Solution NMR Structure and Functional Analysis of the Integral Membrane Protein YgaP from Escherichia coli

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Background: E. coli YgaP is a membrane-bound sulfurtransferase with a cytoplasmic rhodanese domain.
Results: The three-dimensional structure is composed of a cytoplasmic rhodanese domain and two transmembrane helices forming the interface of the homodimer.
Conclusion: The structure-activity relationship of YgaP suggests a sulfurtransferase activity.
Significance: YgaP may have a role in the detoxification of CN− to the less toxic SCN−.

The solution NMR structure of the α-helical integral membrane protein YgaP from Escherichia coli in mixed 1,2-dihexanoyl-sn-glycerol-3-phosphocholine/1-myristoyl-2-hydroxy-sn-glycerol-3-phospho-(1'-rac-glycerol) micelles is presented. In these micelles, YgaP forms a homodimer with the two transmembrane helices being the dimer interface, whereas the N-terminal cytoplasmic domain includes a rhodanese-fold in accordance to its sequence homology to the rhodanese family of sulfurtransferases. The enzymatic sulfur transfer activity of full-length YgaP as well as of the N-terminal rhodanese domain only was investigated performing a series of titrations with sodium thiosulfate and potassium cyanide monitored by NMR and EPR. The data indicate the thiosulfate concentration-dependent addition of several sulfur atoms to the catalytic Cys-63, which process can be reversed by the addition of potassium cyanide. The catalytic reaction induces thereby conformational changes within the rhodanese domain, as well as on the transmembrane α-helices of YgaP. These results provide insights into a potential mechanism of YgaP during the catalytic thiosulfate activity in vivo.

YgaP has a molecular mass of 18.6 kDa and is composed of 174 amino acid residues, of which 119–174 are predicted by the membrane protein topology prediction method TMHMM (1) to form two transmembrane helices, whereas residues 1–118 are predicted to form a cytoplasmic rhodanese domain with sulfurtransferase activity (2, 3). YgaP therefore belongs to the family of rhodanese (thiosulfate-cyanide sulfurtransferase), which is a large superfamily of enzymes found in bacterial, archaeal, and eukaryotic cells. Escherichia coli encodes eight rhodanese-containing proteins, and we studied the enzymatic activity of its rhodanese domain. These enzymes are supposed to have sulfurtransferase activity (3). Rhodanese domain-containing proteins provide both eukaryotic and prokaryotic organisms with labile reactive sulfides. Because sulfur is a functionally important element of living matter, enzymes that involve sulfur transfer reactions generally are of great importance for the homeostasis of the cell and the organism (5). Hence, it is believed that rhodanese-containing proteins are fundamental for various cellular processes, including a response to oxidative stress and cellular redox homeostasis (5). In addition to these physiological functions, rhodanese-containing proteins are also involved in the elimination of toxic cyanogenic compounds (5). Cyanide is a toxic chemical compound, which is synthesized, excreted, and metabolized by many organisms, including bacteria, fungi, plants, and insects to avoid predation or competition (6). Rhodanese-containing proteins play a role in the detoxification of cyanide by catalysis of the sulfane sulfur from thiosulfate to cyanide, leading to the formation of the less toxic thiocyanate (5, 6).

An active site cysteine in a six-amino acid residue loop of the rhodanese domain is thereby essential for the sulfurtransferase activity. The enzymatic reaction takes place in two steps. In the first step, the thiol group of the cysteine reacts with the thiosulfate ion (S2O3−) to form an enzyme-persulfide intermediate, which reacts in a second step with the cyanide ion to produce thiocyanate (SCN−) (7–9). Although the crystal structure of the sulfur-free and the persulfide form of one member of the rhodanese family (GlpE) as well as the solution state NMR structure of another member (PspE) have been determined (3, 4, 10), the molecular mechanism of the sulfurtransferase activity remains unclear (11), but the dynamics appear to be involved (3).

Toward an understanding of the mechanism of action, we determined the three-dimensional structure of the α-helical membrane protein YgaP from E. coli by solution state NMR, and we studied the enzymatic activity of its rhodanese domain.

EXPERIMENTAL PROCEDURES

Sample Preparations—To simplify the resonance assignment of full-length YgaP, we designed a construct expressing the cytoplasmic rhodanese domain alone, hereafter called rhod-

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The atomic coordinates and structure factors (codes 2MOI, 2MOL, and 2MPN) have been deposited in the Protein Data Bank (http://wwpdb.org/).
NMR Structure of YgaP

The abbreviations used are: TROSY, transverse relaxation optimized spectroscopy; ct, constant time; PRE, paramagnetic relaxation enhancement; H-bond, hydrogen bond; r.m.s.d., root mean square deviation; DEER, double electron electron resonance; MTSL, 1-oxyl-(2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate; DML, 1-acetyl-(2.2.5.5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate; LMG, 1-myrystoil-2-hydroxy-sn-glycerol-3- phospho-(1'-rac-glycerol); TM, transmembrane α-helix; BisTris, 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl)propane-1,3-diol; DHPC-7, 1,2-dihexanoyl-sn-glycerol-3-phosphocholine.

3 The abbreviations used are: TROSY, transverse relaxation optimized spectroscopy; ct, constant time; PRE, paramagnetic relaxation enhancement; H-bond, hydrogen bond; r.m.s.d., root mean square deviation; DEER, double electron electron resonance; MTSL, 1-oxyl-(2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate; DML, 1-acetyl-(2.2.5.5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate; LMG, 1-myrystoil-2-hydroxy-sn-glycerol-3-phospho-(1'-rac-glycerol); TM, transmembrane α-helix; BisTris, 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl)propane-1,3-diol; DHPC-7, 1,2-dihexanoyl-sn-glycerol-3-phosphocholine.

NMR Spectroscopy, Sequential Assignment, Distance Restraint Collection, and Detergent-Protein NOE—NMR experiments were performed on a Bruker 700 MHz Avance III spectrometer equipped with a cryoprobe. Sequential 4HN, 13C, and 15N backbone resonances of 13C,15N-labeled rhodanese-F were assigned using triple resonance three-dimensional constant time (ct)-TROSY3-HNCA, three-dimensional ct-TROSY-HNCACB (13), and three-dimensional ct-TROSY-HN(CO)CA (14) experiments. The side-chain 4H resonances were assigned using three-dimensional ct-HCCH-total correlation spectroscopy (mixing time (τm) = 21.7 ms) (15) and three-dimensional 15N-resolved 4H,4H total correlation spectroscopy (τm = 46.6 ms) (16) experiments. Therefore, the previously determined 13C and 4H chemical shifts were used as starting points for the analysis (16). Ambiguities were resolved by three-dimensional 13C/15N-resolved 4H,4H NOE experiments (τm = 60 ms) (17, 18). This approach yielded an almost complete sequential assignment with the exception of Gin-64. Interesting to note is the 1H chemical shift of Arg-24 (9.13 ppm), which indicates that Arg-24 forms a salt bridge. Based on the three-dimensional structure, the salt bridge could be with Glu-28 (Fig. 1A).

An important step in the sequential assignment of YgaP− began with mapping the backbone assignment of rhodanese-F onto the spectra of full-length 2H (~50%), 13C,15N-labeled YgaP+. The sequential backbone assignment of YgaP− was mainly obtained from the three-dimensional ct-TROSY-HNCA. Ambiguities were resolved with the three-dimensional ct-TROSY-HN(CO)CA. The sequential amide-amide NOE connectivities (19) in the three-dimensional 15N-resolved 4H,4H NOE experiments (τm = 100 ms) were used to check and to fix ambiguities in the backbone assignment of the transmembrane domain of YgaP−. Although the membrane protein—typical high percentage (here 31%) of the hydrophobic residues isoleucine, leucine, and valine within the transmembrane helices of YgaP− combined with the lack of 13Cβ chemical shifts posed a challenge for the sequential assignment, the combination of triple resonance and NOE experiments resulted in a nearly complete backbone 1H,13Cα, and 15N resonance assignment. However, the backbone assignments of Ile-124, Leu-125, Val-128, Leu-130, Leu-140, and Leu-141 could not be obtained from the procedure described above. The lack of these resonances could result from conformational exchange dynamics. For example, residues 140 and 141 are in the loop between the two transmembrane α-helices, which may undergo conformational exchange due to a potential interaction of the loop with the micelle surface. Assignment of the missing 4HN, 13Cα, and 15N resonances for the remaining above-mentioned residues could not be obtained from the three-dimensional 15N-resolved 4H,4H NOE spectrum due to severe NOE cross-peak overlap between rhodanese and transmembrane domains close to the diagonal amide proton region and/or due to NOE peak overlap within the two transmembrane helices. A summary of the NMR data obtained for YgaP− is shown schematically in Fig. 2A. Identification of the secondary structure elements was based on the 13Cα chemical shift deviations from the corresponding random coil chemical shifts (20) and the characteristic 4HN,4H NOE connectivities (19). Overall, the collected NMR data indicate the presence of two transmembrane helices, residues Met-113–Phe-138 and residues Ser-142–Met-167 (Fig. 2A). Additionally, the presence of exchange NOE cross-peaks between amide protons and the solvent (water) resonance (4H−H2O cross-peaks in the three-dimensional 15N-resolved 4H,4H NOE spectrum) was analyzed. The shielding from the solvent of these amide protons suggests either involvement in hydrogen bonding, and/or being in a water-free environment, such as the interior of the protein–detergent complex. The difference between the chemical shifts of the amide protons and the corresponding random coil values (19) Δδ4H2O was used for a more detailed characterization of the two transmembrane helices. The 4H chemical shifts were referenced to the water signal at 30 °C according to δ(H2O) = 7.83 − T/96.9 ppm (T is shown in K) (16). Δδ4H2O changed in a periodic pattern along the amino acid sequence within the two transmembrane α-helices. The spacings between the minima/maxima were observed for each third or fourth residue (Fig. 2A). This characteristic periodicity is consistent with the results of Kuntz et al. (21), Shenkarev et al. (22), and Zhou et al. (23) that amide protons in α-helices show a 3–4 repeat periodicity in 4H chemical shifts.

