Structure–function analyses reveal the molecular architecture and neutralization mechanism of a bacterial HEPN–MNT toxin–antitoxin system

Received for publication, February 14, 2018, and in revised form, March 13, 2018 Published Papers in Press, March 19, 2018, DOI 10.1074/jbc.RA118.002421

Xuanyan Jia1,1, Jianyun Yao5,1, Zengqiang Gao6, Guangfeng Liu5, Yu-Hui Dong6, Xiaoxue Wang4,2, and Heng Zhang3

From the 1Institute of Physical Science and Information Technology, Anhui University, Hefei, Anhui 230601, the 2CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, the 3Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing 100049, and the 4National Center for Protein Science Shanghai, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Edited by Norma M. Alliewell

Toxin–antitoxin (TA) loci in bacteria are small genetic modules that regulate various cellular activities, including cell growth and death. The two-gene module encoding a HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domain and a cognate MNT (minimal nucleotidyltransferase) domain have been predicted to represent a novel type II TA system prevalent in archaea and bacteria. However, the neutralization mechanism and cellular targets of the TA family remain unclear. The toxin SO_3166 having a HEPN domain and its cognate antitoxin SO_3165 with an MNT domain constitute a typical type II TA system that regulates cell motility and confers plasmid stability in the bacterium Shewanella oneidensis. Here, we report the crystal structure and solution conformation of the SO_3166–SO_3165 pair, representing the first complex structures in this TA family. The structures revealed that SO_3165 and SO_3166 form a tight hetero-octamer (at a 2:6 ratio), an organization that is very rare in other TA systems. We also observed that SO_3166 dimerization enables the formation of a deep cleft at the HEPN-domain interface harboring a composite RX4–6H active site that functions as an RNA-cleaving RNase. SO_3165 bound SO_3166 mainly through its two α-helices (α2 and α4), functioning as molecular recognition elements. Moreover, their insertion into the SO_3166 cleft sterically blocked the RX4–6H site or narrowed the cleft to inhibit RNA substrate binding. Structure-based mutagenesis confirmed the important roles of these α-helices in SO_3166 binding and inhibition. Our structure–function analysis provides first insights into the neutralization mechanism of the HEPN–MNT TA family.

Toxin–antitoxin (TA) loci are small genetic modules that are widespread in bacterial plasmids and chromosomes and target various cellular functions to regulate cell growth and death (1, 2). Six different types of TA systems (types I–VI) have been characterized so far based on the interaction mode of the TA and the molecular nature of the antitoxin (3). In the most well-characterized and abundant type II TA system, an antitoxin can bind directly to a cognate toxin to form a tight protein–protein complex for inactivating toxicity during normal growth. When the expression from TA loci is impaired by stresses, specific ATP-dependent proteases (such as Lon and ClpXP) will be activated to destroy the labile antitoxin by proteolytic cleavage (4, 5). The subsequently released toxin is activated to regulate cell mortality by blocking DNA replication or translation, or by facilitating mRNA degradation. These key cellular processes are associated with many roles in cell physiology, such as biofilm formation and virulence in bacteria (1, 2, 6, 7).

The HEPN (higher eukaryotes and prokaryotes nucleotide-binding) superfAMILY members adopt an all-helical fold and are distributed in the proteins associated with bacterial drug resistance and human neurodegeneration (8). The MNT (minimal nucleotidyltransferase) domains have been identified as the minimal units of DNA polymerase protein superfamily that are responsible for transferring nucleic acids to an acceptor hydroxyl group in prokaryotes and animal Sacsin proteins (9). The two-gene modules encoding a MNT domain and an accompanying HEPN domain have been proposed to represent a novel, nonconventional type II TA system widely distributed in both archaea and bacteria (10). The MNT- and an accompanying HEPN-containing protein have been predicted as the toxin and the cognate antitoxin, respectively. However, a genome-wide screen for toxins has identified the HEPN

© 2018 Jia et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
domain function as the toxin in the HEPN–MNT module belonging to a TA system (11). A subsequent bioinformatics analysis further predicted that the HEPN-domain functions as toxins that are essential components of numerous TA and abortive infection systems in prokaryotes, and are also tightly associated with many restriction–modification (R–M) and CRISPR–Cas systems (12). Moreover, most HEPN domains contain a conserved R\(X^{4\text{--}6}H\) motif (where \(X\) is any amino acid and the residue immediately after the conserved R is typically a polar amino acid, and 4–6 indicates the number of amino acids between R and H). As a conserved feature of the HEPN domain, this motif may function as a novel RNase active site, but it is usually lost when fused with an MNT domain. Until now, the neutralization mechanism and cellular targets of HEPN–MNT TA family members remain unknown.

Recently, we experimentally characterized SO\(_{3166}\) and SO\(_{3165}\) from \(Shewanella\ oneidensis\) as a novel type II TA pair with a critical role in regulating cell motility and conferring plasmid stability (13). SO\(_{3166}\) is composed of a single HEPN domain and a conserved R\(X^{4\text{--}6}H\) catalytic motif. It can function as a toxin with strong inhibition on cell growth in \(S.\ oneidensis\) and \(Escherichia\ coli\). The toxicity can be neutralized by its cognate antitoxin SO\(_{3165}\) with a MNT domain, which binds to the promoter of the TA operon and repressed its activity (13). However, the inhibition mechanism of SO\(_{3166}\) toxicity by SO\(_{3165}\) remains unclear. To this end, we performed structure–function studies on the SO\(_{3166}–\text{SO}_{3165}\) complex and demonstrate how the HEPN toxin (SO\(_{3166}\)) is recognized and inhibited by the MNT antitoxin (SO\(_{3165}\)). The unique structure and the following mutagenesis study revealed one SO\(_{3165}\) can recognize and inhibit the toxicity of three SO\(_{3166}\) by sterically blocking its RX\(_{4\text{--}6}H\) catalytic domain. These findings may provide novel insights into the neutralization mechanism of HEPN–MNT TA family members, and are useful to further understand the function of HEPNs and MNTs in prokaryotes.

