The enzymes of the thiosulfate dehydrogenase (TsdA) family are wide-spread diheme c-type cytochromes. Here, reodox carriers were studied mediating the flow of electrons arising from thiosulfate oxidation into respiratory or photosynthetic electron chains. In a number of organisms, including *Thiomonas intermedia* and *Sideroxydans lithothrophicus*, the *tsdA* gene is immediately preceded by *tsdB* encoding for another diheme cytochrome. Spectrophotometric experiments in combination with enzymatic assays in solution showed that TsdB acts as an effective electron acceptor of TsdA in vitro when TsdA and TsdB originate from the same source organism. Although TsdA covers a range from −300 to +150 mV, TsdB is redox active between −100 and +300 mV, thus enabling electron transfer between these hemoproteins. The three-dimensional structure of the TsdB-TsdA fusion protein from the purple sulfur bacterium *Marichromatium purpuratum* was solved by X-ray crystallography to 2.75 Å resolution providing insights into internal electron transfer. In the oxidized state, this tetraheme cytochrome *c* contains three hemes with axial His/Met ligation, whereas heme 3 exhibits the His/Cys coordination typical for TsdA active sites. Interestingly, thiosulfate is covalently bound to Cys330 on heme 3. In several bacteria, including *Allochromatium vinosum*, TsdB is not present, precluding a general and essential role for electron flow. Both *Avt*Sda and the *Mpt*SdBA fusion react efficiently in vitro with high potential iron-sulfur protein from *A. vinosum* (*E*<sub>m</sub> +350 mV). High potential iron-sulfur protein not only acts as direct electron donor to the reaction center in anoxicogenic phototrophs but can also be involved in aerobic respiratory chains.

The bifunctional thiosulfate dehydrogenase/tetrathionate reductase TsdA is present in various organisms of different proteobacterial genera (1). In the diheme cytochrome *c* from the purple sulfur bacterium *Allochromatium vinosum*, an axial histidine/cysteine ligation of the central iron atom has been firmly established for the active site heme (2). This type of ligation is rare among prokaryotes, usually leading to a low redox potential of the corresponding heme (3–6), and appears to be of special importance in sulfur-based energy metabolism. TsdA proteins catalyze the reversible formation of a sulfur-sulfur bond between the sulfane atoms of two thiosulfate molecules, yielding tetrathionate and releasing two electrons. TsdA enzymes of various source organisms exhibit different catalytic bias (7). Although the enzyme from the sulfur oxidizer *A. vinosum* is strongly adapted to catalyzing thiosulfate oxidation (2), TsdA from *Campylobacter jejuni* acts primarily as a tetrathionate reductase and enables the organism to use tetrathionate as an alternative electron acceptor for anaerobic respiration (8).

At present, it is largely unclear which reodox carriers mediate the flow of electrons arising from thiosulfate oxidation into respiratory or photosynthetic electron chains. In several organ-
isms, including *Thiomonas intermedia*, *Sideroxydans lithotrophicus*, and *Pseudomonas stutzeri*, *tsdA* is immediately preceded by a gene encoding another diheme cytochrome, *TsdB* (1). *TsdB* itself is not reactive with thiosulfate but accepts electrons from *TsdA* even when *TsdA* and *TsdB* do not originate from the same organism (1). Kinetic data that quantitatively describe the interaction between *TsdA* and *TsdB* have not been published so far. In the anoxygenic phototrophic purple sulfur bacterium *Marichromatium purpuratum*, *TsdA* and *TsdB* form a fusion protein with *TsdB* constituting the N-terminal domain (7). *TsdBA* fusion proteins are also encoded in other members of the family Chromatiaceae, *i.e.*, *Thiorhodococcus* sp. AK35 (D779_1816), *Thiocystis violascens* (Thivi_3993), *Thiorhodococcus drewsii* (ThidrDRAFT_3922), and *Thioflaviococcus mobilis* (Thimo_0460). However, *TsdBA* fusions are not a common trait in purple sulfur bacteria. In *A. vinosum*, a *tsdB* gene is not present (1).

In *A. vinosum*, the protein with the closest relationship to *T. intermedia* or *P. stutzeri* *TsdB* is Alvin_2879. This cytochrome *c*₄ (previously cytochrome *c*₅₅₅₃/₅₅₅₀) is membrane-bound (possibly via the hydrophobic protein Alvin_2880) and has a positive redox potential of +330 mV (9). Another candidate for accepting electrons from *TsdA* in purple anoxygenic phototrophic bacteria is the high potential iron–sulfur protein (HiPIP). *A. vinosum* and *M. purpuratum* produce HiPIP, and as this protein has a quite positive reduction potential (+350 mV (10)) it would be well suited as an electron acceptor for *TsdA*. This proposal is corroborated by a previous report where a protein preparation with thiosulfate dehydrogenase activity from *A. vinosum* reduced HiPIP in vitro (11).

Here, we study *Tsd(B)A* enzymes from dedicated sulfur oxidizers and characterize in detail the interaction of *TsdA* and *TsdB*. Variation of the Soret intensity at 418 nm with electrode potential revealed that the hemes were reduced between approximately +150 and −350 mV (Fig. 1, inset, *closed squares*). The response of reduced *AvTsdA* to a stepwise increase of the electrode potential was assessed in a similar manner. The potential was raised in 50-mV steps, and the spectra were recorded after a 60-s pause at each potential (Fig. 1, *inset, open squares*). Variation of the intensity at 418 nm with electrode potential revealed that the hemes were reduced between approximately +150 and −350 mV (Fig. 1, inset, *open squares*). The hysteretic nature of the plot of absorbance versus potential prevented Nernstian analysis to define the heme reduction.

### Results

**Characterization of *TsdA* and *TsdB***—UV-visible electronic absorbance spectroscopy, X-ray diffraction, and activity assays have revealed a number of characteristic features of *AvTsdA* (1, 2, 12). However, the electrochemical window in which the hemes are redox active remained unknown. To gain insight into this property, we mapped out the redox activity of *AvTsdA* adsorbed as an electroactive film on optically transparent mesoporous nanocrystalline SnO₂ electrodes. The spectrum of the enzyme-coated electrode equilibrated at +302 mV contained features typical of ferric *c*-type hemes superimposed on a small contribution from light scattering by the electrode material (Fig. 1). The Soret maximum at 406 nm and broad lower intensity features in the αβ-region are typical of those displayed by solutions of oxidized *AvTsdA* (1). When the electrode potential was lowered to −648 mV in 50-mV steps with a spectrum recorded after a 60-s pause at each desired potential (Fig. 1), those features were replaced with peaks having maxima at 418, 523, and 553 nm, which are typical of dithionite-reduced enzyme (1). Variation of the Soret intensity at 418 nm with electrode potential revealed that the hemes were reduced between approximately +150 and −350 mV (Fig. 1, inset, *closed squares*).