The side chain assignment of rhodanese-F was used as a starting point in the analysis of the three-dimensional 13C-re insolved 4H,4H NOE and three-dimensional 13C-resolved 4H,4H NOE (τm = 100 ms) of a 2H (~50%), 13C,15N, and a 13C,15N-labeled YgaP− sample. In the 13C-resolved 4H,4H NOESY spectrum, the 6 mm protonated 1,2-dihexanoyl-sn-glycerol-3-phosphocholine (DHPC-7) gave rise to strong t1 noise in the methyl spectral region, which masked vital information. Despite applying a 1-ms spin-lock pulse at the 4H carrier frequency prior to acquisition (24) to suppress signals originating from the detergent, the spectrum remained unreadable in some areas of interest. Consequently, only partial side-chain assignment of the transmembrane domain of YgaP− was achieved, and the side-chain assignment of the rhodanese domain in YgaP− was limited. Additionally, fast T2 relaxation in a 13C,15N-labeled sample caused severe signal loss in the transmembrane region of YgaP− (Fig. 3, C and D).

For the identification of intermolecular NOEs between neighboring subunits, a mixed sample was prepared with a 1:1

JOURNAL OF BIOLOGICAL CHEMISTRY 23483
NMR Structure of YgaP

A

B

ω₂ (1H) [ppm]

ω₁ (15N) [ppm]
ratio of the $^2$H,$^{15}$N-labeled protein and the unlabeled protein. A three-dimensional $^{15}$N-resolved $^1$H,$^1$H NOESY spectrum ($t_m = 220$ ms, time domain data size 128:32:2048 complex points, $t_{1\text{max}}(\text{H}) = 12.8$ ms, $t_{2\text{max}}(\text{N}) = 14.4$ ms, $t_{3\text{max}}(\text{H}) = 204.9$ ms) was recorded with this mixed sample. Because in this spectrum there is a lack of strong cross-peaks observed in the NOESY spectra of homogenously labeled samples, it could be excluded that the NOE cross-peaks observed in the mixed sample are based on residual protonation (Table 1).

Additional intermolecular distance constraints were derived from paramagnetic relaxation enhancement (PRE) measurements on the YgaP(C63D/C158S) variant labeled with the thiol-reactive nitroxide 1-oxyl-(2,2,5,5-tetramethyl-$\Delta_3$-pyrroline-3-methyl) methanethiosulfonate (MTSL) at the position of the natural Cys-158 mixed with a $^3$H (50%), $^{15}$N-labeled full-length YgaP(C63D/C158S) sample in a ratio of 1:1. Two-dimensional $^{15}$N,$^1$H TROSY spectra (time domain data size 256:2048 complex points, $t_{1\text{max}}(\text{N}) = 44.8$ ms and $t_{2\text{max}}(\text{H}) = 204.9$ ms) under paramagnetic MTSL and diamagnetic 1-acetyl-(2,2,5,5-tetramethyl-$\Delta_3$-pyrroline-3-methyl)-methanethiosulfonate (DML, both from Toronto Research Chemicals) conditions were then acquired yielding only intermolecular PRE data. The PRE data were assigned to three different categories as follows: $^{15}$N,$^1$H cross-peaks showing a paramagnetic/diamagnetic intensity ratio of <15% were translated to corresponding PRE upper limit distance restraints of 9–12 Å. Peaks with an intensity ratio between 15 and 85% were translated to upper limit distance restraints of 14–18 Å (25, 26). The atoms involved in the distance restraints were the C\textalpha atom of the spin-labeled Cys-158 and the hydrogen atom of the $^{15}$N,$^1$H moieties of the affected cross-peaks (Table 2).

The interaction of YgaP\textsuperscript{-} with DHPC-7 was studied using the three-dimensional $^{15}$N-resolved $^1$H,$^1$H NOESY spectra ($t_m = 220$ ms) recorded with the $^2$H,$^{15}$N-labeled YgaP\textsuperscript{-} mixed with the unlabeled YgaP\textsuperscript{-} in a ratio of 1:1 in DHPC-7/LMPG (1-myristoyl-2-hydroxy-sn-glycero-3-phospho(1’-rac-glycerol)) micelles at 30 °C, pH 7.0 (Table 3). No intermolecular NOEs between the amide protons and LMPG were observed, probably due to the low concentration of LMPG when compared with DHPC-7. The resonance assignment of DHPC-7 in a 6:1 mixture with LMPG was obtained from a one-dimensional $^1$H NMR spectrum with the hydrophobic \text{CH$_3$} groups at 0.82 ppm, the \text{CH$_3$} groups of the chain at 1.21, 1.52, and 2.30 ppm, and the choline methyl resonances at 3.20 ppm. The proton chemical shifts were referenced to the water signal at 30 °C according to $\delta$(H$_2$O) = 7.83 ppm – $T$/96.9 ppm = 4.70 ppm ($T$ is shown in K). It has to be mentioned that for some residues data were not available or were ambiguous due to NOE peak overlap between the cytoplasmic and the transmembrane domain of YgaP\textsuperscript{+}.

**Measurement of $^{15}$N Relaxation in Rhodanese-F and YgaP\textsuperscript{+}**—Steady-state heteronuclear $^{15}$N-$^1$H NOE data (27), $^{15}$N relaxation parameters of longitudinal relaxation rates ($R_1$) (28), and transverse relaxation rates ($R_2$) (29) in the rotating frame were measured and analyzed from a $^{13}$C,$^{15}$N-labeled rhodanese-F and a $^2$H (50%), $^{15}$N-labeled YgaP\textsuperscript{+} sample. TROSY-based $^{15}$N-$^1$H NOE experiments (time domain data size 128:2048 complex points, $t_{1\text{max}}(\text{N}) = 28.8$ ms, $t_{2\text{max}}(\text{H}) = 204.9$ ms, relaxation delay 2.0 s) were performed in the presence and absence of a 3-s proton presaturation period prior to the $^{15}$N excitation. A total of 16 transients per increment were collected in each experiment. Steady-state NOE values were determined by the ratio of cross-peak heights elucidated from cross-sections through the spectra parallel to the $^{15}$N dimension acquired with and without $^1$H presaturation during the recycle delay. For $R_1$ and $R_{1\text{ip}}$, spectra with 64:2048 complex time domain data points ($R_1$, $R_{1\text{ip}}, t_{1\text{max}}(\text{N}) = 14.4$ ms, $t_{2\text{max}}(\text{H}) = 204.9$ ms) were measured with eight transients and a recycle delay of 1.0 s. $R_1$ and $R_{1\text{ip}}$ relaxation rate constants were obtained by fitting the logarithm of peak intensities to a linear function by the least square method using the following relaxation periods $T$ for $R_1$ and $R_{1\text{ip}}$: $T = 40, 91, 191, 293, 394, 594, 796, and 997$ ms and 10, 20, 30, 40, 50, 60, 80, and 100 ms, respectively.

