Biochemical Studies of a soxF-Encoded Monomeric Flavoprotein Purified from the Green Sulfur Bacterium Chlorobaculum tepidum That Stimulates in Vitro Thiosulfate Oxidation

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Received November 4, 2009; Accepted December 26, 2009; Online Publication, April 7, 2010
[doi:10.1271/bbb.90815]

In the green sulfur bacterium Chlorobaculum tepidum, three sulfur oxidizing enzyme system (Sox) proteins, SoxAXK, SoxYZ, and SoxB (the core TOMES, thiosulfate oxidizing multi-enzyme system) are essential proteins, SoxAXK, SoxYZ, and SoxB (the core TOMES, thiosulfate oxidizing multi-enzyme system) in vitro thiosulfate oxidation. We purified monomeric flavoprotein SoxF from this bacterium, which had sulfide dehydrogenase activity. SoxF enhanced the thiosulfate oxidation activity of the purified core TOMES with various cytochromes as electron acceptors to different degrees without any change in the affinity for thiosulfate. The maximal reaction rates with 50 μM C. tepidum cytochrome c-554 were slightly higher than with horse-heart cytochrome c, and the addition of 0.5 μM SoxF increased the rate by 92%. The rates with 50 μM horse-heart cytochrome c and 50 μM horse-heart cytochrome c plus 0.5 μM cytochrome c-554 were increased by SoxF by 31% and 120% respectively. We conclude that SoxF mediates electron transfer between the components of core TOMES and externally added cytochromes.

Key words: flavocytochrome c; green sulfur bacteria; sox; sulfide dehydrogenase; thiosulfate

Sulfide and thiosulfate are the most abundant forms of reduced sulfur compounds in aquatic environments. Microorganisms in the domain Bacteria that use thiosulfate as an electron donor possess a system of multiple periplasmic proteins, referred to either as the thiosulfate oxidizing multi-enzyme system (TOMES) or as the sulfur oxidizing system (Sox).

Two different biochemical pathways for thiosulfate oxidation are distinguishable among these systems. In one pathway, found in bacteria such as Thermithio- bacillus (formerly Thiobacillus) tepidarius and Acidithiobacillus (formerly Thiobacillus) thiooxidans, thiosulfate is first oxidized to tetrathionate either as the final or an intermediary product. In the other pathway, found in bacteria such as the facultative lithotrophic Paracoccus pantotrophus and the photosynthetic green sulfur Chlorobaculum (formerly Chlorobium) tepidum, the oxidized product of thiosulfate is assumed to be bound to the SoxYZ protein. Some bacteria, such as the acidophilic sulfur-oxidizing Starkeya novella, appear to have both pathways.

TOMES components have been intensively studied in facultative lithotrophic bacteria such as P. pantotrophus and Paracoccus versutus, and in purple sulfur bacteria such as Allochromatium vinosum. In these bacteria, the components of TOMES are largely similar among different phyla, with some variations, notably with respect to sulfur dehydrogenase SoxCD. At a minimum, three proteins (the core TOMES) are indispensable to thiosulfate oxidation. SoxAX, sometimes called cytochrome (cyt) c-551, which mediates electron transfer to external cytochrome and/or high-potential iron-sulfur protein, SoxYZ, which binds the oxidized product of thiosulfate on the cysteinyl-SH group of SoxY as the intermediate,7 and SoxB, which hydrolyzes the latter. For bacteria such as P. pantotrophus, which has SoxCD, the following reaction scheme has been proposed:2

\[
\text{SoxY}(-\text{SH}) + \text{SSO}_3^- \rightarrow \text{SoxY}(-\text{SH}) + \text{SSO}_3^- + 2e^- + H^+ \\
\text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + H_2O \rightarrow \text{SoxY}(-\text{SH}) + \text{SoxY}(-\text{SH}) + 2e^- + H^+ \\
\text{SoxY}(-\text{SH}) + 2\text{H}_2\text{O} \rightarrow \text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + 6e^- + 7H^+ \\
\text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + \text{H}_2\text{O} \rightarrow \text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + 2H^+ \\
\text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + \text{H}_2\text{O} \rightarrow \text{SoxY}(-\text{SH}) + \text{SO}_4^{2-} + 2H^+
\]

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Abbreviations: CBB, Coomassie Brilliant Blue R; cyt, cytochrome; eq, equation; FCSD, flavo-cytochrome c sulfide dehydrogenase; MALDI-TOF, Matrix Assisted Laser Desorption Ionization-Time of Flight; MES, 2-morpholinoethane sulfonic acid; RC, reaction center; Sox, sulfur oxidizing enzyme system; SP Sepharose, sulfopropyl Sepharose; SQR, sulfide-quinone reductase; TOMES, thiosulfate oxidizing multi-enzyme system
Experimental systems using reconstituted TOMES containing SoxFCD generate eight electrons and two sulfate per thiosulfate. In bacteria lacking SoxFCD, such as green sulfur bacteria and some purple bacteria, the reaction proceeds according to eqs. 1 and 2 to yield SoxY(-S)-SH and sulfate, and this reaction cycle is assumed to be repeated to yield a poly-sulfide group on SoxY. Thus the reconstituted TOMES of these bacteria generate two electrons and one sulfate per thiosulfate, leaving an additional sulfur atom on SoxYZ in the periplasmic space. In these bacteria, the sulfate sulfur is ultimately oxidized to sulfate.9) Although the exact biochemical reaction pathways are not yet fully elucidated, a model involving transmembrane transfer of a zero-valence sulfur equivalent has been proposed.9–12) with the participation of a putative quinone-interacting protein, while it is normally a soluble protein in other systems containing SoxCD generate eight electrons and two sulfate per thiosulfate, and this reaction cycle is assumed to be significantly increased. From these results, the authors speculated that SoxF acts on some component or under certain conditions present only in whole cells and crude cell-free extracts, and not in the purified reconstituted system.20) The biochemical basis of the stimulating effects of SoxF in the crude extract remains to be investigated.

