The Sulfur Oxygenase Reductase from the Mesophilic Bacterium *Halothiobacillus neapolitanus* Is a Highly Active Thermozyme

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A biochemical, biophysical, and phylogenetic study of the sulfur oxygenase reductase (SOR) from the mesophilic gammaproteobacterium *Halothiobacillus neapolitanus* (HnSOR) was performed in order to determine the structural and biochemical properties of the enzyme. SOR proteins from 14 predominately chemolithoautotrophic bacterial and archaeal species are currently available in public databases. Sequence alignment and phylogenetic analysis showed that they form a coherent protein family. The HnSOR purified from *Escherichia coli* after heterologous gene expression had a temperature range of activity of 10 to 99°C with an optimum at 80°C (42 U/mg protein). Sulfite, thiosulfate, and hydrogen sulfide were formed at various stoichiometries in a range between pH 5.4 and 11 (optimum pH 8.4). Circular dichroism (CD) spectroscopy and dynamic light scattering showed that the HnSOR adopts secondary and quaternary structures similar to those of the 24-subunit enzyme from the hyperthermophile *Acidianus ambivalens* (AaSOR). The melting point of the HnSOR was ~20°C lower than that of the AaSOR, when analyzed with CD-monitored thermal unfolding. Homology modeling showed that the secondary structure elements of single subunits are conserved. Subtle changes in the pores of the outer shell and increased flexibility might contribute to activity at low temperature. We concluded that the thermostability was the result of a rigid protein core together with the stabilizing effect of the 24-subunit hollow sphere.

Sulfur oxygenase reductases (SORs) catalyze a dioxygen-dependent disproportionation reaction of elemental sulfur with sulfite, thiosulfate, and sulfide as products. External cofactors or electron donors are not required, and the two enzyme activities could not be separated (equations 1 to 3) (19, 20, 30, 41).

\[
\text{Oxygenase} \quad S^0 + O_2 + H_2O \rightarrow HSO_3^- + H^+ \quad (1)
\]

Disproportionation \[
3S^0 + 3H_2O \rightarrow 2H_2S + 2HSO_3^- + 2H^+ \quad (2)
\]

Sum \[
4S^0 + O_2 + 4H_2O \rightarrow 2H_2S + 2HSO_3^- + 2H^+ \quad (3)
\]

Thiosulfate formation \[
S^0 + HSO_3^- \quad \text{pH} > 6 \quad \text{SSO}_3^{2-} \quad (4)
\]

SOR is the initial sulfur-oxidizing enzyme in the chemolithoautotrophic and thermoacidophilic archaea *Acidianus ambivalens* (AaSOR) and *Acidianus tengchongensis* (AaSOR), which grow optimally at 70 to 80°C and pH 1 to 4. The three-dimensional (3D) structures of both highly similar SORs (88% identity) showed that the enzymes form large spherical, hollow oligomers with molecular masses of 845 kDa each composed of 24 identical subunits (25, 46). Each subunit contains a low-potential mononuclear non-heme iron site as the putative redox-active cofactor and an indispensable cysteine, which is persulfurated in the *Ac. ambivalens* SOR (C31) (25, 45, 46). The iron sites are located in smaller pockets within each subunit, which are accessible only from the large inner cavity. We had shown using site-directed mutagenesis that the three Fe-coordinating residues (H96, H99, and E114) and the persulfurated cysteine C31 are essential for catalysis. Most likely, the cysteine persulfide is involved in sulfur binding. Mutation of the other two cysteine residues in the protein did not abolish activity, not even in a double mutant (47). Our current hypothesis about the reaction mechanism of SOR predicts that the catalytic cycle is initiated by covalent sulfur binding to the active site C31 as a polysulfide chain (R-Sn-SH), followed by hydrolytic cleavage of the cysteine polysulfide to sulfide and a polysulfenyl moiety (R-Sn-R-SO). Either the sulfenyl group or Fe2+ would subsequently activate dioxygen for polysulfenyl oxidation to the final product(s) sulfate and/or thiosulfate (19).

SORs or sor genes are not widespread in nature. So far, they have been reported only from some thermoacidophilic members of the domain *Archaea* and (hyper)thermophilic members of the domain *Bacteria*. Native SOR enzymes were initially purified from two *Acidianus* species (8, 20). Later, heterologous gene expression allowed study of the AaSOR and AaSOR in more detail (5, 41, 45).

In addition, the SOR from the hyperthermophilic bacterium *Aquifex aeolicus* was biochemically characterized, resulting in similar properties compared to the AaSOR and AaSOR (30). Surprisingly, sor genes were recently found in the genomes of a number of mesophilic and moderately thermophilic bacteria, showing that SORs are not restricted to hyperthermophiles. The issue is relevant for enzymes metabolizing elemental sulfur because the water solubility of the barely soluble solid substrate decreases significantly with temperature (478 nM at 8°C to 6.1 nM at 4°C).
which raises the question whether substrate availability may limit enzyme activity at mesophilic conditions. There is one report of a SOR-like enzyme activity in soluble extracts of the mesophilic bacterium Acidithiobacillus thiooxidans, but molecular details were not given (43).