**Structure Determination**—Three-dimensional structures of rhodanese-F were calculated using the established procedures having seven cycles of the iterative CANDID approach (30) with 100 dihedral angle constraints for the backbone angles $\phi$ and $\psi$ derived from C\textnu chemical shifts (31, 32) added as input for each cycle of the structure calculation (Table 4). The structure of full-length YgaP\textsuperscript{+} was calculated for the rhodanese domain (residues 1–108) and the transmembrane domain (residues 106–173) separately. The structure determination of the rhodanese domain in full-length YgaP\textsuperscript{+} was calculated as described above for rhodanese-F. In contrast, a two-step approach was used for the structure calculation of the transmembrane domain of full-length YgaP\textsuperscript{+}. First, the monomer structure (Table 5) was calculated using the backbone torsion angle restraints, distance constraints derived from NOEs, and hydrogen bond constraints. The backbone torsion angle restraints were derived from the $^{13}$C\textnu chemical shifts (32). The sequential and medium range NOEs were measured in the three-dimensional $^{15}$N-resolved $^1$H,$^1$H NOESY spectrum, whereas the intramolecular long range NOEs were obtained from the three-dimensional $^{13}$C/$^{15}$N-resolved $^1$H,$^1$H NOESY spectra. The following intramolecular inter-helical NOE restraints were obtained from the three-dimensional $^{13}$C/$^{15}$N-resolved $^1$H,$^1$H NOESY spectra: Gln-108(C$_\text{H}$)–Met-167(H\textnu); Gln-108(C$_\text{H}$)–Trp-169(H\textnu); Leu-110(C$_\text{H}$)–Trp-169(H\textnu); Leu-110(C$_\text{H}$)–Leu-169(H\textnu); Leu-110(C$_\text{H}$)–Leu-169(H\textnu); Leu-110(C$_\text{H}$)–Trp-169(C$_\text{H}$); Leu-110(C$_\text{H}$)–Ile-118(C$_\text{H}$)–Asp-165(H\textnu); Ile-118(C$_\text{H}$)–Ile-118(C$_\text{H}$)–Asp-165(H\textnu); Gly-122(H\textnu)–Ile-154(C$_\text{H}$); Gly-122(H\textnu)–Ile-154(C$_\text{H}$); Gly-131(H\textnu)–Ala-147(C$_\text{H}$); and Tyr-132(H\textnu)–Ala-147(C$_\text{H}$).
NMR Structure of YgaP

In addition, hydrogen bond (H-bond) restraints were added if α-helix-typical $^{13}$C$\alpha$ chemical shifts were observed; helix-typical NOEs were present in the three-dimensional $^{15}$N-resolved $^1$H,$^1$H NOESY ($\tau_m = 100$ ms), and if there was no exchange cross-peak between the amide proton and the $H_2O$ signal in the three-dimensional $^{15}$N-resolved $^1$H,$^1$H NOESY ($\tau_m = 100$ ms) spectrum. Following these requests, α-helical H-bond restraints for residues 114–137 and 144–167 were used for the structure calculation.

For the structure calculation with CYANA, the transmembrane YgaP$^-\$ dimer (Table 6) was constructed by connecting two YgaP$^-$ monomer polypeptide chains with a linker of 200 pseudo-residues. To obtain an initial set of dimer structures, two copies of the upper limit distance restraints, the dihedral
angle restraints and the H-bond restraints, used for the structure calculation of the YgaP monomer, were applied in the CYANA protocol. All NOE cross-peaks from the spectra measured with the $^1$H perdeuterated, $^{15}$N-labeled YgaP mixed with unlabeled YgaP were classified as intermolecular restraints (Table 1). In addition, PRE data obtained from $^2$H ($\sim 50\%$), $^{15}$N-labeled YgaP(C63D/C158S) mixed with the paramagnetic spin-labeled YgaP(C63D) variant at position Cys-158 were used as intermolecular restraints (Table 2). For each experimental intermolecular restraint between two adjacent subunits, two identical distance restraints were assigned, respectively, to the pair of neighboring subunits to satisfy the condition of a C2 rotational symmetry (Fig. 2, D and E). This yields a set of $295 \times 2$ intra- and $15 \times 2$ intermolecular distance restraints derived from NOEs, $8 \times 2$ intermolecular PRE restraints, $74 \times 2$ dihedral angle restraints, $40 \times 2$ H-bond restraints, and $4556 \text{C}^\alpha-\text{C}^\alpha$ distance difference restraints based on equivalent contacts to calculate the three-dimensional structure (Table 6). A total of 100 conformers were calculated. The ensemble of the 10 conformers with the lowest residual target functions represents the three-dimensional structure of the dimeric transmembrane domain of YgaP$^-$. The structures were deposited in the Protein Data Bank with the 2MOI code for rhodanese-F, the 2MOL code for the rhodanese domain of YgaP$^-$, and the 2MPN code for the transmembrane region of YgaP$^-$, respectively.

**NMR Titration Studies, Catalytic Thiosulfate Activity of Rhodanese-F, Full-length YgaP, and YgaP^-**—Established rhodanese in vitro assays use thiosulfate as a substrate and cyanide as an acceptor to regenerate the catalytic Cys (3). Following the general principle of these assays, the potential enzymatic activity of $^{13}$C,$^{15}$N-labeled rhodanese-F (in a buffer containing 20 mM Bis-Tris-HCl, pH 7.0, 97% H$_2$O, 3% D$_2$O in absence of tris(2-carboxyethyl)phosphine) was studied at 30 °C by performing series of titrations with 1–4 mM sodium thiosulfate. The absence of cross-peaks is due to solvent protection and is indicative of the presence of hydrogen bonds and/or a location within the transmembrane helical structure. Chemical shift differences indicate helical/β-strand conformation, respectively. The random coil values are taken from Ref. 20. 12th line, $\Delta^1_{1\text{H}}$N(black), difference between the chemical shifts of the amide protons in the two transmembrane helices with their corresponding random coil value (19). The observed periodicity of $\Delta^1_{1\text{H}}$N is indicative of an amphipathic helical structure. Chemical shift differences $\Delta^3_{1\text{H}}$N(NaSCN) (red) between native YgaP$^-$ and NaSCN-treated YgaP$^-$ are indicated. 13th line, solvent accessibility of $^1$H$_2$O (asterisks) of the transmembrane region determined by the presence of an exchange cross-peak between the frequency of water ($^1$H$_2$O = 4.70 ppm) and the $^1$H frequencies of the amide groups in the three-dimensional $^{15}$N-resolved $^1$H$_2$H NOESY spectrum with a mixing time $\tau_m = 220$ ms of a $^2$H-perdeuterated, $^{15}$N-labeled YgaP$^-$ sample mixed with an unlabeled YgaP$^-$ sample in a ratio of 1:1. The presence of cross-peaks indicates fast exchange of $^1$H$^\alpha$ with the solvent. The absence of cross-peaks is due to solvent protection and is indicative of the presence of hydrogen bonds and/or a location within the transmembrane region protected from solvent. The elucidated secondary structure elements as determined by NMR are shown above the protein sequence, where residues with atoms colored gray lack available data due to signal overlap or missing resonance assignment.
the three-dimensional structure was determined following standard protocols (see under “Experimental Procedures”). The three-dimensional structure of rhodanese-F has a root mean square deviation (r.m.s.d.) of 0.63 Å for the backbone atoms of residues 2–101 (Fig. 4, A and B), small residual constraint violations in the final set of the 10 conformers with the lowest values of the CYANA target function, and small deviations from ideal geometry (Table 4). The input data therefore
represent a self-consistent set, and the restraints are well satisfied in the calculated conformers.

Rhodanese-F adopts the typical fold of a single α/β rhodanese domain (Fig. 4, A and B) as predicted by the significant sequence homology between rhodanese-F and GlpE/PspE. The structure of rhodanese-F consists of five α-helices (residues 10–13 (α1), 27–31 (α2), 42–45 (α3), 70–80 (α4), and 93–97 (α5)) and five β-strands (residues 4–5 (β1), 19–22 (β2), 38–39 (β3), 59–62 (β4), and 84–88 (β5)). The twisted arrangement of the β-strands forms a central parallel β-sheet. The three α-helices α1, α2, and α5 are located on one side of the β-sheet, and the other two α-helices α3 and α4 are found on the other side (Fig. 4A). This “α/β-sandwich” thereby adopts the form of a hemisphere.