The photosynthetic green sulfur bacterium Chlorobaculum tepidum utilizes reduced sulfur compounds such as sulfide, elemental sulfur, and thiosulfate as the electron source for growth.29) The primary donor of the reaction center (RC) is a special pair of bacteriochlorophylls called P840, and its immediate electron donor is RC-bound cyt c-551.30) Itoh et al.31) found that a soluble mono-heme cyt c-554 of about 10 kDa (the CT0075 protein,30) sometimes called a small soluble cytochrome c) donates electrons to bound cyt c-551 rather than directly to oxidized P840. In the reconstituted core TOMES (SoxAX, SoxB, SoxY), the presence of the small soluble protein (the three core TOMES and SoxCD), thiosulfate-oxidizing activity did not significantly increase. From these results, the authors speculated that SoxF acts on some component or under certain conditions present only in whole cells and crude cell-free extracts, and not in the purified reconstituted system.20) The biochemical basis of the stimulating effects of SoxF in the crude extract remains to be investigated.

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C. tepidum cyt c-554 greatly increased the stimulating effects of SoxF on horse-heart cyt c reduction.

Materials and Methods

Bacterial strains, growth conditions, and purification of SoxF. The growth of C. tepidum strain TLS (kindly donated by Dr. M. T. Madigan) cells with thiosulfate and sulfide as electron donors and subsequent preparation of the crude cell extract using a French pressure cell followed by ultra-centrifugation were as described previously. From the supernatant (obtained from about 20 g of cells), the fraction precipitating between 40% and 80% saturated ammonium sulfate was collected by centrifugation, and the pellet was dissolved in about 50 ml of 20 mM Tris–HCl (pH 7.8) and dialyzed against 1 liter of the same buffer with 3 changes. After centrifugation at 10,000 × g for 10 min to remove undissolved particles, the supernatant was applied to an anion-exchange DEAE-Toyopearl 650M column (2.5 × 25 cm, Tosoh, Tokyo). The flow-through fraction contained SoxA, SoxB, and SoxYZ, whereas SoxF (CT1015) was retained on the column. After the column was washed with 200 ml of the same buffer, the fractions eluted with 0.3 M NaCl in the same buffer were saved and dialyzed against 10 mM MES-NaOH (pH 6.0). The diazylated preparation was applied to a cation-exchange SP Sepharose FF column (2.5 × 7 cm; GE Healthcare) equilibrated with 10 mM MES-NaOH (pH 6.0). After the column was washed with 10 mM MES-NaOH (pH 6.0), proteins were eluted with 400 mM NaCl in the same buffer, and eluted fractions were retained.

The thiosulfate oxidation stimulation activity of the eluted fractions was assayed by adding 20 μl of each fraction to the reaction mixture (final volume 100 μl) containing 0.5 mM SoxYZ, 0.5 mM SoxB, 0.5 mM SoxA, 50 μM C. tepidum cyt c-554, 20 mM MES-NaOH (pH 6.0), and 2 mM sodium thiosulfate. The pooled active fractions (typically about 20 ml) were desalted by ultra-filtration (PM-30, Millipore) and applied to a Hitrap™ SP Sepharose column (bed volume 5 ml; GE Healthcare) equilibrated with 20 mM MES-NaOH (pH 6.0). Protein was eluted with a linear gradient of 40 ml of 0–400 mM NaCl in 20 mM MES-NaOH (pH 6.0), and the buffer of the active fractions was changed to 50 mM Tris–HCl (pH 7.8) by ultrafiltration. Following buffer exchange, the active fractions were applied to an anion-exchange HiTrap™ Q column equilibrated with 50 mM Tris–HCl (pH 7.8), and the protein was eluted with linear gradient of 40 ml of 0–500 mM NaCl to yield purified SoxF.

Purification of other Sox proteins. SoxA, SoxB, and SoxYZ were purified as described previously. Recombinant SoxA and SoxX were overexpressed in Escherichia coli, and then purified as described previously.

Enzyme assays. Thiosulfate and sulfide oxidation activities using various cytochrome species as electron acceptors were measured at 25 °C in a 100-μl reaction mixture volume. The standard reaction mixture contained 20 mM MES-NaOH (pH 6.0), 0.5 μM each of the purified core TOMES (SoxYZ, SoxB, SoxAXK), 50 μM various cytochrome species as indicated in the figure legends and tables, and 2 mM sodium thiosulfate or 0.5 mM sodium sulfide, unless otherwise indicated.