In order to investigate the structural and biochemical properties of SORs from a mesophile, we initiated a biochemical, biophysical, and enzymological study of the enzyme from the gammaproteobacterium Halothiobacillus neapolitanus (HnSOR) to resolve this issue. The chemolithoautotrophic microbe grows optimally at 28 to 32°C at more or less neutral pH with thiosulfate or sulfur as electron donors (17). The type strain (NCIMB 8539) was isolated from corroded concrete sewers in Australia (29) and named “pertaining to the seawater at Naples from which this species was probably first isolated by Nathansohn in 1902” (18). It seems to be common in sulfide-rich spas and microbial leaching communities (3, 49). Apart from the sor gene, the H. neapolitanus genome encodes a complete SOX complex, a multisubunit sulfur oxidation complex restricted to Bacteria (10). The same applies to Acidithiobacillus caldus. It had been speculated that two sulfur-oxidizing enzyme systems might be used at different temperatures (26). H. neapolitanus does not grow at temperatures exceeding 42°C (18), and data regarding the temperature regulation effects, which are hypothetical so far, are lacking for both species.

We show here that recombinant HnSOR is a highly active enzyme, with a very broad temperature spectrum of activity ranging from 10°C to more than 95°C. Also, the results of spectroscopy and in silico modeling suggest that the enzyme adopts secondary and quaternary structure similar to those from (hyper)thermophiles. The activity optimization of the HnSOR is discussed as an apparent adaptive response to substrate limitation at mesophilic conditions, which allows efficient sulfur-based energy metabolism.

MATERIALS AND METHODS

Strain and culture conditions. Halothiobacillus neapolitanus DSM 15147 = NCIMB 8539 (1, 18) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany; http://www.dsmz.de). Cells were grown in liquid culture at 28 to 30°C either with 1% (wt/vol) tyndallized elemental sulfur or with 1% (wt/vol) thiosulfate as an electron donor at pH 6.8 in medium 68 recommended on the DSMZ web server (http://www.dsmz.de/catalogues/catalogue-microorganisms/culture-technology/list-of-media.html). Cells were grown until the color of bromocresol purple shifted to yellow, indicating a drop in pH, and subsequently harvested by centrifugation.

DNA procedures and heterologous gene expression. Isolation of chromosomal H. neapolitanus (DSM15147) DNA was carried out according to a modified protocol of Sulfolobus solfataricus DNA extraction by S. V. Albers (http://www.rug.nl/gbb/research/researchGroups/molecularMicrobiology/research/extremophiles/DNAisul.pdf; as of November 2011). Six hundred micrograms of sedimented H. neapolitanus cells was resuspended in 550 μl of TEN buffer (40 mM Tris, 1 mM EDTA, 15 mM NaCl, pH 8). Fifty microliters of a 10% sodium dodecyl sulfate (SDS) (wt/vol) solution was added to the suspension, which was subsequently incubated for 45 min at room temperature. After repeated phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) extractions, the genomic DNA was precipitated by the addition of 0.1 volume of a 3 M sodium acetate solution (pH 5.2) and 0.8 volume of 2-propanol. After incubation at −20°C for 2 h, the tubes were centrifuged for 30 min at 16,000 × g in a microcentrifuge. The pelletted DNA was washed once with 70% ethanol (by volume), centrifuged for 5 min at 16,000 × g, dried at room temperature, and eventually resuspended in 50 μl of TE buffer (10 mM Tris, pH 8, 1 mM EDTA).

The sor gene (GenBank locus tag Hnep_1222) was PCR amplified with the primers HnSOR_fwd (fwd stands for forward) (ACTAGT TA AGCG GGGCAA AAAATG TCAGAT GAAAAT CCAATT ATA) and HnSOR_rev (rev stands for reverse) (GTGACG CCAGG GCTTTG CT TAAG ATGCCT ACGCC) (Biomers, Ulm, Germany) which had been designed from the H. neapolitanus genome sequence (strain ATCC 23641). The PCR product was purified with the GenElute PCR clean-up kit (Sigma, Taufkirchen, Germany) according to the manufacturer’s recommendations. It was subsequently cleaved with the SpeI and Eco47III restriction enzymes and ligated into the XbaI/Eco47III-digested pASK75 vector (38). Positive transformants in Escherichia coli Top 10F cells (Invitrogen, Darmstadt, Germany) were sequenced and finally introduced into E. coli BL21(DE3) CodonPlus RIL cells (Agilent, Böblingen, Germany).