The catalytic residue Cys-63 is the first residue of the active loop Cys-63–Thr-69, which links β-strand β4 and helix α4 (Fig. 4C). Similar to GlpE and PspE (3, 4), the active loop folds in a semicircular cradle-like conformation centered around the Cys-63 Sγ atom defining the enzyme catalytic pocket. All backbone amide protons of residues Glu-64–Thr-69 point toward the Sγ atom of Cys-63, although their side chains point away from the catalytic pocket (Fig. 4C). The backbone amide protons of residues 64–69 from the active loop are in hydrogen bonding distances from the Sγ atom of Cys-63 in the final set of the 10 best CYANA conformers. The minimal Sγ-1HN distances in the set are 2.55 Å (Gln-64), 2.75 Å (Ala-65), 2.96 Å (Gly-66), 2.57 Å (Lys-67), 2.52 Å (Arg-68), and 3.05 Å (Thr-69) (Fig. 4C). Thus, similar to other rhodaneses, the active site}

TABLE 1

| H-peptide, 15N-labeled YgaP- | Unlabeled YgaP- | Upper limit distance restraint |
|-----------------------------|----------------|-----------------------------|
| Gln-108(H*)                 | Gln-108(H*)    | 13.0                        |
| Arg-114(H*)                 | Arg-114(H*)    | 9.0                         |
| Gln-117(H*)                 | Gln-117(H*)    | 9.0                         |
| Ile-118(H*)                 | Ile-118(H*)    | 13.0                        |
| Ala-120(H*)                 | Ala-120(H*)    | 13.0                        |
| Gly-124(H*)                 | Gly-124(H*)    | 13.0                        |
| Gly-131(H*)                 | Gly-131(H*)    | 9.5                         |
| Tyr-132(H*)                 | Tyr-132(H*)    | 9.0                         |
| Thr-133(H*)                 | Thr-133(H*)    | 9.5                         |
| Val-134(H*)                 | Val-134(H*)    | 13.0                        |
| Val-134(H*)                 | Val-134(H*)    | 13.0                        |
| Asn-135(H*)                 | Asn-135(H*)    | 9.0                         |
| Leu-146(H*)                 | Leu-146(H*)    | 13.5                        |
| Asp-165(H*)                 | Asp-165(H*)    | 13.0                        |

TABLE 2

| H- (50%), 15N-labeled YgaP (C63D/C158S) | MTSL-labeled YgaP(C63D) | Upper limit distance restraint |
|----------------------------------------|-------------------------|-----------------------------|
| Leu-110(H*)                            | Cys-158(C63)            | 18.0                        |
| Leu-112(H*)                            | Cys-158(C63)            | 14.0                        |
| Met-113(H*)                            | Cys-158(C63)            | 12.0                        |
| Gln-117(H*)                            | Cys-158(C63)            | 9.0                         |
| Ala-120(H*)                            | Cys-158(C63)            | 11.0                        |
| Gly-121(H*)                            | Cys-158(C63)            | 9.0                         |
| Gly-122(H*)                            | Cys-158(C63)            | 11.0                        |
| Gly-127(H*)                            | Cys-158(C63)            | 11.0                        |

TABLE 3

| Secondary structure element | Residue | NOE with H2O | NOE with DHCPC-7 |
|-----------------------------|---------|--------------|------------------|
| Lys106                      | +       | -            | +                |
| Ser107                      | +       | -            | +                |
| Gly108                      | +       | -            | +                |
| Pro109                      | -       | +            | +                |
| Leu110                      | -       | +            | +                |
| Pro111                      | +       | -            | -                |
| Leu112                      | +       | -            | -                |
| Met113                      | -       | -            | -                |
| Arg114                      | -       | -            | -                |
| Glu115                      | -       | -            | -                |
| Val116                      | -       | -            | -                |
| Gly117                      | +       | -            | -                |
| His118                      | +       | -            | -                |
| Ala119                      | +       | -            | -                |
| Ala120                      | +       | -            | -                |
| Gly121                      | -       | +            | -                |
| Gly122                      | +       | -            | -                |
| Leu123                      | +       | -            | -                |
| His124                      | -       | -            | -                |
| Ala125                      | +       | -            | -                |
| Ala126                      | -       | -            | -                |
| Gly127                      | -       | -            | -                |
| Val128                      | -       | -            | -                |
| Val129                      | -       | -            | -                |
| Leu130                      | -       | -            | -                |
| Gly131                      | -       | -            | -                |
| Tyr132                      | +       | -            | +                |
| Thr133                      | -       | -            | -                |
| Val134                      | +       | -            | +                |
| Asn135                      | +       | -            | -                |
| Ser136                      | -       | -            | -                |
| Gly137                      | +       | -            | -                |
| Phe138                      | -       | -            | -                |
| Leu140                      | -       | -            | -                |
| Leu141                      | -       | -            | -                |

TABLE 4

| Quantity                  | Hydrogen bonds | NOE distances | Intra-residue | Medium range | Long range |
|---------------------------|----------------|---------------|--------------|--------------|------------|
| Number                    | 0              | 481           | 478          | 408          | 506        |
| Dihedral angles           | 100            |               |              |              |            |

Residual upper limit constraint violations
Maximun 0.16 ± 0.11 Å
Residual dihedral angle constraint violations
0
Backbone r.m.s.d.
Residues 2–101 0.63 Å
Heavy atom r.m.s.d.
Residues 2–101 1.04 Å
Ramachandran plot
Residues in most favored regions 56%
Residues in additionally allowed regions 35%
Residues in generously allowed regions 6%
Residues in disallowed regions 3%
appears to be stabilized by a radial hydrogen-bonding network (3, 4, 36). This network is critical for the folding of rhodanese-F, because a replacement of Cys-63 with Ala did result in a misfolded rhodanese-F as evidenced by NMR experiments (data not shown). The presence of positively charged (Lys-67 and Arg-68) and polar (Gln-64 and Thr-69) residues in the active loop of rhodanese-F suggests that this domain of YgaP (see below), 15N spin relaxation rates \( R_{1h}, R_{1h'} \) and heteronuclear 15N-1H NOEs were measured (Fig. 5). Because of overlap in the two-dimensional 15N,1H correlation spectra and insufficient sensitivity in the case of the residues close to the active site, relaxation data were not obtained for all 15N-1H moieties. The uniform distribution of the \( R_{1h}, R_{1h'} \) and 15N-1H NOE values implies an overall quite rigid molecular architecture of rhodanese-F as determined previously for PspE (3). Decreased \( R_{1h} \) and \( R_{1h'} \) values and low 15N-1H NOEs indicate the presence of motion in the N- and C-terminal regions of the molecule and in the loops connecting \( \alpha \)-helix \( \alpha_3 \), \( \beta \)-strand \( \beta_4 \), and \( \alpha \)-helix \( \alpha_4 \). Upon further analysis using the model-free method (37), this interpretation was confirmed by reduced order parameters indicating the presence of picosecond-nanosecond motions (Fig. 5D). A correlation time \( \tau_c \) of 7.5 ns was obtained from 15N relaxation measurements (27). This value is in accordance with the size and shape of a monomeric rhodanese-F.

Structural Comparison between Rhodanese-F of YgaP with GlpE and PspE—The structure of rhodanese-F is very similar to the single rhodanese domain GlpE from \( E. coli \) (Fig. 4D). The following major structural differences to GlpE were observed. The \( \alpha \)-helices \( \alpha_1, \alpha_3, \) and \( \alpha_5 \) in rhodanese-F were shorter than in GlpE, and two short \( \beta \)-strands, after the second helix and on the C terminus of GlpE forming a small anti-parallel \( \beta \)-sheet, are absent in the three-dimensional structure of rhodanese-F. Comparison of rhodanese-F with the single rhodanese domain PspE from \( E. coli \) reveals the following major structural differences. The first N-terminal \( \beta \)-strand and \( \alpha \)-helix are absent in PspE; the C-terminal \( \alpha \)-helix is replaced by a loop in PspE; PspE has the second antiparallel \( \beta \)-sheet formed by two short

### Table 5
Experimental NMR data and structural statistics of the cytoplasmic rhodanese domain and the transmembrane monomer YgaP

| Quantity | Rhodanese domain | YgaP\(^{-}\) monomer |
|----------|------------------|---------------------|
| Restraints Hydrogen bonds | 40 × 2 | 79 × 2 |
| NOE distances Intra-residue | 117 × 2 | 23 × 2 |
| Short range | 76 × 2 | 20 × 2 |
| Medium range | 0.27 ± 0.12 Å | 0.26 ± 0.24 Å |
| Long range | 0.99 Å | 1.54 Å |
| Heavy atom r.m.s.d. | 62% | 76% |
| Backbone r.m.s.d. | 33% | 20% |
| Residues in most favored regions | 4% | 3% |
| Residues in generously allowed regions | 1% | 1% |
| Residues in disallowed regions | 1% | 1% |
| Dihedral angles | 8 | 4,556 |
| Spin label restraints (PRE) | | |
| C-C distance difference restraints | | |

### Table 6
Experimental NMR data and structural statistics of the transmembrane homodimer of YgaP

| Quantity | YgaP homodimer |
|----------|----------------|
| Quantity | | |
| Restraints Hydrogen bonds | 40 × 2 | 79 × 2 |
| NOE distances Intra-residue | 117 × 2 | 23 × 2 |
| Short range | 76 × 2 | 20 × 2 |
| Medium range | 0.27 ± 0.12 Å | 0.26 ± 0.24 Å |
| Long range | 0.99 Å | 1.54 Å |
| Heavy atom r.m.s.d. | 62% | 76% |
| Backbone r.m.s.d. | 33% | 20% |
| Residues in most favored regions | 4% | 3% |
| Residues in generously allowed regions | 1% | 1% |
| Residues in disallowed regions | 1% | 1% |
| Dihedral angles | 8 | 4,556 |
| Spin label restraints (PRE) | | |
| C-C distance difference restraints | | |
NMR Structure of YgaP
β-strands similar to GlpE but absent in rhodanese-F; helix α3 is seven residues shorter in rhodanese-F, and β-strands β4 and β5 in rhodanese-F are shorter than in PspE.