The potential was raised in 50-mV steps, and the spectra were recorded after a 60-s pause at each potential (Fig. 1, inset, *open squares*). Variation of the Soret intensity at 418 nm with electrode potential revealed that the hemes were reduced between approximately +150 and −350 mV (Fig. 1, inset, *open squares*). The hysteretic nature of the plot of absorbance versus potential prevented Nernstian analysis to define the heme reduction.
Electron Accepting Units of TsdA

FIGURE 2. UV-visible spectra of TsdB from S. lithotrophicus. As the protein is partly reduced in the "as isolated" state, up to 170 μM ferricyanide were added to record the oxidized spectrum (black line). For full reduction of the protein, sodium dithionite was added (gray line). 100 mM Tris buffer, pH 8.0, with 150 mM NaCl and 2.5 mM desthiobiotin was used, and spectra are normalized to 750 nm. The oxidized spectrum exhibits a 700-nm peak indicating methionine as heme iron ligand. Protein concentration was 6 μM in the overview and 29 μM in the blowup.

potentials. Nevertheless, some further conclusions regarding the redox activity of AvTsdA can be proposed in light of the crystal structures reported (2, 12). The fully oxidized diheme cytochrome AvTsdA contains His/Cys coordination in heme 1 and His/Lys in heme 2. His/Cys-ligated hemes are typically distinguished from other low-spin c-type hemes by having much lower reduction potentials and smaller changes in extinction coefficient associated with the Fe(III)/(II) couple (4, 13). As a consequence, we propose that reduction of His/Cys-ligated heme 1 occurs reversibly between approximately −100 and −350 mV. Reduction of His/Lys-ligated heme 2 is proposed to occur between approximately +150 and −100 mV. AvTsdA X-ray structures reveal that this reduction is accompanied by a switch of Lys by Met as axial distal ligand to ferrous-heme 2 (2). Such a change of ligation would be expected to raise the reduction potential of heme 2. If the Met ligand is replaced slowly by Lys upon enzyme oxidation, this would account for the hysteretic nature of the redox behavior displayed by AvTsdA.

In contrast to TsdAs, TsdB proteins are not well characterized. The most closely related characterized cytochromes on a sequence level belong to the diheme cytochromes of the c9 family (1). The recombinant TsdB protein from T. intermedia indeed binds two heme groups of 616.5 Da as the mass of 21,792.5 Da determined by MALDI-TOF mass spectrometry almost exactly matched the mass of 21,783.3 Da predicted for the mature recombinant protein, including Strep tag and two hemes. The same held true for recombinant S. lithotrophicus TsdB (measured mass, 22,864 Da, and predicted mass, 22,835 Da). UV-visible spectroscopy of pure recombinant TsdB from S. lithotrophicus is shown in Fig. 2. The spectrum conforms to that of TtTsdB (1).

A sequence alignment of various TsdB proteins (Fig. 3) reveals two conserved methionine residues but no conserved histidines or cysteines indicating that both hemes of TsdB have axial coordination by His/Met. This is underpinned by the 700-nm peak in the spectrum, which is characteristic for methionine as the sixth axial heme iron ligand (14) and corroborated by the MpTsdBA crystal structure determined herein (see below). A shift of the Soret band from 411 to 417 nm upon reduction was observed. Moreover, there is a distinct α band at 359 nm in the oxidized protein spectrum. In the reduced state, the α band was detected at 552 nm and the β band at 523 nm. A split α band characteristic for a number of c9-type cytochromes, including those from purple sulfur bacteria, was not observed (9, 15). The UV-visible spectrum for oxidized TsdB exhibited a low intensity high spin feature at 620 nm similar to that noted for cytochrome c4 from P. stutzeri (16). Obviously, the ferric form of TsdB holds a small fraction of high-spin heme probably caused by weakening of the Fe–S bond at one of the two hemes with concomitant partial dissociation of the methionine and formation of an Fe–aquo bond just as outlined for the P. stutzeri cytochrome (16).

The reduction potential of TtTsdB was determined by potentiometry with a gold electrode (Fig. 4). The potential changes determined upon reduction and reoxidation of TsdB match well. The two hemes are redox active between −100 and +300 mV. Hysteresis was not observed, consistent with both hemes being His/Met-ligated. Ligand changes are not apparent.

TsdB Is an Electron-transferring Unit Tightly Interacting with TsdA—The interaction of TsdA and TsdB proteins was analyzed for the recombinant proteins from S. lithotrophicus. Upon analytical gel permeation chromatography SITsdA alone eluted at a volume corresponding to a molecular mass of 65 kDa, indicating dimerization of the protein under the conditions applied (monomer, 33,042 Da). In contrast, SITsdB behaved as a 22-kDa monomer (predicted molecular mass, 22,835 Da). When both proteins were produced simultaneously in Escherichia coli and purified employing the Strep tag attached to TsdA, a preparation was obtained that exhibited two heme stainable polypeptides. In size, these exactly matched SITsdB and SITsdA (Fig. 5). The co-purification of the two proteins is evidence for significant interaction between them. Upon size exclusion chromatography, SITsdA and SITsdB co-eluted again in fractions corresponding to a mass of 108 kDa, indicating formation of an α5β2 heterodimer.

Characterization of the TsdBA Fusion Protein from M. purpuratum—The protein encoded by tsdB-tsdA gene fusion in M. purpuratum provides an exceptional possibility to study the complete tetraheme cytochrome for catalytic properties and internal electron transfer. A sequence alignment of MpTsdBA with combined TsdB and TsdA sequences from S. lithotrophicus and T. intermedia shows significant similarity between the N-terminal region of MpTsdBA and TsdB (amino acids 1–199 of MpTsdBA and TtTsdB share 45% sequence identity) and between the C-terminal region of MpTsdBA and TsdA (amino acids 224–518 of MpTsdBA compared with AvTsdA or TtTsdA, 39 and 50% sequence identity, respectively). The heme distal ligands cysteine and methionine of TsdA as well as the two putative heme-ligating methionines are strictly conserved (Fig. 3). Therefore, we predicted MpTsdBA to contain three His/Met ligated and one His/Cys ligated heme. UV-visible spectroscopy of MpTsdBA protein is shown in Fig. 6. The presence of His/Met-ligated hemes in MpTsdBA is substantiated by the 700-nm peak in the spectrum of the oxidized
protein. This absorption band is characteristic for methionine as heme iron ligand (14). A shift of the Soret band from 413 to 420 nm was observed upon reduction. Moreover, there is a distinct $\delta$ band at 363 nm in the oxidized protein spectrum. The $\alpha$ band is located at 553 nm, and the $\beta$ band resides at 524 nm.

The partly reduced spectrum exhibits a low intensity high spin feature at 620 nm similar to $A_{\nu TsdA}$ (2). The $A_{413 nm}/A_{280 nm}$ for pure oxidized $M. purpuratum$ TsdBA is 3.4.

The partly reduced spectrum exhibits a low intensity high spin feature at 620 nm similar to $A_{\nu TsdA}$ (2). The $A_{413 nm}/A_{280 nm}$ for pure oxidized $M. purpuratum$ TsdBA is 3.4.