**Results**

**HEPN toxin SO\(_{3166}\) is a RNase that can cleave mRNA**

To evaluate whether toxin SO\(_{3166}\) can function as a RNase similar to many type II toxins, its RNA cleavage activity against different types of RNAs (mRNA, tRNA, and rRNA) was studied. The abundant \(ompA\) mRNA in \(E.\ coli\) was synthesized and used for the \textit{in vitro} RNA cleavage assay. The \(ompA\) mRNA (306 nt, 0.5 \(\mu\text{g}/\text{ml}\)) substrate can be cleaved into smaller fragments by purified SO\(_{3166}\) (Fig. S1) after 20 min (Fig. 1A). However, its cleavage capacity is completely inhibited by SO\(_{3165}\) in this complex, consistent with the previous observation that SO\(_{3166}\) toxicity could be neutralized by SO\(_{3165}\) (13). Meanwhile, no RNA cleavage fragments were detected against \(E.\ coli\) total tRNAs (Fig. 1B), or \(S.\ oneidensis\) rRNA (Fig. 1C). DNA cleavage activities of SO\(_{3166}\) against different types of DNAs (circular dsDNA, linear dsDNA, and circular ssDNA) were also tested, but no activity was detected in the \textit{in vitro} DNA cleavage assay (Fig. S2). These \textit{in vitro} and \textit{in vivo} results suggest that SO\(_{3166}\) can function as a RNase by cleaving mRNA rather than tRNA or rRNA.

**Antitoxin SO\(_{3165}\) and toxin SO\(_{3166}\) can form a heterooctamer structure**

The crystal structure of the SO\(_{3166}–\text{SO}_{3165}\) complex was solved by the single-wavelength anomalous dispersion method from synchrotron data using selenomethionine (Se-Met)-labeled protein in space group \(P2_12_12\) and was refined to a final \(R/R_{	ext{free}}\) factor of 0.26/0.29 at 3.0-Å resolution (Table 1).
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

Table 1
Data collection and refinement statistics

| Data collection |    |
|-----------------|----|
| Wavelength (Å)  | 0.9788 |
| Space group     | P2₁2₁2₁ |
| Unit-cell parameters | a = 56.6 Å, b = 224.4 Å, c = 53.3 Å, α = β = γ = 90° |
| Resolution (Å)  | 3.00 (3.08–3.00) |
| Number of unique reflections | 26,218 (1,904) |
| Completeness (%) | 99.9 (100) |
| Redundancy | 7.6 (7.8) |
| Mean I/σ(I)     | 13.93 (3.51) |
| Molecules in asymmetric unit | 4 |
| Rmerge (%)      | 6.5 (64.8) |
| CC1/2           | 99.9 (94.9) |

Structure refinement

| Reflections used in refinement | 14,273 |
| Resolution range (Å)          | 48.15–3.00 |
| Rwork/Rfree (%)               | 26.4/29.2 |
| Macromolecules                 | 4,145 |
| Protein residues               | 517 |
| Waters                         | 0 |
| Average B factor (Å²)          | 72.95 |
| Main chain                     | 77.56 |
| Side chain                     | 8.06 |
| Clash score                    | 95.6 |
| Ramachandran plot (%)          | 3.8 |
| Most favored                   | 0.6 |
| Allowed                        | 0.03 |
| Disallowed                     | 0.55 |
| R.m.s. deviations              | 0.003 |
| Bond lengths (Å)               | 1.446 |
| Bond angles (°)                | 2.6 |

* The values in parentheses indicate the highest resolution shell.

There are four molecules composed of one SO₃₁₆₅ binding three SO₃₁₆₆ in an asymmetric unit (ASU) to form an heterotetramer (Fig. 2A), with overall dimensions of ~57 × 223 × 54 Å. The residues Met-1–Asn-5 and Asn-128–Ser-139 in SO₃₁₆₅ (139 residues at full-length), were not observed in the electron density map and not included in the current model, whereas all the residues in the three SO₃₁₆₆ molecules (133 residues at full-length) could be built into the model (except for the absence of selenomethionine-substituted-1 (Se-Met-1) in SO₃₁₆₆). The three SO₃₁₆₆ molecules are highly similar with a root mean square deviation (r.m.s. deviation) of 0.70–0.72 Å for 131 aligned Ca atoms. The notable differences among them are the variable conformations of the α₁–α₂ and α₄–α₅ loops (harboring part of the catalytic RX₄–6H motif, discussed below) (Fig. S3). Meanwhile, a tight heteroctamer can be generated by crystal packing with symmetry-related molecules with the organization (SO₃₁₆₆)₅–SO₃₁₆₅–(SO₃₁₆₆)₅, (Fig. 2B).

The SO₃₁₆₆–SO₃₁₆₅ complex migrated on size exclusion chromatography with a molecular mass of ~140 kDa compared with its calculated heterotetramer molecular mass of ~60 kDa (Figs. 2C and Fig. S5). The oligomeric state of the complex in solution was further studied by small-angle X-ray scattering (SAXS). General structural parameters calculated from the SAXS profile using Guinier approximation and indirect Fourier transformation (program GNOM) are shown in Table S3. Experimental SAXS curves after standard preliminary processing are presented in Fig. 2D (curve 1). Independent confirmation of the results above is the ab initio reconstruction of the complex shape in solution by the programs DAMMIN (χ² = 1.272) and GASBOR (χ² = 1.446) (Table S3). The results show that the experimental SAXS curves are in agreement with the heteroctamer theoretical curve (curves 2 and 3), and the available heteroctamer crystal structure can be fit into the respective SAXS-derived low resolution envelopes. Moreover, comparison of the theoretical scattering patterns from the heterotetramer crystal structure in the ASU with the experimental SAXS profile showed that the model curve differs considerably, whereas the crystallographic heteroctamer yields good fit to it (χ = 2.6) (Fig. S4). The results suggest the active form of the complex is a heteroctamer that is composed of six SO₃₁₆₆ molecules binding two SO₃₁₆₅ molecules in solution.