In summary, on the one hand, rhodanese-F represents a typical single rhodanese domain with a higher similarity to the single rhodanese domain GlpE than to PspE, which lacks some

FIGURE 4. Solution NMR structure of rhodanese-F. A, ribbon representation of the three-dimensional structure of rhodanese-F. For the ribbon representation, the mean solution NMR structure was calculated from the 10 CYANA conformers with the lowest residual target functions used to represent the three-dimensional structure. The α-helices are colored red, the β-strands gray, and the remaining regions in gold, respectively. 1–5 indicate the sequential order of the α-helices and the β-strands, respectively. B, backbone bundle representation of rhodanese-F. The 10 CYANA conformers with the lowest residual target functions were superimposed by their Cα atoms of residues 2–101. C, catalytic site of rhodanese-F. The polypeptide backbone (gray) and the side chains of the residues forming the catalytic pocket (His-62–Thr-69) are shown in red (oxygen), yellow (sulfur), blue (nitrogen), green (selected backbone amide protons), and gray (proton), respectively. Broken cyan cylinders indicate the hydrogen-bonding network between the Cys-63 S atom and the backbone amide protons of Gln-64–Thr-69. D, structural comparison of rhodanese-F with the single rhodanese domain GlpE and PspE. Ribbon representation of rhodanese-F (red), GlpE (yellow), and PspE (gray) is shown. The N and C termini are identified.

β-strands similar to GlpE but absent in rhodanese-F; helix α3 is seven residues shorter in rhodanese-F, and β-strands β4 and β5 in rhodanese-F are shorter than in PspE.

In summary, on the one hand, rhodanese-F represents a typical single rhodanese domain with a higher similarity to the single rhodanese domain GlpE than to PspE, which lacks some

FIGURE 5. 15N relaxation measurements of rhodanese-F, YgaP, and multilayer static light scattering of YgaP in DHPC-7/LMPG micelles. R1 rates (A), R1p rates (B), steady-state 15N-{1H} NOEs (C), and 15N generalized order parameters S2 (D) as a function of residue position of a 13C,15N-labeled rhodanese-F sample (2–20 mM). The residues displaying 15N-{1H} NOE <0.8 are subjected to extensive motions on the picosecond to nanosecond time scale. The secondary structure elements as determined by NMR are depicted above the graphs, with β-strands shown as filled arrows and α-helices shown as open rectangles. E and F, 15N relaxation measurements of YgaP reveal dynamic properties of full-length YgaP. R1 rates (E) and R1p rates (F) as a function of residue position of an ~0.3 mM, 2H (~50%), 15N-labeled YgaP sample. The cytoplasmic and transmembrane domains are depicted above the graphs by open rectangles. All NMR experiments were measured at 700 MHz 1H frequency, 30 °C, and pH 7.0. G, molecular mass analysis of the YgaP-detergent complex performed by multilayer static light scattering coupled with size-exclusion gel chromatography and refractive index measurements. Two peaks are observed and identified as the YgaP-detergent complex (peak 1) and the detergent micelle only (peak 2). The extracted average molar masses of peak 1 of the protein-detergent complex (red), the detergent component (green), and the protein component (blue) are shown. Correspondingly, the extracted average molar masses of peak 2 are color-coded: orange for the entire mass, cyan for the protein mass, and magenta for the detergent mass, respectively. Following these investigations, YgaP appeared to be dimeric in DHPC-7/LMPG micelles with an apparent molecular mass of ~40 kDa.
secondary structural elements of rhodanese-F. On the other hand, the small structural differences are profound and highlight different yet unknown roles and/or specificities of rhodanese domains in the homeostasis of *E. coli*.

Catalytic Sulfurtransferase Activity of the Rhodanese Domain of YgaP (Rhodanese-F)—Rhodanese enzymes catalyze the transfer of sulfane sulfur from thiosulfate \((S_2O_3^{2−})\) to cyanide \((CN^{−})\) to form thiocyanate \((SCN^{−})\) (Fig. 6D). The catalytic essential Cys in the active site loop (e.g. Cys-63 in the case of YgaP) is a predominant feature of these enzymes. We monitored chemical shift changes using two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectra upon titration of rhodanese-F with sodium thiosulfate \((\text{Na}_2\text{S}_2\text{O}_3)\) followed by titration with potassium cyanide \((\text{KCN})\) (Fig. 6). The data confirmed the presence of a thiosulfate active site within rhodanese-F. Thiosulfate concentration-dependent chemical shift changes were observed for residues Cys-63, and Ala-65–Asn-71 indicative of fast exchange dynamics at the active site Cys-63 between S-bound and -unbound state reminiscent of protonation events on His, for example (Figs. 3B and 6). In addition, thiosulfate concentration-dependent chemical shift changes appeared in the loop region Leu-87–Ile-92, which is structurally adjacent to the predicted active site Cys-63 (e.g. Leu-87 in Fig. 6), as well as for a total of 22 \(^{15}\text{N},^{1}\text{H}\) moieties distant from the catalytic active Cys-63 but located mainly on the same half of the three-dimensional structure (e.g. Leu-13, Glu-28, Tyr-29, and Glu-32).

Overall, thiosulfate-dependent chemical shift changes of \(^{15}\text{N},^{1}\text{H}\) moieties are detected in five different regions of rhodanese-F as follows: (i) Thr-3, Thr-4, and Ser-6; (ii) Leu-20–Ile-34, Ala-37–Ala-40, and Ser-43–Leu-45; (iii) His-62–Asn-71; (iv) Leu-87–Lys-97; and (v) Val-102 and Val-104 (Fig. 6). Using the chemical shift changes of Ala-65, a \(K_d\) value (38) of ~2 mM was determined. These results are in agreement with the corresponding enzymatic activity of PsPE (3).

Indirect evidence for the covalent addition of several sulfur atoms is the observation of continuous chemical shift changes upon titration with sodium thiosulfate (Fig. 6). A single covalent binding in the slow exchange regime would cause a decrease of existing cross-peaks and an increase of new ones, the latter reflects the new sulfur-bound species. Only if the covalent binding of sulfur would be transient, titration-dependent chemical shift changes would be expected to be caused by fast exchange. To support the latter possibility, the sodium thiosulfate-containing buffer of a thiosulfate-treated rhodanese-F sample was exchanged by a thiosulfate-free buffer using a PD10 desalting column (GE Healthcare). The comparison of the two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectra measured before titration and after buffer exchange are similar (Fig. 3B) indicating sulfur binding under fast exchange.

The critical role of Cys-63 for the action of sulfur binding is further supported by titration measurements on a rhodanese-F variant with a replacement of Cys-63 by Asp. Although rhodanese-F(C63D) is still folded as indicated by a two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY experiment (Fig. 3A), it is unable to bind sulfur as evidenced by NMR (data not shown; please note a corresponding variant with Ala at position 63 was not correctly folded as evidenced by NMR experiments, data not shown).

Next, the regeneration of the catalytic Cys-63 in rhodanese-F was monitored using two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectra in a series of titration experiments with KCN in presence of 4 mM sodium thiosulfate. As expected, with the KCN titration it was possible to reverse almost entirely the chemical shift changes caused by thiosulfate (i.e. Cys-63, Ala-65, Gly-66, Arg-68, and Thr-69) toward recovering the two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectrum of untreated rhodanese-F (Fig. 6B). Interestingly, upon over-titration by 5 mM KCN over the 4 mM sodium thiosulfate, an additional cross-peak was observed for a few residues (i.e. Arg-31, Gln-47, and Ala-78), suggesting a cyanel-binding site in rhodanese-F (data not shown).

In summary, Cys-63 of rhodanese-F binds one to several sulfur atoms upon treatment with sodium thiosulfate inducing a widespread conformational change in rhodanese-F (Fig. 6C). In the presence of KCN, the sulfur atoms are released yielding SCN⁻ into the solvent, although rhodanese-F returns back to its active form ready for a next catalysis cycle (Fig. 6D).

**Full-length YgaP Solubilized in Mixed Detergents Forms a Dimer**—In addition to the water-exposed cytoplasmic single rhodanese domain, YgaP is predicted to contain two lipophilic transmembrane α-helices (1). To perform structural studies of full-length YgaP, it was necessary to reconstitute YgaP, extracted from the membrane fraction of *E. coli*, in a membrane-mimicking environment. An extensive screening of conditions (12) finally yielded a decent quality two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectrum of YgaP in mixed DHPC-7/LMPG detergent micelles. The solubilization of YgaP with as little as possible DHPC-7/LMPG (6:1 mM) mixture was thereby critical in order to not unfold the rhodanese domain and simultaneously to stabilize the structure of the transmembrane domain (12). Furthermore, the variant YgaP(C158S) (called YgaP⁻ in the following) was more stable yielding a sample that was inert for a few days.