The standard reaction mixture for sulfide oxidation contained 50 μM of various cytochrome species as indicated in the figure legends and tables, 0.5 μM SoxF, and 10 mM Tris–HCl (pH 7.8). The reaction was initiated by the addition of 20 μM sulfide. Because sulfide reduces various cytochromes non-enzymatically at significant rates, in the figures and tables presented here, the blank values obtained in the absence of protein factors were subtracted from the ones obtained, which were determined by extrapolating the absorbance change at each initial concentration of sulfide to zero time, as with the thiosulfate oxidation activity measurement. Reactions were carried out in a capped cell under nitrogen gas, and cyt c reductions were followed by a spectrophotometer (UV2500PC; Shimadzu, Kyoto).

The reduction rates of cytochromes were calculated using ΔA554 = 23.8 nm−1 cm−1 for C. tepidum cyt c-554, ΔA550 = 20.0 nm−1 cm−1 for horse-heart cyt c, ΔA550 = 21.2 nm−1 cm−1 for the yeast Saccharomyces cerevisiae cyt c,33 and ΔA550 = 31.0 nm−1 cm−1 for C. tepidum recombinant SoxX and SoxAXK at a redox potential higher than zero volt (as determined in this study). The kinetic constants of the enzymatic reactions were obtained by non-linear regression data analysis using Igor Pro 6.03 software (WaveMetrics, Portland, Oregon).

Substrate solution preparation. Both sodium thiosulfate pentahydrate and sodium sulfite were dissolved in water purged with nitrogen gas. The sulfite stock solution was prepared as follows: H2S gas was generated by adding 1 ml of 4 M sulfuric acid to 10 ml of 100 mM Na2S, and was carried in the flow of nitrogen gas and trapped in 1 ml of 100 mM NaOH solution. The trapped sulfide concentration was determined by the methylene-blue method.

Analytical methods. The following methods were as described in a previous report: polyepptide size determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), protein determination (with CBB), N-terminal amino acid sequence determination (Edman degradation), and molecular mass determination of SoxF (MALDI-TOF mass spectrometry).

Results

Characterization of the SoxF protein

In a previous study with C. tepidum, we reported the purification and characterization of three core TOMES factors, SoxA, SoxYZ, and SoxB, which are indispensable for thiosulfate oxidation in vitro. While purifying the three core TOMES components, we discovered that the fraction adsorbed on a DEAE-Toyopearl 650M column contained a protein factor that was not essential for but nevertheless stimulated thiosulfate oxidation by the core TOMES when cyt c-554 from C. tepidum was the electron acceptor. Unlike this protein factor, the core TOMES components were obtained in the flow-through fractions during this chromatographic separation.

Our current report describes the purification of the protein factor to homogeneity from cell extracts, as detailed in “Materials and Methods” section. The purified protein was yellow in color and had an apparent molecular mass of 42 kDa on SDS–PAGE analysis (Fig. 1A). The protein, eluted as a single peak fraction, had an apparent molecular mass of about 48 kDa on gel-permeation chromatography (TSK gel G3000PWxl 7.8/300, Tosoh, Tokyo) analysis (data not shown), indicating that it is a monomeric protein. The N-terminal amino acid sequence of the 42-kDa polypeptide was SAPKAHVVI, which agrees with the one deduced from the CT1015/soxF/soxX.12 gene of this bacterium (http://www.tigr.org), which begins with Ser33, indicating that a signal peptide targeting the periplasmic space is cleaved between Ala32 and Ser33 (Fig. 1C). The gene occurs between CT1014, encoding a hypothetical protein of unknown function of about 12 kDa, and soxX (CT1016) (Fig. 1D), and encodes a protein which in the mature form has high amino acid sequence identity with the monomeric SoxF from P. pantotrophus (41% identity). C. tepidum SoxF as prepared in our current study was a monomeric protein devoid of a bound cyt c subunit, and our attempts to demonstrate tight binding with cyt c-554 by conventional gel-permeation chromatography have been unsuccessful thus far. SoxF exhibited an absorption spectrum typical of a flavoprotein, with peaks at 278, 359, 451, and 773.
476 nm (Fig. 1B). MALDI-TOF mass spectrometry of the SoxF yielded a mass of 43,756 Da, indicating that the above assumptions are valid and that SoxF occurs as a monomeric flavoprotein.

Effects of SoxF on thiosulfate-dependent cyt c reduction by the core TOMES

Kusai and Yamanaka(26) found that a crude thiosulfate oxidizing enzyme system preparation from C. limicola f. sp. thiosulfatophilum used various cytochromes as electron acceptors, albeit with different efficiencies. In the previous study, we found that the components of the core TOMES, SoxF, SoxB, and SoxY are indispensable to oxidation of thiosulfate with cyt c-554 as the electron acceptor, and we subsequently found that horse-heart cyt c can substitute for cyt c-554 and can function as an electron acceptor in the same systems. As shown in Fig. 2, cyt c-554 was moderately more efficient than horse-heart cyt c as the electron acceptor at 50 μM. The addition of 0.5 μM SoxF to the core TOMES increased the rates of reduction of the two cytochromes, but the degree of increase was significantly higher with cyt c-554 than with horse-heart cyt c (about 92% and 31% increase respectively at 2 mM thiosulfate). On the other hand, the $K_m$ values for thiosulfate (about 0.15 mM) were not significantly affected by the species of cytochrome or by the presence or absence of SoxF.