Such antitoxin to toxin oligomerization was mediated entirely by toxin molecules, and the unique organization (toxin–antitoxin = 6:2) has not been previously reported in a TA system. Moreover, further analysis of the recombinant complex at different concentrations (the same as those used in the SAXS experiment) by size exclusion chromatography showed they have almost identical retention time (Fig. S5). Therefore, we can conclude that the SO₃₁₆₆–SO₃₁₆₅ complex always exists as a heteroctamer and this form is not concentration dependent in vitro. Considering that the SO₃₁₆₆–SO₃₁₆₅ concentration used in the in vitro studies (at the micromolar level) is considerably higher than that in cells, additional studies may be required to confirm the unusual stoichiometry of the complex in vivo.

Toxin SO₃₁₆₆ possesses a typical HEPN domain fused with an RX₄–6H motif

SO₃₁₆₆ is composed of a five-helix bundle, as found in HEPN superfamily members that adopt the all α-helical-fold (12). Notably, the fused RX₄–6H motif (the residues Arg-97–His-102) is located at the end of the α₄ helix and in the beginning of the α₅ helix (Fig. 3, A and B). The conserved fused RX₄–6H motif has been suggested as the primary determinant of a novel RNase active site in the HEPN superfamily (13). This motif is exposed to the solvent (before the antitoxin binding) and may be used to accommodate substrate RNA to trigger the catalysis of RNA cleavage. Moreover, the conformation of the region containing this motif is flexible (Fig. S3), which may be required for substrate-binding or effective catalysis.

The DALI search (http://ekhidna.biocenter.helsinki.fi/dali_server) revealed that SO₃₁₆₆ has remarkable similarities with several uncharacterized proteins with a single HEPN-fold composed of five helices, as well as other proteins containing HEPN domains, such as the aminoglycoside NTs. One of the closest structural homologs is H10074 (PDB ID 1JOG), with a typical HEPN-fold, from Haemophilus influenza, with a DALI Z-score of 12.0 and a r.m.s. deviation of 2.8 Å for 119 Ca atoms (Fig. 3E). Similarly, H10074 with a single HEPN-domain is also a triangular homodimer harboring an RX₄–6H motif (PDB ID 1JOG) (Fig. S6). However, the conformations of the regions containing the RX₄–6H motif are remarkably different in the H10074 and SO₃₁₆₆ structures (especially the catalytic histidine), indicating they have different substrate-binding specificities (Fig. 3E). Moreover, the large positively charged cavity at the dimer inter-

5 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
The face formed by two HEPN domains has also been observed in Sacsin (PDB ID 3O10) and kanamycin nucleotidyltransferase (KNTase, PDB ID 1KNY) for the binding of GTP and other nucleotides (14, 15) (Fig. S6). All these structures suggest that dimerization of the HEPN-domain is conserved in its evolution. Dimerization of toxin SO_3166 may be associated with its catalytic activity.

Two SO_3166 protomers (SO_3166A–SO_3166B) in the ASU assemble as a tight homodimer and the dimerization occurs mainly through the interactions of the α2 helix in both monomers (Fig. 3B). These extensive direct interactions are composed of dozens of hydrogen bonds (H-bonds) and salt bridges between the two subunits (Fig. S6). Another SO_3166 dimer (SO_3166C–SO_3166C’) can be generated by crystallographic symmetry (Fig. 2B). Structural comparisons of these two dimers showed that their overall conformations are highly similar, with a r.m.s. deviation of 1.3 Å for 252 Ca atoms (Fig. S7, A and B). Moreover, they have similar buried dimer surface areas at the dimer interface (with the ration of buried surface area/total surface area of 861 Å²/7,628 Å² and 1,062 Å²/7,701 Å², respectively), as well as very similar interacting residues (Fig. S7, C and D). Therefore, the SO_3166A–SO_3166B dimer (also SO_3166B and SO_3165A in the ASU) was used for detailed analysis below, unless otherwise stated.

The contacting residues at the dimer interface are highly conserved across the HEPN toxin family (Fig. 3A), supporting the importance of the dimerization of SO_3166 from an evolutionary point of view. Dimerization of SO_3166 enables the formation of a deep cleft mainly composed of the α2, α4, and α3 helices between the two subunits (Fig. 3, B and C). The catalytic RX4–6H domain is located in the base of the cleft, which is formed by the interlocking of the α4 helix and the α4–α5 loop of the two HEPN domain protomers. The cleft is ~32 Å (long) × 18 Å (deep) × 13 Å (wide at its narrowest point) and the constituent residues are conserved. The width of the cleft may allow the favorable binding of a single-stranded RNA molecule (ssRNA, ~11 Å width), whereas sterically excluding double-stranded DNA (dsDNA) (Fig. S8), which are consistent with the RNA/DNA cleavage experiment results above (Fig. 1 and Fig. S2). Moreover, inspection of the surface charge distribution of the SO_3166 dimer revealed there are notable positively charged residues at the cleft.
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

Figure 3. Structural characteristics of HEPN toxin SO_3166. A, structure-based sequence alignment of SO_3166 with its representative homologs performed using Clustal X (version 1.81) and ESPript 3. They include SO_3166 from *S. oneidensis* and the homologs from *Pectobacterium carotovorum* (*P. carotovorum*), *Oleibacter marinus* (*O. marinus*), *Marinomonas aquimarina* (*M. aquimarina*), *Clostridium butyricum* (*C. butyricum*), and *Desulfotomaculum gibsoniae* (*D. gibsoniae*). The residues that were previously identified important for SO_3166 toxicity are highlighted using black arrows. The conserved Rx4–6H motif are labeled using a red box.

B, homodimer structure of SO_3166 shown as a schematic from the side view. The two subunits SO_3166A and SO_3166B are shown in cyan and pink, respectively. The Rx4–6H motifs from both subunits are highlighted in red.