The negligible \(^{1}\text{H}\) and \(^{15}\text{N}\) chemical shift deviations for residues 1–100 between rhodanese-F and the rhodanese domain of YgaP⁻ indicate the structural conservation of the cytoplasmic rhodanese domain in full-length YgaP⁻ solubilized in DHPC-7/LMPG micelles (Fig. 1) (12). In contrast to the relatively narrow line widths and large dispersion of \(^{1}\text{H}\) backbone signals of the rhodanese domain in the two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectrum (from 6.8 to 10.4 ppm), relatively broad cross-peaks were observed for the transmembrane segment of YgaP⁻. Several cross-peaks from the transmembrane domain were broadened beyond detection in the two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectrum in the absence of deuterium labeling (Fig. 3, C and D). Furthermore, fast \(R_{1p}(^{15}\text{N})\) relaxation observed for the \(^{15}\text{N},^{1}\text{H}\) moieties of the transmembrane domain (Fig. 5F) indicates a large protein-detergent complex with the possibility that YgaP⁻ might be in an oligomeric state. Indeed, multilange static light scattering coupled with size-exclusion gel chromatography and refractive index measurements (Fig. 5G) show that the YgaP⁻ detergent complex has a molecular mass of ~80 kDa with a detergent mass of ~45 kDa and a protein mass of ~35 kDa. Because the molecular mass of YgaP⁻ is 18.7 kDa, these data indicate that YgaP⁻ forms a dimer in DHPC-7/LMPG micelles. The presence of a single set of amide cross-peaks in the two-dimensional \(^{15}\text{N},^{1}\text{H}\) TROSY spectrum (Fig. 1B) suggests that YgaP⁺ in DHPC-7/
LMPG micelles forms a symmetrical homodimer. Similar data have been obtained for YgaP (data not shown).

**NMR Structure Calculation of Full-length YgaP**—Solubilized in Mixed Detergents—The sequential assignment of YgaP in DHPC-7/LMPG mixed micelles was performed using TROSY-based triple resonance experiments and three-dimensional 13C,15N-resolved 1H,1H NOESY experiments as described in detail under “Experimental Procedures” (Fig. 2A). Structural restraints were obtained from three-dimensional 13C,15N-resolved 1H,1H NOESY experiments, PRE measurements, and 13C chemical shift analysis (for details see under “Experimental Procedures”). Because YgaP forms a dimer, the collection of intermolecular NOE- and PRE-based distance restraints were of particular importance and were obtained from mixed sample preparations (see “Experimental Procedures”). The three-dimensional structure of YgaP was calculated using the software package CYANA in a three-step approach as follows: (i) three-dimensional structure calculation of the rhodanese domain of YgaP; (ii) three-dimensional structure calculation of the monomeric subunit of the transmembrane domain comprising residues 106–173 of full-length YgaP; followed by (iii) three-dimensional structure calculation of the dimer of the transmembrane domain of YgaP using unambiguous intermolecular NOE and PRE restraints derived from mixed samples (for details see “Experimental Procedures” and Tables 1 and 2). This approach yielded a final set of the 10 best conformers of the YgaP dimer, selected from 100 calculations based on the CYANA target function. The 10 best conformers have a backbone atom r.m.s.d. of 0.27 Å for residues 113–167 (Table 6). Small values of residual constraint violations and small deviations from ideal geometry (Tables 5 and 6) confirm a good quality of the final structure. In addition, the input data represent a self-consistent set, and the restraints are well satisfied in the calculated conformers. However, the accuracy of the three-dimensional structure of full-length YgaP is limited because of the relatively small number of long range distance restraints (Fig. 2, D and E, and Tables 1 and 2). This claim may regarded to be in contradiction to the small r.m.s.d., which reflects qualitatively the precision of the three-dimensional structure, but is in part caused by the symmetry requirements of the homodimer.

**Solution NMR Structure of the Rhodanese Domain within Full-length YgaP**—Solubilized in Mixed Detergents—The three-dimensional structure of rhodanese-F with five α-helices and five β-strands is preserved in the N-terminal cytoplasmic domain of YgaP comprising residues 1–108 as shown in Fig. 7, A and B. Similar to the three-dimensional structure of rhodanese-F, the approximate lengths of the helices are from residues 10–13, 27–31, 42–47, 70–78, and 92–97, whereas β-strands are identified for residues 4–5, 20–22, 38–39, 60–62, and 87–88, respectively. As for rhodanese-F, the five β-strands form a central twisted parallel β-sheet with two and three helices packed on each side of the β-sheet. The loop segment with its catalytic residue Cys-63 consists of seven residues, and the C-terminal region is stabilized by its proximity to the residues Glu-32 and His-33 (which is supported by strong long range NOEs) (Fig. 7). However, the structure of the rhodanese domain of full-length YgaP− is less well defined than rhodanese-F, which is reflected in the r.m.s.d. values relative to the mean coordinates of 0.99 Å for the backbone atoms and 1.54 Å for all heavy atoms (Table 5). The lower resolution of the structure is attributed to the smaller amount of structural restraints collected, which is due to the lower quality of the spectra because of a lower protein concentration, t1 noise from the detergent signals, and more signal overlap originated from the overall larger size of YgaP− when compared with its domain. Furthermore, the line width of the proton resonances doubled, which was attributed to the larger size of the complex of full-length YgaP− with detergent when compared with rhodanese-F. Indeed, TROSY-based R1(15N) relaxation measurements indicate that the rhodanese domain has overall a 2-fold increased R1(15N) relaxation rate (Fig. 5F) when compared with rhodanese-F, and correspondingly, the rotational correlation time of the rhodanese domain in YgaP− is twice as large for rhodanese-F. Interestingly, based on the molecular mass of the protein-detergent complex, as determined by multangle static light scattering (Fig. 5G), a 5-fold increase of the rotational correlation time was expected for full-length YgaP−. This discrepancy is explained by a partial independent tumbling of the rhodanese domain in YgaP−. R1(15N) values, which are considerably larger for the transmembrane domain of YgaP− than for the rhodanese domain (Fig. 5F), and the absence of long range NOEs between the cytoplasmic rhodanese domains of YgaP− and the transmembrane domain support this interpretation. In return, the PRE data, which are less sensitive to domain motions, show that MTSL labeling on Cys-158 in the YgaP(C63D) variant affected numerous 15N-1H moieties of the rhodanese domain (i.e. Asp-22, Ile-23, Asp-25, Ala-26, Asp-27, Glu-28, Tyr-29, Arg-31, Glu-32, His-33, Ile-34, Glu-36, Ala-37, Asp-38, Ala-40, Leu-42, Ser-43, Val-44, Leu-45, Glu-46, Gln-47, Ser-48, Gly-49, Leu-50, Ala-52, Lys-53, Leu-54, Arg-55, Val-102, Ala-103, Val-104, Lys-106, and Gln-108). The PRE data together with the relaxation data thus indicate the presence of a dynamic proximity of the rhodanese domain with the transmembrane segment of YgaP−.

**FIGURE 6.** NMR-based observation of the sulftransferase activity of rhodanese-F. Sodium thiosulfate (Na2S2O3) titration followed by potassium cyanide (KCN) titration of 15N-labeled rhodanese-F monitored by two-dimensional 15N,1H TROSY experiments. The spectra of ~1 mM 15N-labeled rhodanese-F were measured at 700 MHz 1H frequency at 30°C, pH 7.0. A, two-dimensional 15N,1H TROSY spectra and close-ups of two-dimensional 15N,1H TROSY spectra of rhodanese-F in the absence of sodium thiosulfate (black) and in the presence of 1 mM (yellow), 2 mM (orange), 3 mM (green), and 4 mM (blue) sodium thiosulfate. B, two-dimensional 13C,15N,1H TROSY spectra and close-ups of two-dimensional 13C,15N,1H TROSY spectra of rhodanese-F in presence of 4 mM sodium thiosulfate in the absence of KCN (blue) and upon addition of 1 mM (green), 2 mM (orange), 3 mM (yellow), and 4 mM KCN (black). Cross-peaks labeled with * indicate a minor population of rhodanese-F. The cross-peak labeled with ** might belong to the unassigned 15N-1H moiety of Gln-64. Cross-peaks of the amino acid residues of the active loop of rhodanese-F are highlighted in bold (i.e. Cys-63, Ala-65, Gly-66, Arg-68, and Thr-69). The 15N-1H cross-peak of Lys-67 was not visible in the titration experiment because of low signal intensity. C, chemical shift changes of the 15N-1H moieties of rhodanese-F upon titration with 4 mM sodium thiosulfate mapped onto the three-dimensional structure of rhodanese-F. Red color corresponds to residues showing chemical shifts changes above 0.02 ppm in the proton dimension, and residues colored in blue did not show any substantial chemical shift changes. No data were obtained for residues in gray. The S^ atom of the catalytic Cys-63 is shown as a yellow sphere. The letters N and C identify the corresponding termini. D, sketch showing the corresponding sulfur-transfer reaction upon titration with thiosulfate followed by cyanide (9).
NMR Structure of YgaP