For heterologous gene expression, 500-ml cultures were grown aerobically at 37°C in 2× LB medium in notched Erlenmeyer flasks. The expression was induced by the addition of 200 μg/liter anhydrotracycline from a 2% (wt/vol) stock solution in dimethylformamide at an optical density at 600 nm (OD600) between 0.6 and 0.8. Ferric citrate (100 μM) was added at the time of induction to ensure sufficient iron incorporation. The cultures were incubated for 20 h after induction with vigorous aeration and stirring. The Acidibacillus ambivalens sor gene (EMBL accession number X56616) was expressed heterologously after ligation into the pASK75 vector with a C-terminal Strep tag fusion (38) as described elsewhere (pASK-SOR.05 plasmid) (45).

Protein purification. The cell pellet obtained by centrifugation was washed once in approximately 10 volumes (vol/wt) of 100 mM Tris-HCl buffer (pH 8) with 150 mM NaCl (buffer W) and afterwards resuspended in 5 volumes of the same buffer. Cells were disrupted with a high-pressure homogenizer (0.18-mm nozzle and 1.35-MPa pressure; Constant Systems, Low March, Daventry, United Kingdom). After the first centrifugation step (10,000 × g for 30 min), the soluble protein-containing supernatant was centrifuged in an ultracentrifuge (100,000 × g for 45 min). The soluble total extract from 10 to 20 g of cells (wt weight) was applied either to a 1-ml Strep-Tactin gravity flow column or to an 8- to 10-ml Strep-Tactin superflow column (both columns from IBA, Göttingen, Germany) connected to an AKTApurifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). This step was performed repeatedly, when the binding capacity of the column was exceeded. The column was equilibrated with 5 column volumes (CV) of buffer W prior to loading. The column was subsequently washed with 6 CV of the same buffer. The protein was eluted by applying 3 CV of buffer E (buffer W with 2.5 mM desthiobiotin; IBA). The column was regenerated with 15 CV of buffer W with 1 mM hydroxyl-azepoxynol benzoic acid (HABA) (Sigma). Alternatively, the column was regenerated with 3 CV each of double-distilled water (ddH2O), 0.5 M NaOH, and ddH2O instead of the regular HABA solution.

SOR activity and inhibition assays. The SOR activity assay was performed as previously described (20, 45). Specific activities were routinely determined after incubation of 0.5 to 5 μg of purified enzyme at 80°C in a 1-ml solution of 0.1 M sodium citrate–0.2 M Na2HPO4 (pH 7.2) (27) containing 2% sulfur (wt/vol) and 0.1% Tween 20 (vol/vol). Samples were taken at appropriate time points (usually after 0, 2, 4, 6, 8, and 10 min), chilled on ice, and centrifuged briefly to sediment elemental sulfur. The concentrations of the reaction products hydrogen sulfide, sulfite, and thiosulfate were determined colorimetrically (20) and quantified with calibration curves. The specific activities were calculated from the linear increase of the reaction products. One unit of enzyme activity was defined as 1 μmol of sulfite plus thiosulfate (oxyanions) or hydrogen sulfide (reductase) formed per minute.

The optimal pH and temperature of HnSOR activity were determined with the same activity assay and different buffer systems. The citrate-phosphate buffer (27) was used to determine the protein activity in a range from pH 5 to 8. For higher pH values, 0.2 M Na2HPO4 was titrated with a
0.1 M Na2HPO4 solution to the desired pH. The pH profile was recorded at 50°C due to extensive nonenzymatic sulfur disproportionation observed at high temperatures combined with high pH values (20, 40). Usually, 0.75 to 2.65 μg of purified HnSOR was added to 1 ml of ice-cold assay buffer. The reaction was started by incubation of the tubes in a heating block at 50°C. The concentrations of reaction products and specific activities were determined as described above.

Different amounts of a freshly prepared ZnCl2 solution resulting in a final concentration of 10 to 100 μM Zn2+ were added to the reaction mixtures in the SOR activity assay. The reaction mixture was kept on ice water for 30 min after 2 to 5 μg of enzyme had been added. The reaction was started by heating the samples to 80°C, and specific SOR activities were measured as described above. An enzymatic reaction mixture without ZnCl2 and a nonenzymatic reaction mixture with inhibitor but without SOR were used as controls. For a second control, 2 mM EDTA was added to the zinc-containing reaction vials to reverse inhibition. All measured specific activities were plotted against the Zn2+ concentration. The slope of the trend line was used for calculation of the appropriate Ki values.

Biochemical procedures. The protein concentration was determined by the Coomassie blue method (2). Iron quantification was performed with pure protein preparations with the 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) method (9). Denaturing SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 10% or 12% polyacrylamide–Tris–tricine gels (35).