**Crystal Structure Determination and Model Quality of $M. purpuratum$ TsdBA**—To compare structural features of TsdA and TsdBA, to get a closer look into ligation of the four heme...
groups and to see how TsdA and TsdB domains are linked to each other, we have determined the X-ray structure of MpTsdBA by Fe-SAD. Crystals belong to the hexagonal space group H32 with cell dimensions \(a = b = 159.75 \text{ Å} \) and \(c = 393.09 \text{ Å} \). There are two molecules in the asymmetric unit corresponding to a Matthews coefficient \(17 \) of \(2.8 \text{ Å}^3 \text{ Da}^{-1} \) and a solvent content around 56%. The “as isolated” structure was refined to 2.75 Å resolution with \(R_{	ext{meas}} \) of 15.7% and \(R_{	ext{free}} \) of 19.8% using a 141°-sweep of data with overall better statistics. Data collection and refinement statistics are depicted in Table 1. The high \(R_{	ext{meas}} \) values observed for both sweeps are due, first, to the fine binning that autoPROC implements during data reduction, and second, to the fact that the crystal has some regions in the rotational space that are of bad quality. This is perfectly clear in the plots \(R_{	ext{meas}} \) versus image number output by autoPROC (data not shown), highlighting that the high \(R_{	ext{meas}} \) values are due to the crystal quality and not radiation damage. No evidence for radiation damage is also observed in a \(m[F_{\text{obs}}] - m[F_{\text{calc}}] \) map calculated using both data sweeps (data not shown). MpTsdBA is numbered without taking into account its 23-amino acid signal peptide that is removed upon transport into the periplasm. The model comprises the following residues of the mature recombinant protein: Pro \(^1\)–Leu \(^{191}\) and Arg \(^{237}\)–Val \(^{515}\) (chain A) and Pro \(^1\)–Ala \(^{192}\) and Ala \(^{240}\)–Ala \(^{516}\) (chain B), eight heme molecules, two thiosulfate ions, two chloride ions, seven ethylene glycols, two 1,2-propanediols and one triethylene glycol, and 113 water molecules. Electron density maps are of good quality except for some parts of the map that are somewhat “noisy” with positive and negative difference map peaks in the solvent region that could not be modeled.

**Overall Fold and Similar Structures of MpTsdBA—**

MpTsdBA is organized into two domains, an N-terminal TsdB domain and a C-terminal TsdA domain (Fig. 7A). Each domain includes two subdomains that are related by a pseudo-2-fold symmetry axis. Each subdomain consists of four \(\alpha\)-helices surrounding a heme group, the typical class I c-type cytochrome topology. This has been previously reported for the AvTsdA crystal structure (2, 12) and is also observed for the \(M.\) purpureatum N-terminal TsdB domain. The four subdomains superimpose with r.m.s.d. of 1.3–3.0 Å for ~70 aligned Ca atoms corresponding to sequence similarities between 10 and 38%.

The final model coordinates were submitted to the DALI server (18), showing no similar structures to the completeMpTsdBA arrangement. However, several hits structurally match each domain of the enzyme separately. The highest matches to MpTsdB domain were cytochrome \(c_4\) from \(P.\) putida \((\text{PDB code 1M6Z})\), cytochrome \(c_{553}\) from \(Acditibio-
cillus ferroxidans\) \((\text{PDB code 1H10})\), the cytochrome subunit of flavocytochrome \(c\) sulfide dehydrogenase from \(A.\) vinosum \((\text{PDB code 1FCF})\), and flavocytochrome \(c\) from \(Thermochromatidium tepidum\) \((\text{PDB code 3VRD})\), with Z-scores of 22.7 to 17.5, r.m.s.d. of 2.0–2.4 Å, and sequence identities between 30 and 23%.

The highest hit for the MpTsdA domain was \(Avt\)sdA (4V2K and 4WQ9) with a Z-score of 30.6 (with 20% sequence identity and r.m.s.d. of 1.4 and 1.2 Å, respectively), followed by SoxAX from \(Rhodovulum sulfidophilum\) (2Z01), the SoxD subunit of SoxCD from \(Paracoccus pantotrophus\) (2XTS), and the SoxA subunits of SoxAX from \(Starkeya novella\) (3OAB) and \(P.\) pantotrophus (2C1D). Z-scores ranged from 12–74 and r.m.s.d.s from 2.1 to 4.7 Å.

**Heme Coordination in the “As Isolated” MpTsdBA—**
The MpTsdBA crystal structure shows four heme groups per chain packed as a wire with closest iron-to-iron distances between 15 and 19 Å and shortest edge-to-edge distances of 3.5 to 6.6 Å (Fig. 7, B and C). This agrees well with other multiheme cytochrome structures that show edge-to-edge distances of 4–8 Å (19, 20).

The four hemes are covalently bound to the polypeptide chain through thioether bonds formed by cysteine residues Cys \(^{21}\) and Cys \(^{24}\) for heme 1, Cys \(^{121}\) and Cys \(^{124}\) for heme 2, Cys \(^{287}\) and Cys \(^{290}\) for heme 3, and Cys \(^{402}\) and Cys \(^{405}\) for heme 4 (Fig. 8, A–D). Moreover, the structure confirmed the spectroscopic evidence gathered showing that this tetraheme cytochrome \(c\) has three hemes (hemes 1, 2, and 4) with His/Met coordination (Fig. 8, A, B, and D). Axial ligation by histidine and cysteine is typical for the active site of TsdA proteins (2, 12). Indeed, heme 3 exhibits axial ligation by His \(^{290}\), and the S\(\bar{y}\) atom of Cys \(^{330}\) is located in close vicinity to the heme iron such that it could serve as the sixth ligand. However, the 2.9 Å distance between the sulfur and the iron atom precludes direct ligation (Fig. 8, C, E, and F). It has been shown earlier that the S\(\bar{y}\) atom of the corresponding active site cysteine (Cys \(^{366}\)) in TsdA from \(A.\) vinosum can adopt two different conformations by rotation of the cysteine C\(\alpha\)–C\(\beta\) bond. Therefore, the sulfur atom switches between iron-ligating and iron-non-ligating states (2). The non-ligating conformation has been proposed as
an essential intermediate step in the catalytic cycle, possibly involving covalent attachment of a substrate molecule (2, 12).

Remarkably, in the “as isolated” structure of TsdBA Cys330 to coordinate the heme when no ligand/substrate is present, similar to what is observed in AvTsdA crystal structures (2, 12).