C, the molecular surface representation of the SO_3166 dimer from the same view as B (blue, /H11001 7.1KT; red, /H11002 7.1KT), colored by its local electrostatic potential. The deep cleft formed by HEPN-domain dimerization of both subunits is highlighted using an ellipse.

D, surface representation of the SO_3166 dimer from the top view. The key residues (except Cys-15 and Leu-107) for SO_3166 toxicity are highlighted in red. E, structural superimposition of SO_3166A (*dark gray*) and the homolog HI0074 (*light gray*, PDB ID 1JOG). The regions containing Rx4–6H motif in SO_3166 (Val94–Asp103) and HI0074 (Asp104–Tyr113) are highlighted in cyan and orange, respectively. The conformations of the regions (especially the catalytic histidine) are remarkably different.
charged protuberances with large continuous areas in and around the cleft (Fig. 3C). These patches with continuously positive surfaces may function as the wrapping path for the substrate binding. Therefore, the cleft in the SO_3166 dimer may provide a platform for the catalytic process as an active center. Our recent site-directed mutagenesis of SO_3166 revealed that three residues (Arg-97, Asn-98, and His-102) belonging to the RX_4–6H motif, as well as six additional residues (Cys-15, His-56, Arg-70, Val-94, Leu-107, and His-118), are critical for the toxicity of SO_3166 (13). Mapping of these residues on SO_3166 showed they are located in the cleft (except Cys-15 and His-118) (Fig. 3D), indicating these residues are closely associated with SO_3166 RNase activity.

Antitoxin SO_3165 possesses an extra helix (α4) compared with its structural homologs

SO_3165 adopts a mixed α/β-fold, composed of a four-stranded twisted β-sheet (β1–β4) in the middle flanked by four helices (α1–α4) at the sides (Figs. 2A and 4A). The overall structure is well-ordered in the complex. Surface charge analysis of SO_3165 showed there is a positively charged protuberance distributed in helix α1 and around (Fig. 4B). Meanwhile, helices α2 and α4 are covered with dominantly negative charges (Fig. 4B), which mediated SO_3166 binding (discussed below). The solution behavior of purified apo SO_3165 using size exclusion chromatography indicated that the corresponding molecular mass (34 kDa) is larger than that of a monomer (15.5 kDa) (Fig. 2C and Fig. S5), suggesting a dimer may be formed in the absence of toxin SO_3166. Considering that SO_3165 exists as a monomer in the complex structure, it may be depolymerized to accommodate toxin binding in the inhibition process.

A DALI search for globally similar proteins revealed that SO_3165 has remarkable similarities with several nucleotidyl transferases (NTs) from archaea and bacteria, including MNTs with a single MNT domain and aminoglycoside NTs with a fused MNT–HEPN domain. The closest structural homolog is HI0073 (PDB ID 1NO5) with a typical MNT-fold from H. influenza, with a DALI Z-score of 8.1 and a r.m.s. deviation of 2.4 Å for 100 Cα atoms (Fig. 4C). One important difference is the extra α4 helix in SO_3165, which play an important role in SO_3166 binding and inhibition (discussed below).
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

Fig. 5. Contacts analysis (H-bonds and salt bridges) between SO_3165 and SO_3166. A, direct interactions between SO_3165A and SO_3165C δ, direct interactions between SO_3166A and SO_3165C. B, direct interactions between SO_3166A and SO_3165C. C, direct interactions between SO_3166A and SO_3165C. The residues that are previously identified important for SO_3166 toxicity are highlighted using ellipses.

The helices α2 and α4 of SO_3165 play a major role in SO_3166 binding

Structural analysis showed that SO_3165 has direct interactions with three SO_3166 molecules simultaneously. The buried surface areas of the three subunits (labeled using the subscripts A–C) at the interface are 565, 786, and 484 Å², which is up to 7.1, 9.9, and 6.1% of the total surface area (7,971 Å²), respectively. These extensive contacts include 19 H-bonds and salt bridges (Fig. 5), as well as ~100 van der Waals (VDW) contacts (not shown).

Notably, the two helices (α2 and α4) from SO_3165 are oriented toward the RX4–6H domains of the three SO_3166 molecules. These helices contribute to the majority of the hydrogen-bonding network and function as the main molecular recognition elements, thereby stabilizing the heterooligomer. The long α4 helix binds to the two subunits SO_3166A and SO_3166B simultaneously, and insert into the deep cleft of the SO_3166 dimer. The corresponding interacting elements in SO_3166 include the α2 helix (Glu-48, Asp-52, Asn-55, His-56, and Arg-59) in SO_3166A, as well as the α2 helix (Asn-55 and Arg-59), the α2–α3 loop (Pro-66, Gln-67, and Ser-69), and the α4 helix (Arg-97) in SO_3166B through extensive direct interactions (Fig. 5, A and B). The α2 helix of SO_3165 also falls close to the RX4–6H domain in one subunit of the symmetry-related SO_3166 dimer. The corresponding interacting elements in SO_3166 include the α4 helix (Lys-91, Lys-92, and Asn-98), the α2–α3 loop (Arg-70) and the α4–α5 loop (Leu-107) through extensive contacts (Fig. 5C). Moreover, the surfaces of the two helices are predominantly distributed with negative charges (Fig. 4B), which complements the positively charged regions in and around the cleft (Fig. 3C).

SO_3166 toxicity inhibition by SO_3165 in the active octameric form

Further structural analyses showed RX4–6H motif residues Arg-97, Asn-98, and His-102, as well as Arg-70 and Val-94, are located in the bottom of the cleft and extend to a continuous large area (Fig. 6, A–D). More importantly, these residues, (such as Arg-70 and Arg-97) interact with helices α2 and α4 of SO_3165, are key residues for SO_3166 toxicity. For example, the side chain (NH1 and NH2) groups of Arg-97 of SO_3166B can form a salt bridge with the side chain carboxyl group (OE2) of Glu-117 (3.0 Å). The side chain (NH2) of Arg-70 in SO_3166C can form two salt bridges with the side chain (OD1 and OD2) of Glu-117 (3.7 and 3.2 Å, respectively).