When a 100-fold lower amount of cyt c-554 (0.5 μM) was added to the core TOMES (0.5 μM each) containing 50 μM horse-heart cyt c, it increased the rate by about 30% and 120% in the absence and the presence of SoxF respectively (Fig. 2). In these assays, the total amounts of cytochrome reduced far exceeded 0.5 μM, and the reaction time course proceeded almost linearly for several min.

The results of using various cytochrome species as electron acceptors are shown in Table 1. Yeast cyt c was a slightly more efficient electron acceptor than cyt c-554 under the assay condition used, and the reaction rate was further increased slightly by the addition of cyt c-554 and significantly by the addition of 0.5 μM SoxF. SoxAXK is a trimeric protein with SoxA and SoxX subunits each binding a single heme. When added at 50 μM to a reaction mixture containing 0.5 μM each of the core TOMES components, SoxAXK itself and its subunit rSoxX (overexpressed in Escherichia coli and purified, see ref. 20) functioned as electron acceptors although not very efficiently, and rSoxA did not function as an electron acceptor at all even though it binds a heme. The reduction rates of SoxAXK and rSoxX were greatly increased by the addition of 0.5 μM cyt c-554, and were further increased by the presence of SoxF.

Effects of SoxF on sulfite-dependent cyt c reduction by the core TOMES

The core TOMES oxidized sulfite with various cytochrome species as electron acceptors, and all their components were indispensable to this reaction. SoxF added to the core TOMES resulted in modest inhibition of sulfite-dependent cyt c-554 reduction (Fig. 3A). The kinetics of inhibition were analyzed (Fig. 3B) with the result that the apparent maximum rate ($V_{max}$) was lowered by SoxF without any significant change in the $K_m$ value for sulfite (24 μM), indicating non-competitive inhibition ($K_i = 0.57$ μM for SoxF). The addition of high concentrations of sulfite (higher than 0.5 mM) decreased the cyt c-554 reduction rate by the core TOMES in a time-dependent manner (data not shown; noticeable within several tens of seconds, and the activity was almost halved after 60 s when the initial sulfite concentration was 0.5 mM).

SoxF-catalyzed sulfide-dependent cyt c-554 reduction

In the absence of the core TOMES, SoxF alone catalyzed sulfide-dependent reduction of C. tepidum cyt c-554. The sulfide oxidation activity of SoxF was low at pH 6.0, increased with increases in pH, and reached a maximum at about pH 8.5 (Fig. 4). Since the $pK_a$ for hydrogen sulfide is 7.06, this pH profile of the activity might reflect a preference of HS⁻ over H₂S as the substrate for the enzyme.

The genome of C. tepidum(10) has three soxF homologs: CT1015 (a soxF/soxF/C. limicola soxF2 homolog), CT1025 (encoding a SoxF-like protein, putative), and a CT2081/fccB(24)/C. limicola soxF1 homolog (encoding the flavoprotein subunit of flavocytochrome c sulfide dehydrogenase (FCSD) contiguous to a CT2080/ fccA(24)/C. limicola soxE homolog encoding the cyto-
Fig. 2. Effects of SoxF and Various Cytochromes on Thiosulfate Oxidation.
The reaction mixture contained 0.5 mM each of SoxAXK, SoxB, and SoxYZ, the indicated concentration of thiosulfate in 20 mM MES-NaOH (pH 6.0), and 0.5 mM each of the cytochromes or SoxF, as indicated. A, cyt c-554 (50 μM) as the electron acceptor, in the absence (triangle) and presence of SoxF (circle), B, horse heart cyt c (50 μM) as the electron acceptor, in the presence of cyt c-554 (diamond), SoxF (circle), and cyt c-554 plus SoxF (square), and in the absence of both (triangle).

Table 1. Effects of SoxF and Various Cytochromes on Thiosulfate-Dependent cyt c Reduction Catalyzed by Core TOMES

<table>
<thead>
<tr>
<th>Electron acceptor (50 μM)</th>
<th>None</th>
<th>SoxF</th>
<th>cyt c-554</th>
<th>SoxF, and cyt c-554</th>
<th>hh* cyt c</th>
<th>SoxF, and hh* cyt c</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt c-554</td>
<td>7.2 ± 0.2</td>
<td>13.8 ± 0.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>hh* cyt c</td>
<td>7.0 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>15.3 ± 0.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>yeast cyt c</td>
<td>9.4 ± 0.2</td>
<td>13.9 ± 0.4</td>
<td>10.7 ± 0.7</td>
<td>15.7 ± 0.6</td>
<td>9.1 ± 0.2</td>
<td>13.2 ± 0.6</td>
</tr>
<tr>
<td>SoxAKK</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>6.5 ± 0.2</td>
<td>12.9 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>rSoX</td>
<td>0.77 ± 0.01</td>
<td>1.0 ± 0.00</td>
<td>7.0 ± 0.00</td>
<td>14.1 ± 0.1</td>
<td>1.2 ± 0.0</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>rSoX</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*ND, not detectable (less than 0.005 absorbance change/min at 551 nm)