CD spectroscopy. Circular dichroism (CD) spectra were measured with a JASCO J-815 spectropolarimeter with Peltier temperature control. Typically, 10 accumulations were recorded in the far-UV region (180 to 260 nm) at 20°C with a data pitch of 0.2 nm and a 0.1-cm-path-length polarized quartz cuvette. A bandwidth of 2 nm was used with a detector response of 1 s and scanning speed of 200 nm/min. The spectra were obtained with 0.1 mg/ml of protein in 20 mM Tris-HCl buffer (pH 7.5) and afterwards corrected by subtracting the spectrum of the buffer solution. Thermal unfolding was assessed by measuring the CD signal at 220 nm while raising the temperature at a 1°C/min rate.

DLS. Dynamic light scattering (DLS) analyses of recombinant Ac. ambivalens and H. neapolitanus SORs were performed on a Zetasizer Nano apparatus (Malvern Instruments, Worcestershire, United Kingdom) at 25°C. Samples (0.3 mg/ml in 20 mM Tris-HCl buffer [pH 7.5]) were filtered through a 0.22-μm-pore-size filter and assayed with a quartz cuvette with 45-μl volume and 3-mm path length (Hellma, Müllheim, Germany). Three measurement cycles were performed for each protein sample. The data were averaged from 14 light scattering periods of 10 s for each cycle. Average protein diameter values were calculated using the corresponding Malvern Instruments DTS software.

Phylogenetic analysis and modeling. SOR homologues from other microorganisms were detected by BLASTP searches with the Ac. ambivalens SOR amino acid sequence as a probe (NCBI accession number CAA39952.1) against the public database at NCBI (www.ncbi.nlm.nih.gov). The deduced Sulfo bacteriaceae and Acidithiobacillus ferrivorans SOR sequences were identified via tBLASTN analysis in the respective genomes available at the DOE Joint Genome Institute (http://www.jgi.doe.gov). All SOR sequences were aligned by the KALIGN algorithm (24) with manual correction. The dendrogram was calculated feeding the alignment into the phylogeny server at the MAFFT site (http://mafft.cbrc.jp/alignment/server/phylogeny.html) with the default parameters and 100 bootstrap repetitions.

3D models of the H. neapolitanus SOR were predicted at the Phyre (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html) (16) and I-Tasser servers (http://zhanglab.ccmb.med.unich.edu/I-TASSER/) (51). Energy minimization of the PHyre model was performed with UCSF Chimera (http://www.cgl.ucsf.edu/chimera) (31). Clustering was done with CLUSPRO 2.0 (http://cluspro.bu.edu/login.php) (6, 22) and a single subunit in the dimer mode. Structure alignments, root mean square deviation (RMSD) readouts, and the preparation of figures were done with PyMol (7).

RESULTS

Phylogenetic analysis of the SOR protein family. SORs form a conserved protein family with high mutual similarity (Fig. 1) (Pf07682; http://pfam.sanger.ac.uk) but with no recognizable similarity to outside proteins. The Halothiobacillus neapolitanus SOR (HnSOR) shared pairwise amino acid identities with other SORs ranging between 76% (Acidithiobacillus caldus) and 40% (Aquifex aeolicus). The archaeal SORs were about 40 to 42% identical relative to the HnSOR, while Sulfo bacteriaceae spp. (47%) were in between.

The aligned bacterial SOR amino acid sequences showed several regions with differences distinguishing them from the archaeal SORs (e.g., positions 91 to 96 and 112 to 117) (see Fig. S1 in the supplemental material). The dendrogram calculated from the alignment reflects a grouping of the enzymes according to phylo-
genetic relationship (Fig. 1). The *Aquifex* SOR was always the least similar enzyme (33 to 40% identity) compared to all other SORs. It contained two insertions and a slightly shortened C terminus compared to the otherwise constant lengths of the SOR sequences. An outgroup was not included, as no paralogous sequence family with even minimal similarity was identified so far.

Cloning, expression, and purification of recombinant *H. neapolitanus* SOR. The *H. neapolitanus* sor gene (open reading frame [ORF] Hneap_1222) was cloned into the pASK75 expression vector with a C-terminal Strep tag (38). When sequenced for validation, the sor genes of strain DSM 15147 used here and of strain ATCC 23641 (genome sequence) were identical. When the resulting plasmid pASK_HnSOR was used to produce recombinant HnSOR from *E. coli* BL21 cells, 20 to 30% of the protein was recovered in the soluble form, while the remainder precipitated in inclusion bodies. The purification yield was 11 to 18 mg of soluble protein per liter of LB medium. SDS-PAGE revealed a major 36-kDa band characteristic of full-length SOR (Fig. 2). The average iron content of the preparations was 1.6 ± 0.3 Fe per protein monomer.