A

B

C

D

E

F
Solution NMR Structure of the Transmembrane Domain of Full-length YgaP—Solubilized in Mixed Detergents—In addition to the rhodanese domain, the three-dimensional structure of YgaP is composed of two slightly tilted (i.e. ~15°) transmembrane α-helices comprising residues 113–138 (i.e. TM1) and 142–167 (i.e. TM2), respectively, building the center of the symmetric dimer interface (Figs. 2A and 7). Hence, the transmembrane region of YgaP is composed of four transmembrane helices. As highlighted in Fig. 7, both the intramolecular (i.e. Leu-125, Ile-126, Val-129, and Leu-130 of TM1 and Leu-150, Phe-151, and Ile-154 of TM2, color-coded in yellow in Fig. 7E) as well as the intermolecular helix-helix packing of TM1 (i.e. Ile-134, Leu-135, Val-138, Val-138, and Tyr-132 of TM1s, color-coded in orange in Fig. 7E) are of hydrophobic nature and the two hydrophobic cores are intertwined with each other. Also, the interface with the membrane as determined by detergent-protein NOEs is of a hydrophobic nature (Fig. 2, B and C, and Table 3). In addition, it is interesting to note that there is a large number of Gly residues present within the TM helices (i.e. Gly-121, Gly-122, Gly-127, Gly-131, Gly-137 of TM1 and Gly-143, Gly-148, Gly-153, and Gly-159 of TM2; highlighted by dark spheres in Fig. 7E). Particularly worth mentioning is the 127GVXXG131 segment, which is a known motif important for transmembrane helix-helix association (39). Indeed, this motif is at the center of the dimer interface. The small residues Gly-127 and Gly-131 allow for a tight packing of the Val-128 methyis across the dimer interface. The bulky residues Leu-125, Ile-126, Leu-130, and Tyr-132 flank this interaction. However, no inter-monomeric/inter-helix C=O–H⋯O hydrogen bonds within the 127GVXXG131 motif are detected, although extensive intermolecular NOE contacts between the two TM1s have been collected and define well the interface. Additionally, on TM2 there are many Gly residues, which alternate with three Phe residues supporting the embedment of the transmembrane helices of YgaP into the mixed DHPC-7/LMPG micelle. Intermolecular NOEs between the choline methyis (with a chemical shift of 3.20 ppm) of DHPC-7 and the amide protons of YgaP were not observed, with the only exception of Trp-169. The indole of Trp-169 has a number of NOE contacts with DHPC-7 protons, namely with the -CH3 (0.82 ppm) and -CH2- (1.21, 1.52, and 2.30 ppm) of the alkyl chain and with the choline methy protons. Except for intra-residual and sequential NOE contacts and the inter-helix NOE contact between Trp-169(H1) and Leu-130(CαH), protein-protein NOEs were not detected for this amino acid residue. These data indicate that Trp-169 might.

FIGURE 7. Solution NMR structure of YgaP—YgaP is a dimeric membrane protein with two transmembrane α-helices per monomer and a cytoplasmic N-terminal rhodanese domain. The two-domain architecture of the dimeric YgaP is used here to split the figure. A, ribbon drawing of the mean solution NMR structure of the rhodanese domain of YgaP. The mean structure was calculated from the 10 CYANA conformers with the lowest residual target functions. The structure contains five α-helices (red) and five β-strands (gray) that are connected by linkers (gold) with nonregular secondary structure. 1–5 indicate the sequential order of the α-helices and the β-strands. B, polypeptide backbone superposition on all Cα atoms (residues 2–101) of the NMR structures of rhodanese-F (magenta) and the rhodanese domain of YgaP (yellow) represented by the corresponding bundles of the 10 CYANA conformers in each case with the lowest residual target functions. C, ribbon drawing of the mean solution NMR structure of the transmembrane segment of the dimeric YgaP. The mean structure was calculated from the 10 CYANA conformers with the lowest residual target functions. The monomer subunits are colored in green and light blue. The N and C termini are identified. D, bundle representation of the ensemble of the 10 CYANA conformers with the lowest residual target functions of the transmembrane segment of the dimeric full-length YgaP. The conformers were superimposed on all Cα atoms of residues 113–138 and 142–167. The r.m.s.d. for backbone and heavy atoms of residues 113–167 are 0.27 and 1.07 Å, respectively. E, some structural properties are highlighted in the present ribbon representation. The hydrophobic intramolecular helix-helix interaction is indicated by a yellow space-filling area. An orange-colored area highlights the corresponding hydrophobic intermolecular interactions. Therein, the dark orange area indicates the location of Val-128 located at the center of the intermembrane contact. Glycine residues are indicated by dark spheres at their Cα positions. The aromatic side chains are shown and color-coded in yellow, although the positively charged side chains of residues Lys and Arg are highlighted in blue. The hydrophilic core residues of Ser-158 (red) and Gin-117 (green) are shown as a ball and stick model, and the potential hydrogen bond between them is indicated with a pink colored line. F, homodimeric structure model of full-length YgaP embedded in a lipid bilayer as sketched. The monomer subunits are colored in green and light blue. A dotted line indicates the structurally nondefined linkers between the transmembrane region and the rhodanese domain.
be located at the micelle-water interface. This finding is in line with statistical analysis of existing high resolution structures of α-helical integral membrane proteins, which shows that Trp and Tyr are enriched near the ends of the transmembrane helices, suggesting that they interact favorably with the lipid headgroups near the membrane-water interface (47). Various model systems support the role for Trp and Tyr residues in positioning transmembrane helices relative to the lipid bilayer (48).

**Sulfurtransferase Activity of Full-length YgaP** — The potential enzymatic activity of full-length YgaP was studied similarly to the sulfurtransferase activity of rhodanese-F by sodium thiosulfate titration monitored by two-dimensional 15N,1H TROSY experiments. The two-dimensional 15N,1H TROSY spectra of YgaP−, recorded in the absence and presence of 20 and 40 mM sodium thiosulfate, indicate that the same 15N,1H moieties of the rhodanese domain of full-length YgaP− (Fig. 8) are affected as observed for rhodanese-F. However, a higher sodium thiosulfate concentration (40 mM versus 4 mM) must be used in full-length YgaP− titration to observe a similar magnitude of chemical shift changes as in the rhodanese-F titration experiments. This difference may suggest a lower apparent enzymatic activity of the rhodanese domain attributed to the presence of detergents, which may affect the structure or even partially unfold the rhodanese domain as it was documented (12), or it may be due to the direct interaction between anionic thiosulfate and the positively charged choline group of the DHPC-7 lowering the free available thiosulfate concentration.

In addition to the chemical shift changes of 15N,1H moieties of the rhodanese domain, chemical shift changes within the second transmembrane helix (i.e. Phe-157, Leu-164, Lys-166, and Trp-169, Fig. 8) were observed, which point toward structural changes of the transmembrane segment of YgaP− upon addition of sodium thiosulfate (Fig. 8, B and D). These chemical shift changes are probably not an artifact of the increased salt concentration or ionic interaction between SO4− and the positively charged choline group of the detergent unless there is significant binding of SO42− to the choline N(+)-(CH3)3, affecting residues at the detergent surface. The latter possibility was ruled out because of the lack of significant chemical shift changes of YgaP− upon addition of corresponding amounts of KCl (data not shown).

**Potential Role of YgaP in the Transport of SCN−** — Rhodanese domain-containing proteins provide organisms with labile sulfurtransferase activity. For example, the process of detoxification by the rhodanese domain involves the catalysis of sulfane sulfur from thiosulfate to cyanide, leading to the formation of the less toxic thiocyanate (SCN−) (47) as demonstrated here for YgaP− (Fig. 9, A and B). The presence of eight rhodanese-containing proteins in E. coli underlines the importance of these enzymes for the homeostasis of the organism as well as the reduction of the CN− toxicity. The structural differences between rhodanese domains in various E. coli proteins, highlighted in Fig. 4D, suggest the evolutionary optimized yet unknown functional specializations of these proteins. To get insights into the possible functions of YgaP, one should take into account that in addition to the presented sulfurtransferase activity of the rhodanese domain, YgaP is the only membrane protein in E. coli that comprises a rhodanese domain. Furthermore, YgaP− forms a homodimer by its transmembrane segment as shown in Fig. 7C. Based on this knowledge, we speculated whether YgaP could be involved directly or indirectly in the transport of SCN− through the membrane from the cytoplasm to the periplasm upon the catalysis from CN− to SCN− by its sulfurtransferase activity.