Fig. 3. Inhibition of Sulfite Oxidation by SoxF.
A, Relationship between reaction rate and sulfite concentration in the presence and the absence of SoxF. The reaction mixture contained 0.5 mM SoxYZ, 0.5 mM SoxB, 0.5 mM SoxAXK, 50 μM cyt c-554, 20 mM MES-NaOH (pH 6.0), the indicated concentration of sulfite, and SoxF: 0 μM (circle), 0.2 μM (triangle), and 0.5 μM (square). B, Data from A were fitted to the following equation: 

\[
\frac{1}{v} = \frac{1}{K_m} \left( \frac{V_{app}}{S} \right) + \frac{1}{V_{app}},
\]

\[v\] being the experimentally obtained reaction rate, \(K_m\) the Michaelis constant, \(V_{app}\) the apparent maximum rate, and \([S]\) the sulfite concentration.

A. The values are means ± SD for three independent determinations.
B. The reaction mixture contained 0.5 mM SoxYZ, 0.5 mM SoxB, 0.5 mM SoxAXK, 50 μM cyt c-554, 20 mM MES-NaOH (pH 6.0), and 0.5 μM each of the cytochromes or SoxF.
C. Cyt c reduction was spectrophotometrically determined at 554 nm for cyt c-554, at 551 nm for SoxAKK, rSoX, and rSoX, and at 550 nm for horse-heart cyt c and yeast cyt c.

*ND, not detectable (less than 0.005 absorbance change/min at 551 nm).
chromosome c subunit). We found that SoxF accounted for no less than half (about 60% in one experiment) of the sulfide dehydrogenase activity in the fraction precipitated between 40–80% saturation of ammonium sulfate of cell-free soluble extracts under our experimental conditions (data not shown).

The core TOMES also catalyzed the sulfide-dependent reduction of cyt c-554, the presence of all three core TOMES components being necessary for this reaction. The sulfide dehydrogenase activities of SoxF, the core TOMES, and a combination of the two as a function of sulfide concentration at pH 7.8 are shown in Fig. 5. SoxF had much higher activity and higher affinity for sulfide than the core TOMES, and the activity of SoxF in the presence of the core TOMES was slightly lower than that catalyzed by SoxF alone.

In SoxF-catalyzed sulfide dehydrogenation reactions, not only cyt c-554 but also horse-heart cyt c and yeast cyt c functioned as electron acceptors. At pH 7.8, the $V_{\text{app}}$ values with the latter cytochromes were much lower than with cyt c-554, although the $K_m$ values for sulfide did not change significantly. The results of nonlinear regression analyses of enzymatic sulfide-dependent reduction kinetics constants with various cytochromes as electron acceptor are shown in Table 2.

**Stoichiometries of sulfur compound oxidation**

With the core TOMES, the stoichiometric amounts of cytochrome reduced were found to be about two for each molecule of thiosulfate, sulfite, or sulfide oxidized irrespective of the cytochrome species present as the acceptor (Table 3). The presence of SoxF did not significantly affect this stoichiometry. In these experiments, the amount of cytochrome reduced usually exceeded by at least 8 times the concentration of the core TOMES components and of SoxF, except for the reduction of cyt c with sulfite, which was rather quickly inactivated in a short time as described above.

**Discussion**

SoxF occurred as a monomeric protein

We have isolated from *C. tepidum* the soluble monomeric flavoprotein SoxF encoded by *SoxF*/*CT1015*/*soxP* found in the *sox* gene cluster. It lacked a bound cyt c (Fig. 1A). Monomeric SoxF devoid of cytochrome appeared to be comparable to the monomeric SoxF from *P. pantotrophus*. Monomeric *C. tepidum* SoxF devoid of bound c-type cytochrome was able to oxidize sulfide (Fig. 4, Table 2), as do dimeric FCSDs in a manner similar to the monomeric SoxF from the facultative chemotrophic *P. pantotrophus*, with small molecule cytochromes added *in vitro* as electron acceptors.

The optimum pH value of the sulfide dehydrogenase activity of *C. tepidum* SoxF was about pH 8.5 (Fig. 4), but it has been reported to be about pH 6.0 for SoxF from *P. pantotrophus*.27
In C. tepidum, soxF (CT1015/soxA) is part of the sox gene cluster along with the soxXYZAKB genes (from CT1016 to CT1021), but the soxE gene is absent (Fig. 1D), as it is in Chlorobium limicola. Within the genome of C. tepidum, three ORFs encoding flavoproteins with significant amino acid sequence identity to the flavoprotein subunit of FCSD have been found. CT2081/fccB occurred in tandem with the CT2080/fccA gene (encoding cyt c), and these genes were found about 1 Mbp away from the sox gene cluster. Gene CT1025 encoding a putative SoxF-like protein was found just downstream of the sox gene cluster. The amino acid sequence identities between the deduced mature proteins (SignalP, http://www.cbs.dtu.dk/services/SignalP/) were 51% between SoxF (CT1015) and FccB (CT2081), 13% between SoxF and SoxF-like protein (CT1025), and 16% between FccB and SoxF-like protein.