Consequently, the α4 helix can insert into the cleft formed by the SO_3166 dimer and may mask the RX4–6H catalytic domain and sterically block access of the substrate (Fig. 6, A and C). On the other side, the α2 helix (as well as the α2–α3 loop) from two SO_3165 molecules can also narrow the cleft formed by the other SO_3166 dimer formed by two symmetry-related molecules (Fig. 6, B and D). This helix may also affect the substrate binding access to the RX4–6H motif. Therefore, under the active octameric status, the complete neutralization of SO_3166 toxicities may require the cooperation of two SO_3165 molecules by their two helices simultaneously.

Functional studies confirm the important roles of the SO_3165 helices α2 and α4 in SO_3166 toxicity inhibition

Structure-based truncations of the helices α2 and α4 of SO_3165 were performed to confirm their roles in SO_3166 toxicity inhibition. The truncations including Δα4 (Gln-98–His-113), Δα4 (Leu-114–Val-125), and Δα2 (Asn-52–Ala-65) (Table S1), were constructed to test their neutralization capacity on SO_3166 toxicity by cell toxicity assays (Fig. 7A). All these variants can be produced in E. coli and have similar secondary structures to the WT (Fig. 59).

The results showed the inhibitory effect on SO_3166 toxicity is significantly affected by the Δα4 (Gln-98–His-113) truncation compared with the WT SO_3165, whereas Δα4 (Leu-114–Val-125) could still repress the toxicity to allow normal cell growth (Fig. 7, B and C). Deletion of the α2 helix also had a notable effect on SO_3166 toxicity inhibition and caused cell growth retardation and low viability. Therefore, these cell toxicity studies revealed the α2 and α4 helices play an important role in SO_3166 toxicity inhibition. The results are in good agreement with the structure above, where the regions responsible for SO_3166 toxicity (the RX4–6H motif and surroundings) are dominantly masked by α4 (Gln-98–His-113) and α2, whereas Δα4 (Leu-114–Val-125) is not involved in this blockade.

Discussion

Although the HEPN–MNT module has been previously predicted as a novel type II TA system, the neutralization mechanism and cellular targets of this family remain unclear. Site-directed mutagenesis studies have shown that the conserved
RX4–6H motif is responsible for SO_3166 toxicity, which is probably associated with its RNase activity (13). In this study, we first identified that HEPN toxin SO_3166 can specifically cleave mRNA, rather than tRNA, rRNA, or DNA. The structure of SO_3166 revealed that dimerization of the HEPN domains brings the RX4–6H motifs of the two HEPN domain protomers into close proximity, generating a composite RNase active site in the cleft. The situation is similar to that observed in type

Figure 6. Structural basis of SO_3166 toxicity suppression by SO_3165. A and B, hetero-octamer structure of the SO_3166–SO_3165 complex from side view (A) and top view (B), respectively. SO_3166 and SO_3165 are shown as surface and schematics, respectively, and their colorings are the same as described in the legend to Fig. 2. C and D, close-up views show the contacts between SO_3166 and SO_3165. The residues important for SO_3166 toxicity are highlighted in red. The helix α4 of SO_3165 can bind into the cleft formed by the SO_3166 dimer (C) and sterically block the two active sites of SO_3166 simultaneously. The helix α2 and α2–α3 loops of the symmetry-related SO_3165 dimer can bind to the edge of the cleft (D), and the narrowed cleft will probably cause the unfavorable binding of the substrate by SO_3166.

Figure 7. The roles of helices α2 and α4 of SO_3165 in the inhibition of SO_3166 toxicity by functional studies. A, three truncations: Δ(98–113) and Δ(114–125) in the helix α4 and Δ(52–65) in the helix α2 of SO_3165 are constructed for SO_3166 toxicity inhibition studies. The regions important for SO_3166 toxicity are highlighted in red as described in the legend to Fig. 6. B, the viability (CFUs/ml) of BL21 hosts carrying the pET28a-based plasmids were induced with 0.3 mM IPTG added at A_600 = 0.1. Three independent cultures were conducted, error bars indicate mean ± S.E. (n = 3). C, viabilities were tested after induced for 4 h. Three independent cultures of each strain were tested and only representative images are shown.

Neutralization mechanism of HEPN-MNT toxin–antitoxin system
III-A CRISPR-associated protein Csm6, composed of the C-terminal HEPN domain and N-terminal CRISPR-associated Rdomain of RNase L and the RNase domains of RloC and the toxicity of SO_3166 (13). Site-directed mutagenesis of the site RNA asymmetrically.

composite symmetric active center in SO_3166 that binds the substrate RNA asymmetrically. The conserved RX4–6H motif has emerged as the most strongly conserved feature of the HEPN domain. Our recent site-directed mutagenesis on SO_3166 revealed three residues (Arg-97, Asn-98, and His-102) within the motif are critical for the toxicity of SO_3166 (13). Site-directed mutagenesis of the KEN domain of RNase L and the RNase domains of RloC and PrrC have shown that the arginine and histidine corresponding to the conserved R and H in the RX4–6H motif are essential for their respective nuclease activities (18–20).

In our complex structure, the two RX4–6H catalytic motifs located in the cleft are sterically blocked by two helices (α2 and α4) of SO_3165. Structure-based mutagenesis further confirmed these helices play an important role in SO_3166-binding and toxicity inhibition. More importantly, the RX4–6H motif is usually lost in HEPN–MNT-fused aminoglycoside NTases (such as PDB IDs 1KNY, 1L8T, and 4CS6). Although the structures of the HEPN and MNT domains in SO_3166 and SO_3165 show remarkable similarities with these NTases, SO_3166–SO_3165 does not cause resistance to aminoglycoside antibiotics. Therefore, the presence of the RX4–6H motif may indicate that SO_3166–SO_3165 functions as a TA system with RNase activity in a HEPN toxin, rather than as an aminoglycoside NTase for antibiotic resistance via the nucleotidylation of antibiotic molecules.