The thiosulfate substrate lies in a cleft accessible from the solvent to Cys330 and heme 3. This cavity is delineated by the side chains of positively charged residues Arg314, Lys316, Arg326, Arg438, and Arg480 (Fig. 8F), which have been previously proposed to be involved in the orientation and stabilization of the substrate for catalysis (2, 12). Some positive electron density (m|Fo| – D|Fc|) is present near the substrate, although no density is observed in 2m|Fo| – D|Fc| maps (even at low contours). This electron density is observed between the Nζ atom of Lys316 and the plane formed by the three oxygen atoms of the thiosulfate ion (although independent from the density observed for the thiosulfate ion itself). Because this electron density was not amenable to refinement, nothing was included in the final 3D structure. Furthermore, heme 3 seems to display another residual conformation with one of the propionates alternating between this cavity and a cleft above the heme plane delineated by Arg377, Arg381, and the N main chain atoms of Gly378 and Tyr379. This motion is illustrated by the different conformation modeled for heme 3 in both chains, either pointing toward the

| TABLE 1 |

**Data reduction and refinement statistics for MptsdBA structure**

| PDB code | SL09 |
|----------|------|
| **Data collection** | | |
| Synchrotron | ESRF (Grenoble, France) |
| Beamline | ID-29 |
| Wavelength (Å) | 1.7236 |
| Space group | H32 |
| Unit cell | a, b, c (Å) |
| a, b, c (Å) | 159.75, 159.75, 393.09 |
| b, c (Å) | 90.0, 90.0, 120.0 |
| Resolution range (Å) | 113.13–2.75 (2.76–2.75) |
| Total no. of reflections | 377,526 (2187) |
| No. of unique reflections | 50,199 (461) |
| Completeness (%) | 99.0 (87.8) |
| Anomalous completeness (%) | 98.7 (84.8) |
| Multiplicity | 7.5 (4.7) |
| Anomalous multiplicity | 3.9 (2.5) |
| (|f|/|I|) | 14.9 (2.0) |
| Rmerge (%) | 11.7 (70.6) |
| Rfree (%) | 5.7 (41.8) |
| Cctg (%) | 99.7 (63.8) |
| **Refinement** | | |
| Rwork (%) | 15.7 (25.9) |
| Rfree (%) | 19.8 (30.0) |
| No. of non-H atoms | 7028 |
| Protein | 330 |
| Ligands | 113 |
| Waters | 1.50 |
| r.m.s.d. bonds (Å) | 0.013 |
| r.m.s.d. angles (%) | 113 |
| Protein residues | 52.04 |
| Most favored (%) | 97.6 |
| Allowed (%) | 2.4 |
| Outliers (%) | 0.9 |
| Ramachandran plot | 2.69 |
| Clashscore | 1.28 |
| B-Factors (Å²) | 50.43 |
| Protein | 45.06 |
| Ligands/ions | 7028 |
| Waters | 113 |
| MolProbity score' | 142.65 |
| Electron Accepting Units of TsdA | 5126 |

* Information in parentheses refers to the last resolution shell.
* Rmerge = ΣhΣq|Ihq| − |Ihq|)/ΣhΣq|Ihq|, where |Ihq| is the ith observation of reflection h and |Iq| is the average counts for reflection h.
* Cctg is as described previously (57).
* Rfree = ΣhΣq|Fo||Fc|)/ΣhΣq|Fo|, where |Fo| = the observed and calculated structure factors for reflection h, respectively.
* MolProbity score provides a single number that represents the central MolProbity protein quality statistics; it is a log-weighted combination of clashscore, Ramachandran not favored and bad side-chain rotamers, giving one number that reflects the crystallographic resolution at which those values would be expected.
active site cavity, in chain A (Fig. 8E), or toward the cleft above the heme plane, in chain B (Fig. 8, C and F). However, this possible alternate conformation could not be properly refined, and therefore it was not added to the crystallographic model.

Reactivity with External Electron Acceptors—To test different electron acceptors for TsdA and TsdBA, we performed enzyme activity assays with TsdB from *T. intermedia*, *A. vinosum* cytochrome *c*₄ (Alvin_2879), and *A. vinosum* HiPIP as electron acceptors. All three potential electron acceptors were produced as recombinant proteins in *E. coli*.

Previously, it had been shown that TsdB from *T. intermedia* is not reactive with thiosulfate but that it is instantaneously reduced when TsdA is added in the presence of thiosulfate (1). Here, we succeeded in obtaining quantitative kinetic data for a homologous system by analyzing electron transfer between TsdA and TsdB from *S. lithotrophicus*. Just as TTsDb, STsDb alone is not reduced by addition of thiosulfate. An enzyme activity assay with STsDb as the catalyst and STsDb as the electron acceptor resulted in a specific activity of 6.3 units mg⁻¹ for STsDb. *S*₀.₅ for thiosulfate was determined to be 0.04 mM. This unambiguously identified STsDb as an effective electron acceptor for TsdA from the same organism. However, a heterologous approach yielded a different result; with 0.6 units mg⁻¹ the specific activity of *A*₃*V*₃TsdA with TsdB from *T. intermedia* amounted to only one-tenth that determined for the homologous system, whereas *S*₀.₅ for thiosulfate (0.03 mM) resided in a similar range.

In a further series of experiments, *A*₃*V*₃TsdA activity was tested with Cyt *c*₄ originating from the same host. It should be noted that the recombinant cytochrome was electrophoretically pure and that it exhibited exactly the same spectral features as Cyt *c*₄ purified from *A. vinosum* cells, including the characteristic split α-band (9). The specific activity of *A*₃*V*₃TsdA with *A*₃*V*₃Cyt *c*₄ as the electron acceptor amounted to 0.6 units mg⁻¹ and was thus not found to be higher than with TsdB from a different source organism. Therefore, we exclude those diheme cytochromes as efficient electron acceptors for *A*₃*V*₃TsdA *in vitro* as well as *in vivo*.

HiPIP from *A. vinosum*, a protein with a positive reduction potential (+350 mV (10)), was tested as another potential candidate for accepting electrons from *A*₃*V*₃TsdA as well as from *M*₃pTsDbA. Indeed, both thiosulfate dehydrogenases reacted efficiently with *A. vinosum* HiPIP (Fig. 9 and Table 2). *A*₃*V*₃TsdA exhibited a higher *V*ₚₙₐₓ with HiPIP as electron acceptor, whereas *M*₃pTsDb exhibited an especially low *S*₀.₅ value for thiosulfate when the reaction was measured with HiPIP as electron acceptor. In both cases, *S*₀.₅ for thiosulfate was much lower with HiPIP than with ferricyanide as the electron acceptor indicating cooperativity between the electron-transferring heme 2 and the active site heme 1. *A. vinosum* and *M. purpuratum* both encode HiPIP in their genome, and both thiosulfate dehydrogenases exhibit substantial specific activity with HiPIP as electron acceptor *in vitro*, leading us to conclude that HiPIP also serves as an efficient *in vivo* electron acceptor for Tsd(B)A in both organisms.

Discussion

In our approach to find suitable electron acceptors for TsdA-type thiosulfate dehydrogenases, we first focused on TsdB, a diheme cytochrome encoded upstream of TsdA in a number of different organisms. As demonstrated here for the proteins from *S. lithotrophicus* and earlier for those from *T. intermedia* (1), TsdA and TsdB enzymes interact strongly with each other and form an α₂β₂ heterodimer. The same arrangement has been described for thiosulfate dehydrogenase from *Halothiobacillus neapolitanus* (21), which consists of heme *c* binding subunits of 27 and 33 kDa conforming in size with TsdA and TsdB, respectively.

