000 × g for 20 min. The collected cells were resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 2.0 mM EDTA and 150 μg of lysozyme per ml. The mixture was incubated for 1 h at room temperature and then centrifuged at 15,000 × g for 20 min to remove the cells. The supernatant was ultracentrifuged at 100,000 × g for 2 h. The insoluble sediment of ultracentrifugation was taken as the crude lysate.

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Thiol groups of the protein in the crude lysate were detected by fluorescence under UV irradiation.

**Solubilization of sulfur-binding protein into CS$_2$**. The sulfur-grown cells were digested by the lysozyme-EDTA method described above. The digested solution was centrifuged at 15,000 × g for 20 min to obtain the crude lysate. The pH of the lysate was adjusted to 2.0 by addition of 0.25 M sulfuric acid solution. The lysate was treated according to the scheme shown in Fig. 1. The lysate was mixed with 1.0 g of elemental sulfur powder and allowed to stand for 10 min at room temperature to induce adhesion of the sulfur-binding protein to elemental sulfur. The mixture was then centrifuged at 15,000 × g for 20 min. The collected powder was washed three times with 5 ml of sulfuric acid solution (pH 2.0) to remove the proteins which did not adhere. The washed powder was transferred into a small glass vessel with 5.0 ml of sulfuric acid solution (pH 2.0). Carbon disulfide (CS$_2$) was added into the vessel until the sulfur powder was solubilized completely to form a water-CS$_2$ biphasic. The water phase was collected by a pipette and then ultracentrifuged at 100,000 × g for 2 h. The sediment was denatured at 100°C for 5 min for SDS-PAGE. Adhesion of proteins to elemental sulfur. The sulfur-grown cells were suspended in sulfuric acid solution (pH 2.0) containing 1.0% Triton X-100. The cells in the suspension were sonicated at 120 W for 1 min. The suspension was then centrifuged at 15,000 × g for 15 min to remove the cells. The supernatant was taken as the crude extract. This crude extract was used for the experiment testing the adhesion of the sulfur-binding protein to elemental sulfur as shown in Fig. 2. The crude extract was incubated with 2.0 g of elemental sulfur powder for 10 min. The mixture was then centrifuged at 15,000 × g for 20 min to collect elemental sulfur. The collected powder was washed three times with 5 ml of sulfuric acid solution (pH 2.0) to remove the proteins which did not adhere. The washed powder was further washed with Laemmli buffer (15) containing 5 mM 2-mercaptoethanol. The washed powder was subjected to in situ proteolysis with protease K (data not shown). The difference in the culture substrates induced a change in protein synthesis on the cell surface of *T. ferrooxidans*. Because the difference in the surface proteins might affect bacterial adhesion, the adhesion of sulfur-grown cells to elemental sulfur was compared with that of iron-grown cells. Iron-grown (1.59 × 10$^9$) or sulfur-grown (1.90 × 10$^9$) cells were added to an elemental sulfur suspension. The iron-grown cells scarcely adhered to elemental sulfur, whereas 40% of the sulfur-grown cells adhered (Table 1). More of the sulfur-grown than of the iron-grown cells adhered to elemental sulfur. The change in specific surface proteins would contribute to the adhesion of sulfur-grown cells to elemental sulfur.

**Detection of thiol groups.** To further examine the adhesion of the specific proteins to elemental sulfur, the existence of thiol groups in the proteins was investigated. The cells were treated with iodoacetamide to mask the thiol groups of the proteins on the cell surface. The number of adhered cells decreased from 7.55 × 10$^6$ to 1.54 × 10$^6$ cells after iodoacetamide treatment (Table 1). The thiol masking of the cells disturbed the adhesion, which suggested that thiol groups were responsible for the adhesion of the cells to elemental sulfur.

**FIG. 1.** Experimental scheme for the separation of the sulfur-binding protein by solubilization of elemental sulfur with CS$_2$. Sup., supernatant; soln., solution.

**FIG. 2.** Experimental scheme for the separation of the sulfur-binding protein by adhesion to elemental sulfur. Sup., supernatant; soln., solution.

**FIG. 3.** Comparison of cell envelope proteins between sulfur- and iron-grown cells. (A) SDS-PAGE of cell envelope proteins of sulfur-grown (lane 1) and iron-grown (lane 2) cells. Electrophoresis was done with an SDS–5 to 20% gradient polyacrylamide gel. The closed and open arrows indicate specific bands in sulfur- and iron-grown cells, respectively. (B) Detection of thiol groups in proteins of the crude lysate from sulfur-grown (lanes 2 and 4) and iron-grown (lanes 1 and 3) cells by thiol-binding reagent. Lanes 1 and 2, Coomassie blue staining; lanes 3 and 4, UV irradiation. Electrophoresis was done with an SDS–12.5% polyacrylamide gel. The arrow indicates the band emitted under UV irradiation. Numbers at the left indicate positions of molecular mass markers in kilodaltons.
The thiol-binding reagent DACM was added to each suspension of sulfur- and iron-grown cells to detect thiol groups in the proteins. The cells treated with DACM were lysed with lysozyme, and the lysate was analyzed by SDS-PAGE (Fig. 3B). Although there were many protein bands in the lysate of sulfur-grown cells (Fig. 3B, lane 2), only the 40-kDa protein showed detectable fluorescence light from DACM under UV irradiation (lane 4). Only the 40-kDa protein of the sulfur-grown cells had thiol groups. The proteins in the lysate of the iron-grown cells did not emit fluorescence (lanes 1 and 3). These results showed that the adhesion of *T. ferrooxidans* to elemental sulfur was due to the thiol groups of the 40-kDa protein which was synthesized by the sulfur-grown cells.