Our structure-functional studies on SO_3166–SO_3165 showed the antitoxin SO_3165 may efficiently inhibit SO_3166 toxicity using its two helices, especially the C-terminal α4, which mediate the formation of the octameric complex at a ratio of 2:6. The α4 helix can directly insert into the cleft, and may block the composite RNase active site in the catalytic RX4–6H motifs of the two HEPN domain protomers. The α2 helix from two symmetry-related SO_3165 molecules can bind to a region close to the RX4–6H motifs on the edge of the cleft from opposite directions to reduce the cleft size. Therefore, the α2 helix may also affect the substrate RNA binding. Our functional studies on truncations of the two helices confirmed the strategies in SO_3166 toxicity inhibition under the active octameric status. Meanwhile, the N-terminal region distributed with dominantly positive charges (Fig. 4B) may be associated with the promoter-binding of the TA operon to repress its activity (13). The relative contributions of neutralization and transcriptional regulation to the inhibition of the toxic phenotype under different conditions warrant further investigations.

The closest structural homologue of SO_3165–SO_3166 is HI0073–HI0074 from H. influenza, which harbors a typical MNT-fold and HEPT-fold, respectively (Figs. 3E and 4C). Therefore, the HI0073–HI0074 pair may constitute a type II TA system like SO_3165–SO_3166. The previous study showed HI0073–HI0074 can form a tetramer with a 2:2 molecular ratio determined by size exclusion chromatography (21). Structural comparison of SO_3165 with HI0073 revealed the presence of the extra helix α4 in SO_3165 that can bind two SO_3166 simultaneously. Unlike the dual role of helices α4 and α2 in SO_3166 binding, only the helix α2 of HI0073 is probably responsible for HI0074 toxicity inhibition. Therefore, variable neutralization mechanisms of the HEPN–MNT TA family may exist depending on the presence or absence of the C-terminal α4 in MNT antitoxin.

Taken together, our structure–function studies on the SO_3166–SO_3165 HEPN–MNT pair present for the first time the structure of the complex with a unique organization, and reveal that one SO_3165 molecule can recognize and inhibit the toxicity of three SO_3166 molecules by sterically blocking the RX4–6H catalytic domains simultaneously. This work sheds light on the molecular architecture of the HEPN–MNT TA complex and the functional organization of its constituent domains.

**Experimental procedures**

**Construction of plasmids and bacterial strains**

Bacterial strains and plasmids used in this study are listed in Table S1. The pCA24N-SO_3166 and pHGE-SO_3166 plasmids were transformed into E. coli BW25113 and S. oneidensis MR-1 for SO_3166 production for RNA cleavage assay, respectively (13). The recombinant plasmids pET28b-SO_3165–SO_3166 and pET28b-SO_3165 were transformed into BL21(DE3) cells, respectively, for the expression and purification used in crystallography, small-angle X-ray scattering, and CD. Truncations of SO_3165 on the plasmid pET28a-SO_3165–SO_3166 (or pET28a-SO_3165) were performed by one-step PCR according to the QuiikChange site-directed mutagenesis strategy (Stratagene). The primers are listed in Table S2.

**SO_3166 RNA cleavage assay**

To conduct mRNA cleavage assay, ompA mRNA was used as substrate for in vitro assay. For the synthesis of ompA mRNAs (1–306 nt from ompA), PCR products were obtained using the primers in Table S2. The PCR products were used as template for in vitro transcription with T7 RNA polymerase (New England Biolabs). The T7 RNA polymerase promoter sequence was included in the forward primers of ompA-T7-F. PCR products were gel-purified, and 0.5 to 1 µg of the PCR product was used as the template for the in vitro RNA reaction following the instructions. SO_3166 and the SO_3165–SO_3166 complex were purified as previously described (13). The reaction mixture for the SO_3166 RNase cleavage assay (8 µl) contained 0.5 µg of RNA, 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 25 mM EDTA, 60 mM KCl, 30 mM MgCl2, and 2 µg of purified SO_3166 protein or SO_3165–SO_3166 complex. For the RNA cleavage assay, E. coli total tRNAs (Roche Applied Science) were used as substrate. The reaction mixture was incubated at 37 °C for 20 or 40 min separately. To inactivate the SO_3166 and SO_3165–SO_3165 complexes, protein samples were heated at 95 °C for 10 min and were cooled before adding to the reactions. The reaction products from ompA mRNA and tRNAs were resolved by 15% TBE-urea gels (Invitrogen). To conduct rRNA cleavage
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

To conduct DNA cleavage assay, plasmid pCA24N, λ dsDNA, and M13 ssDNA were used as substrate for the in vitro assay, respectively. Plasmid DNA (pCA24N) was isolated from strain E. coli BW25113/pCA24N, and λ dsDNA and M13 ssDNA were ordered from New England Biolabs. The reaction mixture for the SO_3166 DNA cleavage assay (15 μl) contained 1.0 μg of DNA, 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 25 mM EDTA, 20 mM CaCl₂, 20 mM MgCl₂, and 2 μg of purified SO_3166 protein or 1 μl of 2000 units/ml of DNase I (reaction devoid of EDTA). The reaction mixture was incubated at 37 °C for 0, 20, or 40 min separately. To inactivate SO_3166, protein samples were heated at 95 °C for 10 min and were cooled before adding to the reactions. The reaction was stopped by the addition of a stop solution (25% glycerol, 0.5% SDS, 0.05% bromphenol blue, and 50 mM EDTA) and was analyzed by electrophoresis on 1% agarose gels stained with SYBR safe (Invitrogen).