In this work, a redox range of −300 to +150 mV was determined for *A*₃*V*₃TsdA, whereas *T*TsDb is redox active between −100 and +300 mV. Generalizing this finding, we state that the
overall reduction potential of TsdB is more positive than that of TsdA, which should enable electron flow from TsdA to TsdB. Indeed, reduction of \( \text{Ti}^{2+} \) by \( \text{Av}^{2-} \) had been shown previously (1) and was verified here for the proteins from \( S. \text{lithotrophicus} \). Enzyme activity assays further revealed \( \text{Sl}^{2+} \) TsdB as an effective electron acceptor for \( \text{Sl}^{2+} \) TsdA but not for \( \text{Av}^{2-} \) TsdA. This was not surprising as \( A. \text{vinosum} \) does not contain a gene encoding TsdB (Table 3).

In \( A. \text{vinosum} \), the gene with strongest similarity to \( \text{tsdB} \) is \( \text{Avlin}_2879 \). The encoded high potential diheme cytochrome \( c_4 \) has been suggested to play a role in transferring electrons to the photosynthetic reaction center (9). The \( M. \text{purpuratum} \) genome also encodes a protein (Marpu_15750) with high similarity to \( A. \text{vinosum} \) Cyt \( c_4 \) (78% identity on the sequence basis; see Table 3). In the anoxygenic phototroph \( \text{Rubrivivax gelatinosus} \), a related cytochrome \( c_4 \) indeed has an established func-

**FIGURE 8. Heme coordination of “as isolated” \( M_p \text{TsdBA} \) (PDB code 5LO9).** A, heme 1 is coordinated by His\(^{25} \) and Met\(^{65} \). B, heme 2 is coordinated by His\(^{125} \) and Met\(^{167} \). C, heme 3 is ligated to His\(^{291} \) but not to Cys\(^{330} \). The distance of \( S_y \) to the heme iron is 2.9 Å and thus not close enough for direct ligation. Thiosulfate covalently bound to \( S_y \) of Cys\(^{330} \) is not shown here for clarity. Presence of thiosulfate is illustrated in detail in E and F. D, heme 4 is ligated by His\(^{406} \) and Met\(^{450} \). E, heme 3 with \( S_y \) of Cys\(^{330} \) covalently bound to thiosulfate, displayed in ball and stick, and polder map electron density contoured at 6σ level depicted as a black mesh. F, heme 3 in a similar view as in E but with positively charged residues surrounding the substrate cleft depicted as sticks. Scheme representation is shown in pale gray with heme moieties and coordinating amino acid residues shown as sticks; color code as in Fig. 7 with sulfur atoms in green.
Electron Accepting Units of TsdA

In many anoxygenic photosynthetic bacteria, the periplasmic high potential iron–sulfur protein is well known to shuttle electrons between the cytochrome bc₁ complex and the photosynthetic reaction center during cyclic electron flow (23–26). This function has also been firmly established for the protein from A. vinosum (24, 27). Here, we demonstrate that HiPIP is a suitable electron acceptor for Tsd(B)A from A. vinosum and M. purpuratum in vitro, identifying this protein as the most likely electron carrier between the thiosulfate-oxidizing enzyme and the reaction center during growth in the light. It should be noted that a direct interaction between Tsd(B)A and the photosynthetic reaction center cannot be completely excluded so far.

Many purple sulfur bacteria, including A. vinosum, are capable of chemolithotrophic growth on reduced sulfur compounds and oxygen under microaerobic conditions (28). Accordingly, cbb₃, as well as ubiquinol oxidases are encoded in their genomes. Although the standard reduction potential of the thiosulfate/tetrathionate couple (+198 mV (7)) appears too positive to feed electrons directly into the quinone pool, and from there to oxygen, delivery of electrons originating from the thiosulfate to tetrathionate conversion to cbb₃ oxidase is certainly feasible. In fact, HiPIP has been reported to be involved in bacterial respiratory chains (29, 30) and is a prime candidate for electron transport between Tsd(B)A and the terminal oxidase in those organisms where it is present. However, in chemotrophs like T. intermedia or S. lithotrophicus, the situation must be different because these bacteria do not contain HiPIP. In fact, for these organisms, it is not exactly established so far which periplasmic proteins deliver electrons to cbb₃ oxidase, regardless of the electron donor oxidized. For S. lithotrophicus it is assumed that the c-type cytochrome MtoD (Slit_2948) can transfer electrons stemming from iron oxidation to cbb₃ and the cytochrome bc₁ complex (31). In the T. intermedia genome, there are two c-type cytochromes (Tint_2575 and Tint_3060) with 36 and 42% sequence identity to S. lithotrophicus MtoD, respectively, which may serve a similar function. It may be possible that thiosulfate dehydrogenase delivers electrons to the MtoD(-like) cytochrome, which then shuttles the electrons to the terminal oxidase.

Nevertheless, an alternative scenario is also possible when we consider the similarity between TsdB and cytochromes of the c₁-type (about 49% identity between TITsdB and Cyt c₁ from Achromobacter xylosoxidans or Pseudomonas pseudoglobens). Cytochromes of the c₁-type have been reported to donate electrons to cbb₃-type cytochrome c oxidases in various oxygen-respiring bacteria (32–34), and we therefore consider the possibility that TsdB serves as a direct electron donor to cbb₃ oxidase at least in tetrathionate-forming thiosulfate oxidizers that neither contain HiPIP nor any cytochrome c₁ homolog except TsdB.

We have determined the first three-dimensional structure of M. purpuratum TsdA, where TsdA is fused with its electron acceptor TsdB. It showed heme arrangement with characteristics of c-type cytochrome topology, unveiling their relative heme spatial disposition and providing insights into the electron flow during enzymatic reaction. In the MpTsdBA structure, a thiosulfate ion is covalently bound to S₈ of Cys₃⁴⁰ in heme 3, although the protein was produced in and purified from E. coli without the addition of thiosulfate to media or buffers. This implies high affinity of the enzyme to thiosulfate, which is possibly present in the complex growth medium in

![Figure 9. Thiosulfate oxidation catalyzed by AvTsdA and MpTsdBA with HiPIP as electron acceptor. Enzyme assays with AvTsdA were performed in 100 mM ammonium acetate buffer, pH 4, at 30 °C with 8 mM enzyme. Activity measurements with MpTsdBA were performed in 100 mM ammonium acetate buffer, pH 5.2, with 200 mM NaCl at 25 °C and with 3.9 mM enzyme. In both assays 10 μM HiPIP and 40 μM ferricyanide were used. A change in absorbance was measured at 480 nm. versus [S] plots were fitted to the Hill equation.](image_url)

**TABLE 2**

| Electron acceptor | Enzyme | Vₘₐₓ | S₅₀ | kₘₐₓ | cat/S₅₀ |
|------------------|-------|------|-----|-------|--------|
| Ferricyanide      | AvTsdA| 31.419 ± 2408 | 835 ± 119 | 14,091 | 16,875 |
|                  | MpTsdBA| 301.1 ± 108 | 179 ± 21 | 2794 | 15,611 |
| HiPIP            | AvTsdA| 96 ± 3 | 27 ± 2 | 43 | 1595 |
|                  | MpTsdBA| 26 ± 1 | 6 ± 0 | 24 | 4000 |

**TABLE 3**

Occurrence of genes encoding TsdA and putative electron acceptors in the genome sequenced organisms relevant to this study

| Organism | TsdA | TsdB | Cyt c₁ | HiPIP |
|----------|------|------|--------|-------|
| A. vinosum DSM 180⁷ | Alvin_0091 | Marpu_15750 | Alvin_2879 | Alvin_2274 |
| M. purpuratum 984 (DSM 1591⁷) | Marpu_02550 (TsdBA fusion) | Marpu_11560 |
| S. lithotrophicus ES-1 (ATCC 700298⁷) | Slit_1877 | Slit_2983 |
| T. intermedia K12 (DSM 18155⁷) | Tint_2892 | Tint_2893 |

References [23–26], [24, 27]
very low concentrations. It should be noted that recombinant AvTsdA and also several SoxA proteins have been isolated with the active site cysteine in a partially or fully persulfurated state (2, 35–37). This has been interpreted as indication for temporary binding of thiosulfate and subsequent incomplete catalysis. Just as proposed here, thiosulfate was assumed to originate from the E. coli growth medium (2).