Enzymatic properties of recombinant *H. neapolitanus* SOR. The pH and temperature profiles of HnSOR activity were determined with an enzyme buffer that was modified from those used previously for the *Acidianus* and *Aquifex* SORs (20, 30, 45). Optimal activity was observed when a 100 mM citrate-phosphate buffer was used (instead of Tris-acetate), resulting in 42.4 U/mg oxygenase and 4.1 U/mg reductase activities at pH 8 and 80°C. Both oxygenase and reductase activities were measured at temperatures between 10 and 95°C with an optimal activity at 80°C (Fig. 3B and C). At moderate temperatures (i.e., 10 to 40°C), both enzyme activities were at similar levels. At temperatures higher than 40°C, oxygenase activity was up to 10-fold higher than reductase activity. The same effect was previously observed for the *Aq. aeolicus* SOR (30). An equal ratio between both oxygenase products (sulfite and thiosulfate) was observed at moderate temperatures (10 to 40°C). The ratio drastically changed when temperatures exceeded 50°C, resulting in an up to sixfold-higher level of thiosulfate at 75°C.

The activity assays used to determine the optimal pH for protein activity (Fig. 3A) were carried out at the suboptimal temperature of 50°C in order to avoid the interference of nonenzymatic sulfur disproportionation observed at higher temperatures combined with a pH of >8 (20, 40). Oxygenase and reductase activities were observed between pH 5.4 and 11 with an optimum at pH 8.4 (Fig. 3A). At pH 12, it was no longer possible to distinguish be-
TABLE 1 Properties of microorganisms and their sulfur oxygenase reductases

<table>
<thead>
<tr>
<th>Property</th>
<th>H. neapolitanus</th>
<th>Ag. aeolicus</th>
<th>Ac. ambivalens</th>
<th>Ac. brierleyi</th>
<th>Ac. tengchongensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topt (°C)</td>
<td>Microorganism</td>
<td>30</td>
<td>85</td>
<td>80</td>
<td>65–70</td>
</tr>
<tr>
<td></td>
<td>Enzyme</td>
<td>80</td>
<td>80</td>
<td>85</td>
<td>65</td>
</tr>
<tr>
<td>Sp act (U/mg) at Topt</td>
<td>Oxygenase</td>
<td>42.1</td>
<td>78.8</td>
<td>10.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Reductase</td>
<td>4.1</td>
<td>3.05</td>
<td>2.6</td>
<td>NR</td>
</tr>
<tr>
<td>T range (°C) of enzyme</td>
<td>10–99</td>
<td>20–90</td>
<td>50–108</td>
<td>55–±80</td>
<td>50–90</td>
</tr>
<tr>
<td>pHopt</td>
<td>Microorganism</td>
<td>6.5–6.9</td>
<td>6.8</td>
<td>2.5</td>
<td>1.5–2.0</td>
</tr>
<tr>
<td></td>
<td>Enzyme</td>
<td>8.4</td>
<td>NR</td>
<td>6.5–7.4</td>
<td>7.0</td>
</tr>
<tr>
<td>pH range of enzyme</td>
<td>5.4–11</td>
<td>5.5–8</td>
<td>5–8.4</td>
<td>NR</td>
<td>3.5–9</td>
</tr>
<tr>
<td>M&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Subunit</td>
<td>35,300</td>
<td>37,674</td>
<td>35,318</td>
<td>35,000&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Holoenzyme (method)</td>
<td>848,000 (DLS)</td>
<td>602,000 (native gel)</td>
<td>844,000 (X-ray)</td>
<td>560,000 (native gel)</td>
<td>845,000 (X-ray)</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt; inhibition of oxygenase/reductase</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 46/36 μM</td>
<td>NR</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 45/39 μM</td>
<td>NR</td>
<td>27%&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a Properties of five microorganisms (Halothiobacillus neapolitanus, Aquifex aeolicus, Acidaminobactrum ambivalens, Acidaminobacter brierleyi, and Acidaminobacter tengchongensis) and their sulfur oxygenase reductases are shown.

b Topt, optimum temperature; T range, temperature range; pHopt, optimum pH; NR, not reported; DLS, dynamic light scattering.

c Data from this work.

d Data from reference 30.

e Data from references 20, 45, and 46.

f Data from reference 8.

g Data from references 11, 25, and 41.

h Apparent molecular mass on SDS gel; the gene is not known.
i Residual activity with 1 mM Zn<sup>2+</sup>.