Protein expression and purification

Bacterial cells harboring pET28b-SO_3165–SO_3166 were grown to A_600 of 0.6 in LB media at 37 °C in the presence of 50 mg/ml of kanamycin. Induction of protein expression was initiated by adding IPTG to the culture to a final concentration of 1 mM, and cells were grown at 16 °C. Cells were pelleted after 18 h by centrifugation at 3500 rpm for 35 min at 4 °C. Cell pellets were suspended in the buffer containing 20 mM Tris (pH 8.0), 250 mM NaCl, 10 μg/ml of DNase, and 1 mM β-mercaptoethanol. The cell suspension was disrupted by a high-pressure homogenizer and then centrifuged at 16,000 × g for 50 min at 4 °C. The supernatant was then loaded onto a nickel-nitritoltri-acetic acid column that was pre-equilibrated with 20 mM Tris (pH 8.0), 500 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol buffer. The His-tagged protein was eluted in 20 mM Tris (pH 8.0), 250 mM NaCl, and 250 mM imidazole. The complex was further purified by a Hitrap Q column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol (DTT), pH 8.0, with a linear gradient of 100–1000 mM NaCl in 20 mM Tris-HCl (pH 8.0). Next the protein was purified by Superdex-200 chromatography on an ÄKTA Prime system (GE Healthcare) to obtain highly pure SO_3165–SO_3166 complex. The gel filtration buffer contained 20 mM Tris (pH 8.0), 100 mM NaCl and 1 mM DTT. The eluted fractions in all purification steps were analyzed by SDS-PAGE. The full-length SO_3165 gene was inserted into expression vectors pET28b, then pET28b-SO_3165 was transformed into BL21(DE3). SO_3165 was overexpressed and purified using the same producers described above.

The Se-Met SO_3165–SO_3166 complex were produced in minimal medium supplemented with 100 mg/liter of lysine, phenylalanine, and threonine, and 50 mg/liter of isoleucine, leucine, valine, and selenomethionine. The Se-Met protein production and purification were the same as described above.

Crystallization, data collection, structure determination, and refinement

The purified complex was concentrated to ~ 10 mg/ml using a Millipore Amicon Ultra apparatus. The initial crystallization conditions were obtained through utilization of several sparse matrix screens (Hampton Research, USA) with the sitting drop vapor diffusion method at room temperature after 2–3 days. Crystal quality was optimized by adjust the concentration of the precipitant and buffer. The best crystal of SO_3165–SO_3166 was obtained in solution with 0.1 M Bicine (pH 8.6), 14% PEG6000, and 8% ethylene glycol at 20 °C.

The diffraction data from a single crystal of selenomethionine-substituted protein were collected on the beamline station BL19 U of SSRF (Shanghai Synchrotron Radiation Facility) using a Pilatus 6 M detector at a wavelength of 0.9788 Å. The total oscillation was 360° with 1° per image and the exposure time was 1-s per image. Before data collection, the crystals were soaked in the reservoir solution supplemented with 20% (v/v) glycerol for a few seconds and then flash-frozen in liquid nitrogen. All the data were processed by XDS (22). The Se-Met crystal structure of the SO_3166–SO_3165 complex was determined by the single wavelength anomalous dispersion method. The selenium atoms were located by the program Shelxd and then used to calculate the initial phases in Shelxe (23). The phases from Shelxe were improved in Resolve (24) and then used in Buccaneer (25) for model building. All structures above were refined with the program Phenix.refine (26) and manually corrected in Coot (27). The qualities of the final models were validated with the program MolProbity (28). Refinement statistics and model parameters are given in Table 1. The program PyMOL was used to prepare structural figures.

SAXS and low resolution model building

Synchrotron SAXS experiments were performed on the BL19U2 station of SSRF, equipped with a PILATUS 1 M detector (DECTRIS, Switzerland) (Table S3). The scattering was recorded in the range of the momentum transfer 0.018 Å⁻¹ < s < 0.321 Å⁻¹, where s = 4πsinθ/λ, 2θ is the scattering angle, and λ = 1.03 Å is the X-ray wavelength. The solutions were loaded in a flow-through quartz capillary cell with a diameter of 1 mm and a wall thickness of 10 μm, temperature controlled at 22 °C. The radiation damage was checked with 20 successive exposures of 1 s. To exclude concentration dependence, three different concentrations, 1, 3, and 5 mg/ml of purified SO_3166–SO_3165 complex (corresponding to 8.3, 25.0, and 41.7 μM, respectively) were prepared and measured. All SAXS data were processed with the program package ATSAS (29). The scattering of buffers were subtracted from that of the samples, and the forward scattering I(0) and the radius of gyration R_g were derived by the Guinier approximation I(q) = I(0) exp(−q² R_g²/3) for q R_g < 1.3 using PRIMUS (30). The pair-distance distribution functions, p(r) and the maximal dimension of the macromolecule, D_max were calculated using indirect Fourier transformation and the program GNOM (31). To model the structures of the SO_3165 and SO_3166–SO_3165,
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

10 independent models were generated with the programs DAMMIF or GASBOR (32) in fast mode, compared and aligned with SUPCOMB (33), and averaged with DAMAVER (34) to determine common structural features and representative shapes. Theoretical scattering patterns I(\theta) from the available high resolution crystal structures were calculated by a program CRYSOL (35).

Circular dichroism (CD) spectroscopy

Purified SO_3165 (WT and its variants) was loaded onto a Superdex 200 column (GE Healthcare) equilibrated with PBS buffer (pH 8.0). The elution containing SO_3165 was subsequently concentrated to 0.5–1.0 mg/ml. The CD spectra were measured on the 4B8 station of Beijing Synchrotron Radiation Facility (BSRF) at 1-nm bandwidth with a 1-nm step resolution from 170 to 250 nm at 25 °C. The data were averaged over eight accumulations. The data are processed by CDtool (36) and further analyzed by Dichwr web (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

Cell toxicity assay

E. coli BL21 harboring pET28a-based plasmids were grown in Luria-Bertani (LB) medium supplemented with 50 μg/ml of kanamycin at 37 °C and 0.3 mM IPTG were added at A_600nm = 0.1. The viability (CFUs/ml) was measured at different time points for individual cell cultures. Viability was calculated by serially diluting the cells in 10-fold steps and plated onto the LB agar. The plates were prepared for pictures after incubation at 37 °C for 12 h. Triplicate measurements were performed and similar results were obtained for each measurement unless stated.