Regardless of its source, the covalent attachment of a complete thiosulfate molecule to the MpTsdBA active site cysteine strongly supports the hypothesis that tetrathionate formation from two thiosulfate molecules proceeds via a rhodanese-like reaction mechanism involving a thiosulfate transfer reaction with a thiosulfate molecule covalently bound to the active site cysteine as an essential intermediate in the catalytic cycle (2, 12). This type of mechanism has been illustrated in detail by Grabarczyk et al. (12) for TsdA from A. vinosum. A rhodanese-like reaction cycle has also repeatedly been depicted and discussed for the closely related SoxXA protein, but it could not be unambiguously proven before (4, 35). The MpTsdBA structure provides conclusive evidence that the reactions catalyzed by TsdA as well as SoxXA enzymes indeed involve a cysteine S-thiosulfonate intermediate that is formed once the first thiosulfate molecule is positioned in the substrate binding pocket by positively charged amino acid side chains (Arg314, Lys316, Arg326, and Arg438 in MpTsdBA, Fig. 8). The latter also stabilized the cysteine S-thiosulfonate group once it had formed. Formation of the cysteine S-thiosulfonate releases two electrons that reduce the iron atoms of the two hemes in TsdA to the Fe(II) state. Heme reoxidation by an external electron acceptor is then likely to be followed by a thiol-disulfide exchange reaction that proceeds via an attack of the sulfane atom of a second thiosulfate molecule on the thiosulfonate group (12).

We conclude that catalysis of thiosulfate oxidation by Tsd(B)A enzymes and very probably also that by SoxXA proteins involves formation of a covalent adduct between the sulfane sulfur atom of thiosulfate and the Sγ of the active site cysteine. When present, TsdB is the immediate electron acceptor of TsdA. TsdB is very likely able to transfer electrons directly to the cbb3 terminal oxidase. In organisms containing HiPIP, this electron carrier is likely to act as an additional shuttle not only between Tsd(B)A and the terminal oxidase during oxygen respiration but also between Tsd(B)A and the photosynthetic reaction center during photolithotrophic growth in the light.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Growth Conditions**—Table 4 lists the bacterial strains and plasmids used for this study. E. coli BL21 (DE3) was used for recombinant protein production and was grown in LB medium. E. coli DH5α was used for molecular cloning.

**Recombinant DNA Techniques**—All general molecular genetics techniques were described earlier (38). Restriction enzymes, T4 ligase, and Pfu DNA polymerase were obtained from Thermo Scientific (Schwerte, Germany) and used according to the manufacturer’s instructions. Oligonucleotides for cloning were obtained from Eurofins MWG (Ebersberg, Germany).

**Construction of Expression Plasmids**—A. vinosum tsdA and T. intermedia tsdB genes coding for the mature proteins without the signal peptides were amplified and cloned as described.

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**FIGURE 10.** Role of periplasmic electron transfer proteins in aerobic respiration or photosynthesis of A. vinosum, M. purpuratum, S. lithotrophicus, and T. intermedia. All organisms contain genes for NuoA-N (Alvin_2418–2430 + Alvin_2412, Marpu_04365–04430, Slit_1070–1083, and Tint_2255–2268), the cytochrome bc1 complex (Alvin_0068–0070, Marpu_01465–01475, Slit_0130–0132, and Tint_2192–2194), and cbb3 oxidase (Alvin_0781–0784, Marpu_02795–02810, Slit_0411–0414, and Tint_1070–1073). Moreover, A. vinosum and M. purpuratum can gain energy by photosynthetic growth. HiPIP can transfer electrons to the photosynthetic reaction center (23, 24) as well as to cbb3 oxidase (29, 30). Cytochrome c4, also known to transfer electrons to the photosynthetic reaction center (22) as well as to cbb3 oxidase (32–34) in some bacteria. For S. lithotrophicus, it is assumed that MtoD (Slit_2498) can transfer electrons to cbb3 oxidase and the cytochrome bc1 complex (31).
Table 4: E. coli strains and plasmids used in this study

| Strains          | Description                                                                 | Ref. or source |
|------------------|-----------------------------------------------------------------------------|----------------|
| E. coli DH5a     | F<sup>−</sup> gptD lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1hisD17(rk<sup>−</sup> m<sup>k</sup>) supE44 λ thi-1 gyrA relA1 | 56             |
| E. coli BL21 (DE3) | F<sup>−</sup> ompT hsdS<sub>gr− m<sup>+</sup></sub> gal dcm met(DE3)           | Novagen        |

Plasmids

| Plasmids                  | Description                                                                 | Ref. or source |
|---------------------------|-----------------------------------------------------------------------------|----------------|
| pEC86                     | Cm<sup>+</sup>, product from pEC66 and pACYC184 with E. coli cemABCDEFGH genes | 40             |
| pET-22b (+)               | Ap<sup>+</sup>, T7 promoter, lac operator, C-terminal His tag, pelB leader   | Novagen        |
| pASK-IBA3 plus            | Ap<sup>+</sup>, tetA promoter/operator, C-terminal Strep tag                 | IBA (Göttingen) |
| pET_MarpuDRAFT_1194       | Ap<sup>+</sup>, tsdA from M. purpuratum (Marpu_02550) cloned into pET-22b (+) with Ndel and Xhol, C-terminal His tag | 7              |
| pASK-IBAInt_tsdB           | Ap<sup>+</sup>; tsdB from T. intermedia cloned into pASK-IBA3 plus with BsaI, C-terminal Strep tag | This study     |
| pASK-IBA3plus-slit1877     | Ap<sup>+</sup>; tsdA from S. lithotrophicus (Slit_1877) cloned into pASK-IBA3 plus with BsaI, C-terminal Strep tag | This study     |
| pASK-IBA3plus-slit1878     | Ap<sup>+</sup>; tsdA from S. lithotrophicus (Slit_1878) cloned into pASK-IBA3 plus with BsaI, C-terminal Strep tag | This study     |
| pET-soxXAK-Strep          | Ap<sup>+</sup>, soxXAK, including fragment cloned into pET-22b (+) together with C-terminal Strep tag by use of Xbal and HindIII | This study     |
| pET-Alvin2274-C-Strep     | Ap<sup>+</sup>, hip gene coding for HiPIP from A. vinosum (Alvin_2274) cloned into pET-soxXAK-Strep with Ndel and Ncol, C-terminal Strep tag | This study     |
| pPR-IBAAvtsdA             | Ap<sup>+</sup>; tsdA from A. vinosum (Alvin_0091) cloned into pASK-IBA3, C-terminal Strep tag | This study     |
| pASK-IBA3plus_Avin_2879    | Ap<sup>+</sup>; Alvin_2879 from A. vinosum cloned into pASK-IBA3 plus with BsaI, C-terminal Strep tag | This study     |