C. tepidum SoxF shared relatively high amino acid sequence identity with other deduced SoxFs found in the sox gene clusters of various sulfur-oxidizing bacteria and with the flavin subunits of various FCSDs. In the following list, the percent amino acid sequence identity of the deduced mature protein is underlined where at least one of the soxA, B, C, D, K, X, Y, and Z genes was found within three ORFs from soxF, and is shown in bold when soxF was contiguous with soxZ, followed by the strain name, and (the GenBank accession number): 80% Chlorobium limicola (Q8RLX4), 72% Chlorobaculum parvum (Q8RLX4), 72% Prosthecochloris vibrioformis (A4SCG4), 61% Chlorobium phaeobacteroides (A1BDPS), 61% Pelodictyon phaeocladuthraformae (B4SEK8), 59% Chlorobium phaeobacteroides (A1BCG5), 59% Prosthecochloris vibrioformis (A4SCA2), 59% Pelodictyon phaeocladuthraformae (B4SAK2), 58% Chlorobium limicola (Q8RLW4), 56% Chlorobaculum parvum (B3QQX4), 51% Chlorobium tepidum (Q8KAS4), 50% Allochromatium vinosum (Q06530), 41% Paracoccus pantotrophus GB17 (O07821), 16% Chlorobium tepidum (Q8KDM1).

These results indicate that within green sulfur bacteria, some SoxFs, encoded by genes within the sox gene cluster, tend to show higher sequence identity with each other than with the SoxFs of FCSD, but this was not always the case (Chlorobium phaeobacteroides). When we extended the similarity search over various bacteria, we found it difficult to distinguish between the monomeric SoxF and the flavoprotein portion of FCSD by simply comparing amino acid sequences.

### Table 3. Stoichiometries of cyt c and cyt c-554 Reduction

<table>
<thead>
<tr>
<th>Electron source</th>
<th>Sox protein</th>
<th>Ratio of mol hh⁺ cyt c reduced/mol of electron donor oxidized</th>
<th>Ratio of mol cyt c-554 reduced/mol of electron donor oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AXK, B, YZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiosulfate</td>
<td>+</td>
<td>2.1 ± 0.0</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.1 ± 0.0</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>ND⁺⁺</td>
<td>ND⁺⁺</td>
</tr>
<tr>
<td>Sulfide</td>
<td>+</td>
<td>1.9 ± 0.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.0 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.9 ± 0.1</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td>Sulfite</td>
<td>−</td>
<td>1.9 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

*Horse heart
**not detectable (less than 0.005 absorbance change/min of a peak of cyt c)

The reaction mixture contained 0.5 μM SoxY, 0.5 μM SoxB, 0.5 μM SoxAAXK, 50 μM horse-heart cyt c or cyt c-554, 10 μM Tris-HCl (pH 7.8), and 5 μM thiosulfate, sulfite, or sulfide. Where indicated, SoxF was added at 0.5 μM.

Stimulating effects of SoxF on thiosulfate oxidation by the core TOMES

Based on genomic sequence comparisons, the majority of thiosulfate oxidizing bacteria have a soxF homolog within the sox gene cluster but not necessarily a soxE homolog (roughly about half of them are devoid of soxE). This suggests that the soxF-encoded protein (monomeric SoxF in C. tepidum) has some role in thiosulfate oxidation, the biochemical function of which had yet to be established.

As reported by Kusai and Yamanaka, who used partially purified thiosulfate oxidizing preparations from C. limicola f. sp. thiouosulfotrophilum (which we now speculate probably consisted of the core TOMES), we found that various soluble cytochromes could function as electron acceptors in thiosulfate oxidation reactions catalyzed by the core TOMES (Fig. 2, Table 1). In our reconstituted system, various cytochromes at 50 μM differed in their apparent efficiencies as electron acceptors. The addition of 0.5 μM SoxF increased the reactions with C. tepidum cyt c-554, yeast cyt c, and horse-heart cyt c by 92, 48, and 31% respectively. This is the first report that clearly indicates significant stimulation of thiosulfate oxidation by SoxF in vitro with a reconstituted reaction system composed of purified core TOMES.