between nonenzymatic sulfur disproportionation and the enzymatic reaction. Under slightly acidic conditions (pH 5.4 to 6.4), reductase and oxygenase activities were at comparable levels. The oxygenase and reductase activity ratios changed with increasing pH: at pH 7, a 2-fold excess of oxygenase over reductase activity was observed, which increased to almost 5-fold at the optimal pH of 8.4 (15.9 U/mg oxygenase and 3.27 U/mg reductase activities, respectively, at 50°C). Both products of the oxygenase reaction, sulfite and thiosulfate, were detected at all pH values tested. Sulfite was the major product under slightly acidic conditions (pH 5.4 to 6.4) (not shown). Thiosulfate formation was predominant under slightly acidic conditions (pH 5.4 to 11 5.5–8 5–8.4 NR 3.5–9 pH range of enzyme). Reductase 4.1 3.05 2.6 NR 45.2 NR 27%<sup>e</sup> 35,172 Holoenzyme (method) 848,000 (DLS) 602,000 (native gel) 844,000 (X-ray) 560,000 (native gel) 845,000 (X-ray) Zn<sup>2+</sup> inhibition of oxygenase/reductase K<sub>i</sub> = 46/36 μM NR K<sub>i</sub> = 45/39 μM NR 27%<sup>e</sup> 35,172 Holoenzyme (method) 848,000 (DLS) 602,000 (native gel) 844,000 (X-ray) 560,000 (native gel) 845,000 (X-ray) Zn<sup>2+</sup> inhibition of oxygenase/reductase K<sub>i</sub> = 46/36 μM NR K<sub>i</sub> = 45/39 μM NR 27%<sup>e</sup>

The enzymatic activity of HnSOR was lost when the protein or protein extracts were stored at −20°C. Consequently, preparations were kept either at 4°C or at −20°C after the addition of 50% glycerol, which prevented loss of activity. Previous reports about the AaSOR and AaTSOR had shown that Zn<sup>2+</sup> is a potent inhibitor of these enzymes (4, 20, 45, 48). K<sub>i</sub> values of HnSOR were 46 μM for the oxygenase and 36 μM for the reductase activity, which are values comparable with the AaTSOR (Table 1) (48).

Structural properties of recombinant H. neapolitanus SOR. In order to compare the folding of mesophilic and (hyper)thermophilic SORs, we performed a circular dichroism (CD) study of the proteins from H. neapolitanus and Acidaminobacter ambivalens (Fig. 4). The CD spectra of both proteins were rather similar, with minima around 220 nm and a shoulder at 209 nm (Fig. 4A). The low wavelength maximum of HnSOR is not identifiable in the accessible spectral window. The signal intensity difference is likely due to quantification error. The conservation of spectral features suggests highly similar secondary structure content in both proteins. In addition, the hydrodynamic diameters as determined by dynamic light scattering were almost identical for both proteins (17.3 ± 1.0 nm for HnSOR and 18.0 ± 0.8 nm for AaSOR), showing that both proteins adopt comparable oligomeric states. HnSOR was ~20°C less thermostable than the enzyme from the hyperthermophile (Fig. 4B).

3D modeling of the H. neapolitanus SOR. We performed homology modeling of the HnSOR with the two Acidaminobacter SOR structures as templates (Protein Data Bank [PDB] identifiers 2cb2 and 3bxv) (25, 46) in order to identify features that could give clues to the activity at low temperature and to temperature stability. The results obtained with the H. neapolitanus SOR were reproducible giving similar models, regardless of the modeling server and the template. When the modeled HnSOR subunit was superimposed on each of the templates, the central beta barrel, the nine α-helices (longer than 5 amino acids [aa]) and the active site pocket were almost identical (Fig. 5A). Minor deviations were seen in loop or coil regions. The RMSD of the Ca chain was 0.16 to 0.38 Å.

The two Acidaminobacter SORs are built from 24 subunits, each forming a large (15-nm-diameter) hollow sphere with a 432-point group symmetry and an almost impervious surface. The minimal building block is a subunit dimer, so that the holoenzymes repre-
sent dodecamers of dimers (45, 46). The dynamic light scattering results suggest that the HnSOR adopts a comparable multimerization state. When two of the modeled HnSOR subunits were docked to each other, a dimer was formed, which could be superimposed on the AaSOR structure with an RMSD of 1.7 Å (not shown).

Narrow pores at the 4-fold and 3-fold symmetry axes of the AaSOR provide entrance to the inner cavity of the sphere (Fig. 5B and D). The question arose whether multimerization of the single subunit might lead to changes in the pore structures of the enzyme. When replacing one of the pore-forming subunits at the 4-fold symmetry axis of the AaSOR with a copy of the modeled HnSOR, changes in the pore structure became apparent. The phenylalanine residue F141 (in AaSOR numbering) was conserved, which forms the outer ring of the two Phe rings. The inner Phe ring was not present due to replacement of F133 with a valine residue (Fig. 5B; see Fig. S1 in the supplemental material). In consequence, only one of the two access-restricting residues is retained in the HnSOR.