Overproduction, Purification, and Preparation of Recombinant Proteins—AvTsdA and TtTsdB were produced as described before (1). For production of S. lithotrophicus TsdA, STTsdB and the simultaneous production of STTsdB and STTsA, E. coli BL21 (DE3) cells containing pASK-IBA3plus-slit1877, pASK-IBA3plus-slit1878, or pASK-IBA3plus-slit1877-slit1878 and pEC86 (40) were cultured in 700 ml of LB media supplemented with 100 µg ml<sup>−1</sup> ampicillin and 25 µg ml<sup>−1</sup> chloramphenicol at 37 °C and 180 rpm after inoculation with an overnight pre-incubation in a (1:50) dilution. At an OD<sub>600</sub> of 0.4 to 0.6, 200 ng ml<sup>−1</sup> anhydrotetracycline were added, and the appropriate culture was switched to 25 °C and 90 rpm in case of TsdB production. Cells were harvested after 18 h. MpTsdBA and HiPIP were produced in E. coli BL21(DE3) cells containing pET_MarpuDRAFT_1194 or pET-Alvin2274 and pEC86 (40). After 2 or 0.5% inoculation with a pre-culture, the cells were grown in 700 ml of LB medium containing 100 µg ml<sup>−1</sup> ampicillin and 25 µg ml<sup>−1</sup> chloramphenicol at 37 °C and 180 rpm. At an OD<sub>600</sub> of 0.5 to 0.6, the cultures were switched to 25 °C and 120 rpm for about 18 h. For production of AvCyt<sub>c</sub> (Alvin_2879), E. coli BL21(DE3) cells containing pASK-IBA3plus_Avin_2879 and pEC86 (40) were cultured in 400 ml of LB medium, 100 µg ml<sup>−1</sup> ampicillin, and 25 µg ml<sup>−1</sup> chloramphenicol at 37 °C and 180 rpm after 2% inoculation with a preculture. At OD<sub>600</sub> of 0.5, 200 ng ml<sup>−1</sup> anhydrotetracycline were added, and the culture was switched to 25 °C and 90 rpm for 18 h. Harvested cells were resuspended in 100 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and lysed by sonication. After removal of insoluble cell material by centrifugation (10,000 g for 25 min at 4 °C), STTsA, STTsB, and STTsdB were separated from the culture supernatants by size-exclusion chromatography and then subjected to a size-exclusion chromatography step performed on a HiLoad 16/60 Superdex 75-<wbr/>pg column (GE Healthcare) using an AKTAapu-
The Superdex 75 column was equilibrated with 100 mM ammonium acetate buffer, pH 5, and 150 mM NaCl. The column was calibrated with the molecular weight marker kit MW-GF-70 (GE Healthcare). All purified proteins were desalted with 5 ml of HiTrap Desalting columns (GE Healthcare). The column was equilibrated with 100 mM ammonium acetate buffer, pH 5, at 30 °C, whereas TsdB and Tsda from *S. lithotrophicus* were analyzed by the same procedure either separately or as a mixture of both proteins. In this case, the Superdex 75 column was equilibrated with 100 mM Tris-HCl buffer, pH 7.5, and 150 mM NaCl. The column was calibrated with the molecular weight marker kit MW-GF-70 (GE Healthcare). All purified proteins were desalted with 5 ml of HiTrap Desalting columns (GE Healthcare) and concentrated with Amicon Ultra-15 centrifugal filter units (Merck Millipore). Recombinant *S. lithotrophicus* proteins were stored in 100 mM sodium acetate buffer pH 5 at −70 °C, *A. vinosum Cyt c₁* in 20 mM Tris-HCl buffer, pH 7.5, at 4 °C, *mpTsdBA* in 20 mM Tris-HCl buffer, pH 7.5, with 150 mM NaCl at −20 °C, and HiPIP in 20 mM Tris-HCl, pH 7, at 4 °C. The concentration of purified proteins was determined with the BCA kit from Pierce. For assessment of purity, SDS-PAGE was performed, and the proteins were visualized either by Coomassie or here staining techniques.

**UV-visible Spectroscopy with TsdA in Solution**—UV-visible spectra were recorded between 250 and 750 nm with an Analytical Jena Specord 210 (Analytical Jena, Jena, Germany).

**Assay of Thiosulfate Oxidase Activity with Ferricyanide**—Thiosulfate-dependent ferricyanide reduction was measured by following the decrease in absorbance at 420 nm (ε = 1.09 mM⁻¹ cm⁻¹). Enzyme activity measurements with AvTsdA at pH 4 are described in Ref. 2. Activity measurements with *mpTsdBA* were performed with 1 mM ferricyanide at 25 °C in 100 mM ammonium acetate buffer, pH 5.2, with 200 mM NaCl. Assays were started by addition of TsdA, and data were recorded in a Specord 210 spectrophotometer (Analytical Jena, Jena, Germany). Activity is expressed as micromoles of tetra-thionate produced per min and milligram of protein on the basis of one tetra-thionate formed per two ferricyanides reduced. In the case of enzymes that use two molecules of the same substrate (here thiosulfate) primary v versus [S] plots provide the best way to examine the data (41). Data were fitted to the empirical Hill equation (Equation 1) using GraphPad Prism (version 6; GraphPad).

\[
v = \frac{V_{\text{max}}[S]^n}{K + [S]^n} \quad \text{(Eq. 1)}
\]

The Hill equation resembles the classical Henri-Michaelis-Menten equation; however, the *n* term allows accounting for non-hyperbolic shapes. A substrate concentration [S]₀.₅ can be reported that yields half-maximal velocity and is characteristic of the process. The constant *K*, which is not equivalent to *Kₘ*, characterizes enzyme-substrate interaction. The relationship between *K* and [S]₀.₅ is *K = [S]₀.₅^n*.

**Assay of Thiosulfate Oxidase Activity with HiPIP**—For assays of electron transfer from thiosulfate to the electron acceptor HiPIP, 10 μM HiPIP preoxidized with 40 μM ferricyanide were used. The reaction was started by addition of enzyme and followed by the absorbance decrease at 480 nm. A molar extinction coefficient at 480 nm of 10.7 mM⁻¹ cm⁻¹ (10) was used. Measurements with AvTsdA were performed in 100 mM ammonium acetate buffer, pH 5, at 30 °C and with *mpTsdBA* in 100 mM ammonium acetate buffer, pH 5.2, with 200 mM NaCl at 25 °C.