### Solubilization of sulfur-binding protein by CS2

To clarify whether the 40-kDa protein is involved in bacterial adhesion to elemental sulfur, direct adhesion of the protein to sulfur powder was investigated by the scheme shown in Fig. 1. The organic solvent CS2 was used to dissolve the sulfur particles, releasing the proteins which had reversibly adhered to sulfur from sulfur into the water phase. The water phase was analyzed by SDS-PAGE (Fig. 4A). The band pattern of the water phase (Fig. 4A, lane 2) was similar to that of the original crude lysate (lane 1). Most of the proteins in the crude lysate adhered to elemental sulfur, and these adhered proteins were released into the water phase by solubilization of elemental sulfur. However, the 40-kDa band disappeared from the water phase (lane 2). The protein could irreversibly adhere to elemental sulfur by a disulfide bond, and so it must be solubilized into the CS2 phase with elemental sulfur. On the other hand, the 38-kDa band, like the 40-kDa band, disappeared from the water phase (lane 2) as a result of solubilization into the CS2 phase by irreversible adhesion. However, the 38-kDa protein was not detected as the surface protein, unlike the 40-kDa protein (Fig. 3), and so the protein is not related to adhesion of the bacterium to elemental sulfur.

### Sulfur-binding protein in the presence of surfactant

The irreversible adhesion was reconfirmed by quenching the hydrophobic interaction as shown in Fig. 4B. Triton X-100 was used to prevent adhesion by hydrophobic interaction. The crude extract of sulfur-grown cells containing Triton X-100 was directly added to elemental sulfur to induce adhesion to the sulfur-binding protein. After the adhesion, the powder was washed with 2-mercaptoethanol solution to release the proteins adhered by the disulfide bond. In the 2-mercaptoethanol washout solution, only the 40-kDa protein was detected (Fig. 4B, lane 2). After the wash with 2-mercaptoethanol, the sulfur powder was boiled in denaturing solution to detect the proteins which were not washed out by 2-mercaptoethanol. In the denaturing solution, no proteins were detected upon SDS-PAGE. These results showed that the 40-kDa protein was the only protein which adhered to elemental sulfur in the presence of the surfactant. The protein has the same electrophoretic mobility on SDS-PAGE as DACM-labeled protein and as the proteins in the CS2 phase.

### Location of sulfur-binding protein in the cell envelope

To identify the location of the 40-kDa protein, a flagellum solution was prepared from sulfur-grown and iron-grown cells. Many of the flagella were observed in the solution prepared from sulfur-grown cells under a transmission electron microscope (Fig. 5A). However, they were not observed in the solution from the iron-grown cells. The flagella were located on the surface of the sulfur-grown cells (Fig. 5B). The flagellum solution was analyzed by SDS-PAGE. A single band of the 40-kDa protein was detected from the flagella (Fig. 5C). The 40-kDa protein is synthesized as a part of the flagella of sulfur-grown cells.

### Adhesion by sulfur-binding protein of flagella

A DACM-labeling experiment was carried out to investigate whether the 40-kDa protein in flagella contained thiol groups. The flagella were removed from sulfur-grown cells by shearing. Both flagella and the deflagellated cells were treated with DACM to label thiol groups. The flagella showed detectable fluorescence light from DACM under UV irradiation, whereas the deflagellated cells did not (Fig. 6). Adhesion of the deflagellated cells to elemental sulfur was quantified to investigate the mediation of adhesion by the flagella. When $1.32 \times 10^7$ deflagellated cells were added to an elemental sulfur suspension, $1.05 \times 10^7$ cells adhered to elemental sulfur. This amount was clearly smaller than the amount obtained from the cells with flagella (Table 1). These results suggested that the flagella contained the thiol groups in a 40-kDa protein which mediated the adhesion to elemental sulfur.

### DISCUSSION

It is widely known that bacterial adhesion is mediated by hydrophobic interaction. In this study, it was determined that the adhesion of *T. ferrooxidans* ATCC 23270 to elemental sulfur is mediated not only by hydrophobic interaction but also by a chemical bond. The 40-kDa protein in the flagella of the bacterium is responsible for this chemical bond. The thiol groups in the protein react with elemental sulfur to form a disulfide bond. The existence of the thiol group was demonstrated by DACM staining (Fig. 3B). The contribution of thiol groups to adhesion to sulfur powders was examined by iodoac-
etamidetreatment of whole cells (Table 1). The contribution was also examined by two methods using the 40-kDa protein directly. One was dissolution of the protein into CS2 solution with elemental sulfur (Fig. 4A), while the other was a fission reaction with mercaptoethanol (Fig. 4B). All of these results supported the existence of a disulfide bond of the 40-kDa protein to elemental sulfur.