In P. pantotrophus, SoxF deletion mutant cells grew slower than the wild type on thiosulfate, and the rate of oxidation of thiosulfate in crude cell-free extracts prepared from mutant cells using horse heart-cyt c as the electron acceptor significantly increased when the reaction mixture was supplemented with SoxF isolated from wild type cells. However, in a reconstituted system composed of four purified sox proteins (including SoxCD), thiosulfate-oxidizing activity was not significantly increased by the addition of SoxF, and the cause of this discrepancy remains to be investigated. Because the degree of stimulation by SoxF depends greatly on the species of cytochrome used as the electron acceptor in the C. tepidum reconstituted system, and because the addition of a small amount of C. tepidum cyt c-554 to the assay system with horse-heart cyt c as the electron acceptor greatly accelerated the reaction rate (Fig. 2B, Table 1), it will be interesting to study the effects of SoxF on the reconstituted system of P. pantotrophus using the indigenous cytochrome and/or high-potential iron-sulfur protein from this bacterium as the electron acceptor, as well as the effects of these additions on horse-heart cyt c reduction.
Comparison of various cytochromes as electron acceptors in thiosulfate oxidation catalyzed by core TOMES

In a previous study, we found that both SoxA and SoxX bind a single heme c using purified preparations of recombinant proteins (rSoxA and rSoxX) overexpressed in *E. coli* cells. The midpoint redox potentials of rSoxX (at pH 7.0) and rSoxA (at pH 10) were determined to be +153 mV and lower than −550 mV respectively. Native SoxAXK had two midpoint redox potentials, one at +161 mV and the other lower than −550 mV, the former to be ascribed to the heme in SoxX and the latter to the c-devoid of the cyt c. We found that SoxAXK and rSoxX functioned as electron acceptors though not very efficiently, and that rSoxA did not function at all as the acceptor (Table 1), as expected from the redox potentials of the two hemes. These results indicate that the redox active heme of SoxAXK, in thiosulfate oxidation is that of SoxX, not that of SoxA.

Azai et al. reported that a mutant of *C. tepidum* devoid of the cyt c-554 gene grew on thiosulfate at slower rates than the wild type, and that a double mutant devoid of both cyt c-554 and SoxB did not grow on thiosulfate. These results indicate that SoxB is indispensable to the in vivo oxidation of thiosulfate, and that in the absence of cyt c-554 some factor(s) mediate electron transport between core TOMES and RC-bound cyt c-551, which serves as an immediate electron donor to the oxidized primary donor P840⁺. The factor might be some cytochrome, because a variety of cytochromes, including SoxAXK, can function as electron acceptors in the thiosulfate oxidation reaction catalyzed by core TOMES (Table 1), and because some putative cytochrome c genes without identified functions are present within the genome of *C. tepidum*.

On the addition of 0.5 μM *C. tepidum* cyt c-554, the reaction rates of both SoxAXK and rSoxX as electron acceptors dramatically increased, indicating that cyt c-554 is a good direct electron acceptor in the core TOMES catalyzed reaction, and that reduced cyt c-554 is easily oxidized by many of the externally added cytochromes. The addition of SoxF increased the reaction further (Table 1). The midpoint potentials of *C. tepidum* cyt c-554, horse-heart cyt c, and yeast cyt c (29) are reported to be +148, +248, and +290 mV respectively. The potential of *C. tepidum* cyt c-554 is slightly lower than that of SoxAXK (+161 mV), the heme in SoxX, (20) and the ratio of the forward reaction kinetic constant (k_f) of SoxF (SoxAXK → cyt c-554) to that of the back one (k_b) is calculated by the equation 0.148–0.161 = k_f/k_b to be about 0.60. The back reaction can affect the apparent rates with cyt c-554 in Table 1, but we did not pursue these possibilities any further because it is difficult to determine the forward rate at low concentrations of cyt c-554 by conventional measurements.

SoxF increased the oxidation of thiosulfate by core TOMES without changing the affinity for the substrate (Fig. 2). Based on the mid-point redox potentials at pH 7.0, only the SoxX heme (+161 mV), not the SoxA one (−550 mV), appears to be redox-active under physiological conditions, and the oxidation reaction of thiosulfate should proceed in two steps. SoxF increased in vitro electron transfer between externally added cytochromes and some of the reduced core TOMES components (possibly SoxAXK or SoxB) in one of the two oxidation steps. The effects of SoxF were more pronounced on cyt c-554 than on horse-heart or yeast cyt c, and the addition of a low concentration (0.5 μM) of cyt c-554 greatly accelerated the reduction of the other two. In SoxF-catalyzed sulfide oxidation that did not involve core TOMES, cyt c-554 was a much better electron acceptor than the other two (Table 2). These results suggest that SoxF directly mediates electron transfer between the components of core TOMES and externally added cytochromes.

Effects of SoxF on the oxidation of sulfide and sulfate by core TOMES

The complete TOMES of *P. pantotrophus*, which contains SoxCD, can oxidize not only thiosulfate, but also sulfide (23) and sulfate (7, 23). The SoxB from this bacterium was reported to catalyze sulfide oxidation without the participation of SoxA and SoxYZ. In contrast to that report, we found that although *C. tepidum* core TOMES catalyzed sulfide oxidation (Table 2, Fig. 5), SoxB alone was unable to do so (data not shown), indicating that the participation of all the TOMES components is required for this reaction.

The sulfite dehydrogenase activity of *P. pantotrophus* TOMES was only slightly inhibited by SoxF (23) but we found that the activity of *C. tepidum* TOMES was greatly inhibited by SoxF (Fig. 3). Green sulfur bacteria cannot grow on sulfite as the sole electron donor, but purified core TOMES showed sulfite dehydrogenase activity (Fig. 3). As described in “Results,” we found that the sulfite dehydrogenase activity of core TOMES was rather quickly inactivated by sulfite (noticeably at concentrations higher than 0.5 mM), and this might be the reason green sulfur bacteria apparently cannot grow on sulfite as the sole electron donor.