The amino acids R99 and S226 define the pore at the 3-fold symmetry axis of the AaSOR (Fig. 5D). They are connected by hydrogen bonds within and across the subunits. A salt bridge between R99 and E228 of the same subunit also might play a role in stabilization (48). Neither of these residues is conserved in the HnSOR; R99 is replaced by a glutamate (E101), S226 is replaced by stabilization (48). Neither of these residues is conserved in the subunit.

**DISCUSSION**

**Sources of SORs or sor genes.** Once thought to be restricted to some hyperthermophiles and thermophiles, the sor genes identified in genomes of mesophilic bacteria gave credit to a report by Tano and Imai from 1968 (43) on a SOR-like, glutathione-independent, and sulfur-dismutating enzyme activity in cell extracts of the bacterium Acidithiobacillus thiooxidans (formerly Thiobacillus thiooxidans). The sulfur-dismutating enzyme reaction was measured at 30°C in a Warburg apparatus. The products were H2S and thiosulfate. These results were neither repeated nor confirmed, nor was the enzyme ever purified. In contrast, the GSH-dependent sulfur dioxygenases known from other acidithio-bacilli represent a different and unrelated type of sulfur-oxidizing enzymes, which remained elusive so far (32, 42). Therefore, the molecular basis of sulfur oxidation in these microorganisms is not known despite their importance in bioremediation and formation of acid mine drainage (3). The preliminary shotgun genome sequence of Acidithiobacillus thiooxidans ATCC 19377 available at GenBank does not contain a sor homologue (GenBank accession number AFOH00000000.1). We chose Halothiobacillus neapolitanus (formerly Thiobacillus neapolitanus) as a representative mesophilic microbe carrying a SOR gene to demonstrate enzyme activity and to study the properties of the protein.

Like the two Acidithiobacillus species (Fig. 1), many but not all of the microorganisms with sor genes are (facultatively) chemolithoautotrophic sulfur oxidizers originating from sulfide or sulfur-rich springs, solfataras, or bioremediating environments. Others would not have been expected to carry a sor gene: for example, Picrophilus torridus is a thermoacidophile described as a strict heterotroph and Desulfomicrobium baculatum (formerly Desulfovibrio baculatus) is, as far as we know, a strictly anaerobic sulfate and sulfur reducer (34, 36). The physiological role of the SOR in these microorganisms is unknown.

**HnSOR activity within a broad pH and temperature range.** The HnSOR is active over a broad pH range (pH 5.4 to 11) with an optimum at pH 8.4, when measured at 50°C. Measurements at the optimal temperature at alkaline pH were not possible because of the much higher rates of nonenzymatic sulfur disproportionation.

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**FIG 4** (A) Far-UV circular dichroism spectra of the SOR proteins from Halothiobacillus neapolitanus and Acidianus ambivalens at 0.1 mg/ml protein concentration and 25°C. (B) Thermal unfolding curves for the H. neapolitanus and A. ambivalens SOR, as measured from the CD variation at 220 nm. The lines show sigmoidal fits to the CD data. Values have been offset to facilitate comparison.
The pH range of the \( H_n \)SOR extends further into the alkaline region than for any of the SORs characterized so far (Table 1) (4, 20, 30, 41). The pH preference correlates with the slightly alkaline internal pH of \( H. neapolitanus \) (pH of \( 7.8 \)) (44). In contrast, the pH optimum of the \( Aa \)SOR is 7 to 7.4, correlating with an internal pH of around 6.5 (28).

The enzymatic activity of the \( Hn \)SOR covered a temperature span of almost 90 K, ranging from 10°C to 99°C (Fig. 3), which makes it a thermozyme. Two questions arise from these observations, namely, how the protein maintains its thermostability and how flexibility is retained sufficiently at low temperatures and low substrate concentrations to enable catalysis.

Even though the \( Hn \)SOR originates from a mesophilic microorganism with an optimal growth temperature around 30°C (maximum 42°C) (17), both oxygenase and reductase activities peaked at 80°C. As a comparison, the SOR from the hyperthermophile \( Ac. ambivalens \) (maximal growth temperature of 88°C) was active from 50°C to 108°C (Table 1) (20, 52). Despite the structural similarity between the enzymes from the hyperthermophile and mesophile observed by spectroscopy, the distinct environmental conditions of the organisms result in specific adaptations, including the level of stability and their catalytic properties. High environmental temperatures impose the need for thermostabilization of the enzymes, resulting in a higher rigidity, less flexibility and low or no activity at ambient temperatures (50). However, when the comparison is done at their respective optimal catalytic
temperature, the enzymes tend to have identical flexibilities under their particular optimal working conditions so that a “corresponding state” is maintained regarding conformational flexibility (50). The HnSOR is a remarkable exception to this well-established trend. In fact, the specific HnSOR activity exceeded the (hyperthermophilic) AaSOR activity about 10-fold under optimal conditions (20, 45, 48).