**Assay of Thiosulfate Oxidase Activity with TsdB or AvCyt c₄**—Thiosulfate-dependent reduction of *T. intermedia TsdB* or *A. vinosum Cyt c₄* was measured by following the increase in absorbance at 417 nm (ε₄₃₁₇ nm = 99 mM⁻¹ cm⁻¹) for *TtTsdB* or *SITsdB* and at 420 nm (ε₄₄₂₀ nm = 55 mM⁻¹ cm⁻¹) (9) for *AvCyt c₄*. The extinction coefficient for TsdB was calculated with the help of the Beer–Lambert law using distinct concentrations of *TtTsdB* and the differences in absorbance at 417 nm in the reduced and oxidized spectra. A value averaged from measurements with three different protein concentrations was derived. Assays of *SITsdA* activity with *SITsdB* as electron acceptor were carried out in 100 mM ammonium acetate buffer, pH 4, at 25 °C. *AvTsdA* activity with *TtTsdB* was assayed in 100 mM ammonium acetate buffer, pH 5, at 30 °C, whereas *AvTsdA* activity with *AvCyt c₄* was determined in 100 mM ammonium acetate buffer, pH 5.5, at 25 °C.

**Determination of Redox Properties of AvTsdA Adsorbed on a Mesoporous Nanocrystalline SnO₂ Electrode**—An optically transparent mesoporous nanocrystalline SnO₂ electrode coated with AvTsdA was prepared using the previously described method (42) with adsorption from a solution of 10 μM AvTsdA, 2 mM neomycin, 50 mM NaCl, 50 mM HEPES, pH 7. The enzyme-coated electrode was rinsed with 2 mM neomycin, 50 mM NaCl, 50 mM HEPES, pH 7, to remove unbound protein, taken into a N₂-filled chamber (atmospheric O₂ < 2 ppm) and immersed in an anaerobic solution of the same composition within a previously described spectroelectrochemical cell (42). The cell was sealed, removed from the anaerobic chamber, and inserted into a Jasco V650 UV-visible spectrophotometer thermostated at 4 °C and flushed with argon to maintain anaerobic status. Spectral contributions from light
scattering by the electrode were minimized by placing a bare SnO\textsubscript{2} electrode (i.e. having no adsorbed enzyme) in the reference beam of the spectrophotometer. The electronic absorbance of the as prepared enzyme-coated electrode revealed features indicative of a mixture of ferric and ferrous hemes. After the electrode had been poised at $+302$ mV for 45 min, the spectrum revealed that the enzyme had been converted to the fully oxidized all ferric state. To determine the redox activity of AvTsdA, the electrode potential was swept from $+302$ to $-648$ mV at a scan rate of 5 mV s$^{-1}$ with a pause of 150 s every 50 mV. At 60 s into each pause, a spectrum was measured before the scan continued. Reoxidation of the sample was performed in a similar manner. Spectra are presented after equating absolute absorbance at 600 nm to account for potential dependent changes in the spectral contributions that arise from scattering by the electrode material.

**Redox Potentiometry with TsdB in Solution Measured with a Gold Electrode**—The reduction potential of TiTsdB was measured with help of a gold–platinum electrode system under anoxic conditions. The electrode extended into a cuvette containing the protein solution (20 \mu l) and redox mediators (\textit{N,N}-dimethyl-1,4-phenylenediamine, p-benzoquinone, trimethylhydroquinone, phenazine, 1,4-naphthoquinone, and 1,2-naphthoquinone at 2 \mu l each) in 20 mM MOPS buffer, pH 6, and was connected to a potentiometer. TiTsdB was reduced by changing the applied potential from $-150$ to 450 mV. Subsequent decrease of the potential again to $-150$ mV led to reoxidation of the protein. A spectrum was recorded every 2 min and potentials converted to values versus standard hydrogen electrode.

**Crystallography, Data Collection, Structure Determination, and Refinement—MpTsdBA at a concentration of 3.2 mg ml$^{-1}$ in 20 mM BisTris-HCl, pH 6.5, and 150 mM NaCl was crystallized in 10% (w/v) PEG 8000, 0.1 M Tris-HCl, pH 7.0, 0.2 M MgCl\textsubscript{2}, and 10 mM trimethylamine hydrochloride (as additive) by vapor-diffusion hanging-drop method at 20 °C. Crystallization droplets contained 1.0 \mu l of protein, 0.8 \mu l of precipitant, and 0.2 \mu l of additive and were equilibrated against a 200-\mu l reservoir solution (26% (w/v) PEG 3350). Crystals were cryoprotected with No. 2 solution of CryoProT\textsubscript{X} screen (Molecular Dimensions), consisting of 25% (v/v) diethylene glycol, 25% (v/v) 1,2-propanediol, and 25% (v/v) glycerol. X-ray diffraction data were collected at a wavelength of 1.7236 \AA{} on beamline ID-29 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Data were indexed and integrated with XDS (43), and the space group was determined with POINTLESS (44) and scaled with AIMLESS (45, 46), all within the autoPROC (47) data processing pipeline. An R$_{free}$ flag was created at this stage corresponding to 5% of the measured reflections of the data set. The structure was determined by single wavelength anomalous dispersion method around the iron edge (Fe-SAD), employing a high multiplicity data collection strategy using the autoSHARP module (48), within the SHARP package (49). Iterative model building and refinement cycles were performed with COOT (50) and BUSTER-TNT (51) (at early stages of refinement), followed by phenix.refine (52), until a complete model was built and refinement convergence achieved. Friedel mates were kept separately and refinement was carried out against l(+)/SIGl(+) ,l(−)/SIGl(−). A polder map (an omit map that excludes the bulk solvent around the omitted region) and mF$_{o}$ − mF$_{c}$ map was calculated within the PHENIX package of programs. The Ramachandran diagram was assessed with RAMPAGE (53), and the model was validated with MolProbity (54) as implemented in PHENIX. All figures were rendered with PyMOL, Schrödinger LLC (55).

**Author Contributions**—J. M. K., J. A. B., J. N. B., M. A., and C. D. wrote the manuscript. C. D. conceived and coordinated all experiments except the MpTsdBA crystallization and determination of reduction potentials. J. M. K. analyzed and compiled the data for those experiments. J. R. constructed the vector for production of MpTsdBA and T. F. the vector for production of HiPIP. A. F. and J. R. performed activity assays with MpTsdBA, AvTsdA, and HiPIP (Fig. 9; Table 2) and recorded UV-visible spectra of MpTsdBA (Fig. 6). T. K. produced AvCyt c and measured activity of AvTsdA with this protein. E. K. and J. M. K. produced and analyzed proteins from \textit{S. lithothrophicus} (Figs. 2 and 5). K. D. determined the redox activity of TiTsdB (Fig. 4) under the supervision of I. A. C. P. and S. F. R. examined the redox activity of AvTsdA (Fig. 1) under the supervision of J. N. B. J. A. B. crystallized MpTsdBA, processed the X-ray data, determined the crystal structure, and performed model building and refinement (Figs. 7 and 8; Table 1). J. A. B. and M. A. analyzed the crystal structure.

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