Requirement of SoxB in the initial reaction of thiosulfate oxidation

A reaction model of thiosulfate oxidation by TOMES has been proposed by Friedrich et al. The initial steps are as follows in outline: In the presence of SoxYZ, SoXAX (the protein of this bacterium does not bind SoxX) initiates oxidation, resulting in covalent attachment of oxidized thiosulfate to a conserved cysteine of SoxY (eq. 1 in “Introduction”). SoxB then hydrolytically releases sulfate, leaving the sulfane sulfur atom of thiosulfate on SoxY to yield persulfide (eq. 2). In contrast to this scheme, we found that all three core TOMES components, SoxAXK, SoxB, and SoxYZ were absolutely necessary for the initiation of thiosulfate oxidation, and that no reduction of cytochromes (even as low as the stoichiometric amount of SoxYZ and SoxAXK) occurred in the absence of SoxB (data not shown), indicating that the participation of SoxB is also required at the initial reaction step.

The same is true for sulfite oxidation catalyzed by core TOMES (data not shown).

Possible physiological functions of SoxF

The genome of *C. tepidum* has soxF but not soxE within the sox gene cluster, as with *C. limicola* f. sp.
Physiological function of SoxF.

In the discussion below we consider several possible functions of SoxFs encoded by sox genes that are not confined to sulfide dehydrogenase. Based on analyses of the genomes of various green sulfur bacteria, Fridgaard and Bryant
\cite{11,12} annotated soxF for soxF (CT1015) in C. tepidum sox gene cluster, though no specific functions have yet been assigned to the encoded protein. In the discussion below we consider several possible functions of SoxFs encoded by soxF genes in the sox gene cluster:

(i) Stimulation of thiosulfate oxidation catalyzed by core TOMES. Above, we explain that SoxF from C. tepidum increased thiosulfate oxidation in vitro by purified core TOMES, especially when cyt c-554 was the electron acceptor (Table 1). The results strongly indicate this stimulating activity as an important physiological function of SoxF.

(ii) Activation of SoxYZ. Quentmeier et al. \cite{41} reported that SoxYZ prepared from P. pantotrophus was an inhibited state, and that preincubation with sulfide greatly increased the activity, occasionally higher than 100-fold. We too found that preincubation with C. tepidum SoxYZ with SoxF and sulfide led to stimulation of subsequent thiosulfate oxidation catalyzed by core TOMES, but the degree of stimulation was modest, normally 10% at most. However, we observed that a recombinant C. tepidum SoxYZ preparation purified from E. coli was almost inactive in thiosulfate oxidation, and that the activity was greatly increased by incubation with sulfide (data not shown), as has been found for P. pantotrophus SoxYZ. \cite{41} Recently, Friedrich et al. \cite{13} quoted in a review unpublished results of their research group that SoxF reactivated inactivated SoxYZ. It appears that one of the in vivo functions of SoxF that remains to be investigated is stimulation of inactivated SoxYZ.

(iii) Sulfide dehydrogenase activity of SoxF. SoxF had high sulfide dehydrogenase activity at pH values greater than 6.0 (Fig. 4), and correspondingly had high affinity for sulfide (a K_M value of about 2 μM at pH 7.8) (Fig. 5, Table 2). In the genome of C. tepidum, three soxF homologs are found, and SoxF (CT1015) accounted for the major sulfide dehydrogenase activity in the soluble cell-free fraction under our experimental conditions (data not shown). Of course SoxF is probably not the only source of the sulfide dehydrogenase activity present since the C. tepidum genome contains genes encoding membrane-bound sulfide-quinone reductase (SQR). \cite{42} Core TOMES has significant sulfide dehydrogenase activity, although the specific activity and the affinity for sulfide were lower than those of SoxF at pH 7.8 (Fig. 5, Table 2). Of the multiple sulfide oxidation electron transport pathways with photooxidized P840 as the terminal electron acceptor in C. tepidum, the one involving SQR appears to be more efficient in terms of energy conversion than the others, because it should generate a proton-motive force through a cyt b-Rieske-type iron-sulfur protein complex during the oxidation of sulfide by photooxidized P840, and the others do not. \cite{30} Although the FCSD and SoxF pathways are energetically inefficient as compared with the SQR pathway and the affinities for sulfide did not differ very much, retention of these activities is likely favored under certain environmental conditions, because sulfide is a diffusible substrate required by many bacterial species occupying the same ecological niche. \cite{6} Oxidation of sulfide by FCSD or SoxF, or even by core TOMES to elemental sulfur, allows an individual bacterium to utilize sulfide and subsequently generate six electrons as electron donors for further reactions. Further studies, as on competition in growth in media of varying pH levels of mutants deficient in SoxF, FCSD, and core TOMES pathway in mixed cultures are promising to address the question why less energetically efficient sulfide oxidation pathways have been retained.

Acknowledgment

This work was supported in part by the Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms) and by High-tech Research Center Project of MEXT, Japan (to KI).

References