To obtain experimental support for this hypothesis, we performed chemical shift perturbation experiments of the following: (i) 15N thiosulfate-treated YgaP− by addition of 1, 3, 5, and 10 mM KCN and (ii) wild-type YgaP or YgaP− by addition of 2–7, 9, and 10 mM NaSCN, respectively. In the negative control experiment with 10 mM KCl, no 1H chemical shift changes above 0.01 ppm were detected in the two-dimensional 15N,1H TROSY spectra of 2H (~50%), 15N-labeled YgaP (data not shown) and YgaP− (Fig. 9C). In turn, in the KCN and NaSCN titration experiments, pronounced chemical shift perturbations (above 0.07 ppm) were observed for the 15N,1H moieties of the positively charged Arg-162 and Lys-166 located at the end of the second transmembrane α-helix for both YgaP and YgaP− (Fig. 9, A–D). Upon the KCN titration, additional less pronounced chemical shift changes of 15N,1H moieties of YgaP− were observed for Val-116, Ala-120, and Gly-121 (first transmembrane α-helix) as well as for Ala-147, Ala-152, Gly-153, Gly-156, Phe-157, Leu-163, and Leu-164 (second transmembrane α-helix) (Fig. 9, A and B). In the presence of NaSCN, the 15N,1H chemical shift changes were detected in the first (Val-116, Ile-118, Ala-120, Gly-121, Ile-126 in YgaP; Met-113, Val-116, Ile-118, Ala-120, Gly-121, Leu-123, and Gly-131 in YgaP−) and second (Ser-142, Gly-148, Gly-153, Gly-156, Arg-162, Leu-164, Lys-166, and Met-167 in YgaP; Phe-144, Ala-147, Gly-148, Leu-149, Ala-152, Gly-153, Gly-156, Phe-157, Ala-161, Arg-162, Leu-163, Leu-164, Lys-166, and Met-167 in YgaP−) transmembrane α-helices (Fig. 9, C and D). No substantial chemical shift perturbations upon addition of

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**FIGURE 8. Chemical shift perturbation of full-length YgaP− upon thiosulfate titration.** The two-dimensional 15N,1H TROSY spectra of 2H (~50%), 15N-labeled YgaP− samples (~0.3 mM) were measured on a Bruker 700 MHz Avance III at 30 °C, pH 7.0. A, two-dimensional 15N,1H TROSY spectra and close-ups of two-dimensional 15N,1H TROSY spectra of YgaP− in the absence of sodium thiosulfate (black) and in the presence of 20 mM (blue) and 40 mM sodium thiosulfate (red), respectively. Peaks identified with asterisk numbers correspond to amino acid residues from the His tag. The cross-peak labeled with ** might belong to the unassigned 15N,1H moiety of Gln-64. B, selection of individual cross-peaks of the two-dimensional 15N,1H TROSY spectra displaying Phe-157, Leu-164, Lys-166, and Trp-169 cross-peaks under 0–15 mM sodium thiosulfate addition as indicated (from left to right). The red broken lines show the corresponding chemical shifts of untreated YgaP−. C and D, chemical shift changes of the 15N,1H cross-peaks in the two-dimensional 15N,1H TROSY spectra upon addition of 40 mM sodium thiosulfate and the transmembrane domain of YgaP−. Residues that undergo chemical shift changes are shown in red, and no chemical shift perturbations upon ligand binding were observed for residues colored in blue. No data were obtained for residues in gray. The S+ atom of the catalytic Cys-63 in the active loop is colored in yellow. The letters N and C identify the termini of the rhodanese and transmembrane domain of YgaP−.
NMR Structure of YgaP

A

B

C

D

E

F

Periplasm

Cytoplasm
TABLE 7
NMR spectra for resonance assignments, conformational restraints, and backbone dynamics of rhodanese-F and YgaP−

| Sample | Experiment (mixing time) | Information |
|--------|--------------------------|-------------|
| 12C,15N-Labeled rhodanese-F | Two-dimensional 15N,1H TROSY | Optimization of buffer and detergent composition, catalytic thiosulfate activity |
| 12C,15N-Labeled YgaP− | Two-dimensional 15N,1H TROSY | Sequential backbone assignment |
| 2H (~50%), 13C,15N-labeled YgaP | Two-dimensional 15N,1H TROSY | Side-chain assignment |
| 13C,15N-Labeled YgaP− | Combined three-dimensional 13C/15N-resolved 1H,1H NOESY (100 ms) | Resonance assignment, conformational restraints of the rhodanese domain of full-length YgaP− |
| MTSL-labeled YgaP(C63D) variant at the natural Cys-158 mixed with 2H (~50%), 15N-labeled YgaP in a 1:1 ratio | Three-dimensional 15N,1H TROSY (220 ms) | Intermolecular distance restraints, detergent/solvent-protein interaction |
| | Two-dimensional 15N,1H TROSY | PRE data for intermolecular distance restraints |

NaSCN were observed in the rhodanese domain (data not shown). The perturbation of the chemical shifts upon addition of SCN− suggests that SCN− induced conformational changes in the transmembrane domains of YgaP and YgaP− similar to the changes observed upon titration with Na2S2O3 followed by titration with KCN. Therefore, the changes in the transmembrane domain of YgaP appear to be specific to the product of the sulfotransferase enzyme domain of this protein. A detailed analysis of the chemical shift changes on TM2 upon titration with SCN− shows that the amide protons of residues 157, 162, 166, and 169 located on one side of the helix did shift upfield, whereas the corresponding chemical shifts of residues 153, 156, 166, and 169 located on the other side of the helix shifted downfield (Fig. 2A), indicating a bending of the helix from its second half.

To get further evidence on the SCN−-induced conformational change in the transmembrane domain, we measured EPR spectroscopy-based distance restraints in the YgaP dimer between two MTSLs covalently bound to Cys-158 in YgaP(C63D) either solubilized in detergent or reconstituted in proteoliposomes. As we mentioned before, this variant has only one Cys per monomer, and its three-dimensional structure is preserved as indicated by a comparison of the two-dimensional 15N,1H TROSY spectra between YgaP(C63D) and YgaP. The detection of a dipolar oscillation in the DEER traces (Fig. 9E) confirms that YgaP(C63D) forms dimers both in detergent micelles and in a membrane environment. In the absence of SCN−, a broad distance distribution is observed between the labels attached at position 158 (1.3–5 nm) in the detergent.
solubilized YgaP(C63D). The lower distance fraction of the distribution is in line with the simulated interspin distances using the NMR structure of YgaP (Fig. 9E, dotted black lines), whereas the additional long distances up to 5 nm may arise from dynamic conformations of YgaP(C63D) trapped in the frozen state. Most interestingly is that upon addition of SCN^{-}, the overall distance distribution shifts toward shorter distances (1.3–3 nm), indicative of a major rearrangement induced by SCN^{-}. Next, a spin label was placed at position 139 by the generation of a mutant YgaP^{C} (C63D/F139C) having a single Cys at position 139. EPR measurements on the MTSL-labeled YgaP^{C} (C63D/F139C) variant show a distribution of less than 2 nm, which is in line with the NMR structure. Interestingly, upon addition of SCN^{-}, the distance is not affected. The conformational changes detected in detergent-solubilized proteins using the spin labels at position 158 could be reproduced also in proteoliposomes (1.3- and 2.3-nm peaks), which could indicate an existing equilibrium between two conformations. The 2.3-nm peak agrees with the simulated distances in the NMR structure (Fig. 9E, dotted black lines). Addition of SCN^{-} shifted the distribution almost completely toward the short distance peak indicative of a large conformational change.

The discussed EPR data together with the chemical shift changes indicate an SCN^{-}-induced conformational change of YgaP at the second half of TM2 and in close vicinity to the highly conserved Cys-158, which is at the center of the hydrophilic core. In combination with the knowledge that YgaP is the only membrane protein of the rhodanese family of membrane proteins in E. coli, it is interesting to speculate whether YgaP may be involved in the transmembrane transport of SCN^{-} via Cys-158 upon catalytic processing of potassium cyanide into SCN^{-} at the rhodanese domain side as illustrated in Fig. 9F. Because we failed to show such a transport in an experiment with YgaP reconstituted into liposomes (data not shown), another yet unknown factor (for example a membrane protein partner) interacting with YgaP may be required for the transmembrane transport speculated here (Fig. 9F).

Conclusion—Following state of the art solution state NMR experiments on mostly triple-labeled protein samples (Table 7), a low resolution three-dimensional structure of the bacterial membrane protein YgaP^− in complex with detergent was obtained. The three-dimensional structure is composed of a cytoplasmic rhodanese domain and two transmembrane helices forming the interface of the homodimer. The catalytic activity of the rhodanese domain was monitored both by sodium thiosulfate and KCN titration experiments suggesting a sulfurtransferase activity of YgaP with the active center Cys-63. Based on observed conformational changes of the transmembrane domain of YgaP upon SCN^{−} addition, it was speculated that YgaP may not only have a role in the detoxification of CN^{−} to the less toxic SCN^{−} (Fig. 6D) but may also be involved in a yet to be demonstrated transmembrane transport of SCN^{−} ions (Fig. 9F).

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