Hydrophobic interaction and ionic bonds can mediate bacterial adhesion. However, the involvement of the ionic bond was disproved by the CS2 experiment. If adhesion of the 40-kDa protein to sulfur powders involves an ionic bond, the protein would be partly extracted into the water phase by substitution reactions with ions existing in the water phase. However, the protein was not extracted and was dissolved into the CS2 solution with elemental sulfur (Fig. 4A). The involvement of the ionic bond was also disproved by further examination. The 40-kDa protein that adhered to sulfur powder was not released with 500 mM NaCl solution by the substitution reactions (data not shown). On the other hand, the possibility that the 40-kDa protein adhered to elemental sulfur by hydrophobic interaction was disproved by adhesion in the presence of Triton X-100. The 40-kDa protein could adhere to elemental sulfur powders when the hydrophobic interaction was quenched (Fig. 4B). Therefore, the adhesion of T. ferrooxidans to elemental sulfur is mediated by the disulfide bond between thiol groups in the 40-kDa protein and elemental sulfur.

It was previously found that growth conditions such as the pH of the medium (1), temperature (10), and osmolarity (11) affected the biosynthesis of cell surface proteins of T. ferrooxidans. In our experiments, the pH of the sulfur medium was adjusted to 2.5 for the iron medium by the addition of sodium hydroxide solution during cultivation. The change in osmolarity did not affect the cell surface proteins up to a hydroxide concentration of 66 mM. The band pattern of SDS-PAGE at the logarithmic phase did not differ from that at the stationary phase for both sulfur- and iron-grown cells (data not shown). On the other hand, the sulfur-grown cells in the sulfur medium were classified into two kinds, planktonic-phase cells and cells adherent to sulfur powders. It is possible that the surface proteins of the planktonic-phase cells differ from those of the adherent cells. However, the protein profiles of the two kinds of cells were the same (data not shown). These results showed that the change in cell surface protein synthesis of T. ferrooxidans was caused by the difference in culture substrate and not by the other factors. The change in the surface proteins may be regulated by an elaborate mechanism to adapt to the utilization of growth substrates (22), but the mechanism has been not determined.

Rodriguez et al. have reported on cell envelope proteins of iron-grown T. ferrooxidans (20). The band pattern that they found upon SDS-PAGE agreed well with our results (Fig. 3A). On the other hand, recent research has demonstrated that sulfur-grown T. ferrooxidans strains synthesize new 47- and 55-kDa proteins (3). The 46- and 50-kDa proteins of sulfur-grown cells in this study (Fig. 3A) might correspond to the 47- and 55-kDa proteins, respectively. However, the physiological function of these proteins of sulfur- and iron-grown cells has not yet been well defined.

The adhesion of bacteria to solid surfaces has been modeled on the DLVO theory in colloidal chemistry (17). The theory says that bacterial cells cannot approach a solid surface close enough to form a chemical bond, because there is a high energy barrier between the solid surface and the bacterial cells. If the 40-kDa protein is located on the outer membrane of the cells, it cannot form a chemical bond with solid sulfur. However, extracellular appendages can break through the energy barrier and make close contact with the solid surface (9, 17). As the 40-kDa protein was located on the flagella (Fig. 4C and 6B), the 40-kDa protein would be able to come into closer contact with the surface of the elemental sulfur. This closer contact of the flagella would allow the formation of disulfide bonds, resulting in tight adhesion of the bacteria to elemental sulfur.

Gromova et al. described the extracellular appendages of sulfur-grown T. ferrooxidans cells (7). We also observed the existence of appendages on the bacterium (Fig. 5). The appendages found in this study were flagella because sulfur-grown cells showed motility in stand culture under light microscopy (data not shown). The flagella that we observed had a diameter of 16 nm, the diameter of typical flagella of eubacteria. In addition, the molecular masses of flagellins of Roseburia cecicola (18), Bacillus firmus (8), Rhizobium meliloti (14), and some species of genus Pseudomonas (28) range from 38 to 43 kDa. On the other hand, the molecular masses of pilins of Escherichia coli (12), Pseudomonas cepacia (21), Klebsiella...
pneumoniae (5), and Xenorhabdus nematophilus (19) ranged from 16 to 21.5 kDa. The molecular mass of the flagellum protein in this study was within the range of those of flagellins rather than those of pilins.

The question of how *T. ferrooxidans* can utilize insoluble elemental sulfur remains unanswered. This study determined that *T. ferrooxidans* can closely approach elemental sulfur to effectively utilize the soluble polysulfide chain produced by the attack of reduced glutathione on insoluble elemental sulfur. In this way, *T. ferrooxidans* would incorporate insoluble elemental sulfur into the cells.

REFERENCES