The second question to be answered was about the factors that make the HnSOR active at low temperatures. We had observed that the enzyme should be partially melted at the optimal reaction temperature. The apparent thermal stability was about 20 K lower than the AaSOR (Fig. 4B). The results suggest that the HnSOR has a higher flexibility compared to the more rigid AaSOR, which is not active below 50°C (20). The results also suggest that the thermal denaturation is irreversible; however, these conclusions have not yet been verified independently by other methods.

In addition, we had shown in a previous study that the widths of the pores at the 4-fold and 3-fold symmetry axes are crucial for specific AaSOR activities: the wider the pores are, the higher the activity becomes (48). In this context, the changes seen in the pores of the outer shell of the HnSOR (Fig. 5B to D) suggest that the barriers are less restrictive: at the 4-fold axis, the inner ring of the two Phe rings is missing. At the 3-fold axis, the salt bridge-forming arginine and glutamate residues are not present, while the H-bond-forming serine is replaced in favor of an alanine. This should be flexible enough to allow easy passage of substrate and/or products. In the HnSOR, a novel and wider hydrogen-bonding network centered at N122 replaced the salt bridges (and the hydrogen bonds provided by S226 [Fig. 5C and D]) that are considered to stabilize subunit interactions and confer rigidity to the 3-fold axis of the AaSOR.

**SORs are structurally conserved.** The difference of 40 to 50°C between the maximal or optimal growth temperature and the enzyme activity optimum is much higher than what would have been expected from the overall protein stability. The question of how this came about may be tentatively explained by one (or more) of the following hypotheses. (i) The high optimal temperature of HnSOR is a trait that persisted (e.g., AaSOR) due to lack of evolutionary pressure. (ii) The activity-temperature profile is a consequence of the coupling between enzyme activity and protein structure: any SOR protein will have such a high optimal temperature and intrinsic thermostability. (iii) The existence of HnSOR activity over broad pH and temperature ranges is an adaptive mechanism, which allows metabolic homeostasis upon environmental changes. Comparison of the biochemical properties and the sequences (Table 1; see Fig. S1 in the supplemental material) suggests that the SORs have similar properties independent of adaptations to the host organism.

We used spectroscopy and a homology modeling approach to make some predictions regarding the reasons for the thermostability and temperature range of the enzyme. The different catalytic properties of HnSOR and AaSOR do not reside in major differences in the secondary structure content. The similar far-UV CD spectra indicated that the overall fold is preserved among enzymes from mesophilic and thermophilic microorganisms (Fig. 4A). This interpretation is supported by the modeling results, which showed little changes in the main secondary structure features of the subunit (Fig. 5A). In addition, the oligomeric assembly also seems to be conserved, as noted from the similar hydrodynamic properties of both enzymes. The slightly larger hydrodynamic diameter compared to the diameter obtained by electron microscopy and X-ray crystallography (∼15 nm [20, 25, 45, 46]) reflects the conformational dynamics in solution, the effect of the solvation shell, and the averaging inherent in an ensemble measurement.

SORs feature conserved amino acid sequences regardless of SORs or any exception. Furthermore, the enzyme structure can be elucidated by comparison of the biochemical properties and mechanism, which allows metabolic homeostasis upon environmental changes. The SORs are structurally conserved.

**Conclusion.** SORs were once thought to be restricted to some extremophiles and thermophiles, because the enzyme activity had not been recognized in mesophiles with one exception (43). The data presented here showed that the HnSOR possesses folding and quaternary structure properties similar to the SORs from (hyper)thermophiles. However, the activity at low temperatures seems to result from a combination of a less thermostable and more flexible oligomer with less restrictive access. In the context of the limited bioavailability of sulfur at mesophilic temperatures, the basal activity and flexibility of the HnSOR effectively contribute to adequate metabolic flows of sulfur metabolites for the microorganism.

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The Sulfur Oxygenase Reductase from the Mesophilic Bacterium *Halothiobacillus neapolitanus* is a Highly Active Thermozyme

**Supplementary Material**

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Supplementary figure S1 (following page): Multiple alignment of the SOR sequences available in public databases. Genbank identification (GI) numbers are given at the end; *derived from the genome sequences available at the Joint Genome Institute (http://www.jgi.doe.gov). The horizontal line separates Archaea from Bacteria below. Black bar, chimney-like protrusion at the four-fold symmetry axes with the residues forming the two phenylalanine rings in the AaSOR; cylinders, α-helices; arrows, beta sheets; light green cylinder, additional α-helix in the *Aquifex* SOR; abbreviations: Css, cysteine persulfide; Fe, iron-coordinating residues; Zn, zinc-binding residues; other residues are discussed in the Homology Modeling section (Fig. 5); residues above the alignment, AaSOR; residues below the alignment, HnSOR.