Data availability

The atomic coordinates and structure factors of the SO_3166–SO_3165 complex have been deposited in the RCSB Protein Data Bank with PDB code 5YEP.

Acknowledgments—We thank the staff of the beamline stations 1W2B and 4B8 of Beijing Synchrotron Radiation Facility (BSRF), and BL17U and BL19U2 stations of Shanghai Synchrotron Radiation Facility (SSRF) for providing technical support and for many fruitful discussions.

References

1. Gerdes, K., Christensen, S. K., and Løbner-Olesen, A. (2005) Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. 3, 371–382 CrossRef Medline
2. Hayes, F. (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301, 1496–1499 CrossRef Medline
3. Page, R., and Peti, W. (2016) Toxin-antitoxin systems in bacterial growth arrest and persistence. Nat. Chem. Biol. 12, 208–214 CrossRef Medline
4. Hayes, F., and Van Melderen, L. (2011) Toxins-antitoxins: diversity, evolution and function. Crit. Rev. Biochem. Mol. Biol. 46, 386–408 CrossRef Medline
5. Yamaguchi, Y., and Inouye, M. (2011) Regulation of growth and death in Escherichia coli by toxin-antitoxin systems. Nat. Rev. Microbiol. 9, 779–790 CrossRef Medline
6. Lobato-Márquez, D., Diaz-Orejas, R., and García- Del Portillo, F. (2016) Toxin-antitoxin and bacterial virulence. FEMS Microbiol. Rev. 40, 592–609 CrossRef Medline
7. Wang, X., and Wood, T. K. (2011) Toxin-antitoxin systems influence biofilm and persist cell formation and the general stress response. Appl. Environ. Microbiol. 77, 5577–5583 CrossRef Medline
8. Grygorenko, M., Erlandsen, H., and Godzik, A. (2003) HEPN: a common domain in bacterial drug resistance and human neurodegenerative proteins. Trends Biochem. Sci. 28, 224–226 CrossRef Medline
9. Aravind, L., and Koonin, E. V. (1999) DNA polymerase β-like nucleotidyltransferase superfamily: identification of three new families, classification and evolutionary history. Nucleic Acids Res. 27, 1609–1618 CrossRef Medline
10. Makarova, K. S., Wolf, Y. I., and Koonin, E. V. (2009) Comprehensive comparative-genomic analysis of type 2 toxin-antitoxin systems and related mobile stress response systems in prokaryotes. Biol. Direct 4, 19 CrossRef Medline
11. Kimelman, A., Levy, A., Sbero, H., Kidron, S., Leavitt, A., Amitai, G., Yoder-Himes, D. R., Wurtzel, O., Zhu, Y., Rubin, E. M., and Sorek, R. (2012) A vast collection of microbiological that are toxic to bacteria. Genome Res. 22, 802–809 CrossRef Medline
12. Anantharaman, V., Makarova, K. S., Burroughs, A. M., Koonin, E. V., and Aravind, L. (2013) Comprehensive analysis of the HEPN superfamily: identification of novel roles in intra-genomic conflicts, defense, pathogenesis and RNA processing. Biol. Direct. 8, 15
13. Yao, J., Guo, Y., Zeng, Z., Liu, X., Shi, F., and Wang, X. (2015) Identification and characterization of a HEPN-MNT family type II toxin-antitoxin in Shewanella oneidensis. Microbiol. Biotechnol. 8, 961–973 CrossRef Medline
14. Kozlov, G., Denisov, A. Y., Girard, M., Dicaire, M. J., Hamlin, J., McPherson, P. S., Brais, B., and Gehring, K. (2011) Structural basis of defects in the sasC HEps domain responsible for autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS). J. Biol. Chem. 286, 20497–20412 CrossRef Medline
15. Pedersen, L. C., Benning, M. M., and Holden, H. M. (1995) Structural investigation of the antibiotic and ATP-binding sites in kanamycin nucleotidyltransferase. Biochemistry 34, 13050–13111 CrossRef Medline
16. Lehmann, C., Pullalarevu, S., Krajevski, W., Willis, M. A., Galkin, A., Howard, A., and Herzberg, O. (2005) Structure of H10073 from Haemophilus influenzae, the nucleotide-binding domain of a two-protein nucleotidyl transferase. Proteins 60, 807–811 CrossRef Medline
17. Niewoehner, O., and Jinek, M. (2016) Structural basis for the endoribonuclease activity of the type III-A CRISPR-associated protein Cas9. RNA 22, 318–329 CrossRef Medline
18. Davidov, E., and Kaufmann, G. (2008) RloC: a wobble nucleotide-excising and zinc-responsive bacterial tRNase. Mol. Microbiol. 69, 1560–1574 CrossRef Medline
19. Lee, K. P., Dey, M., Neculai, D., Cao, C., Dever, T. E., and Sicheri, F. (2008) Structure of the dual enzyme Ire1 reveals the basis for catalysis and regulation in nonconventional RNA splicing. Cell 132, 89–100 CrossRef Medline
20. Meineke, B., and Shuman, S. (2012) Structure-function relations in the NTase domain of the antiviral tRNA ribotoxin Escherichia coli PcrC. Virology 427, 144–150 CrossRef Medline
21. Lehmann, C., Lim, K., Chalamesetty, V. R., Krajevski, W., Melamud, E., Galkin, A., Howard, A., Kelman, Z., Reddy, P. T., Murzin A. G., and Herzberg, O. (2003) The H10073/H10074 protein pair from Haemophilus influenzae, the nucleotide-binding domain of a two-protein nucleotidyl transferase. Proteins 50, 286–299 CrossRef Medline
22. Kabsch, W. (2010) Integration, scaling, space-group assignment and post-refinement. Acta Crystallogr. Sect. D Biol. Crystallogr. 66, 133–144 CrossRef
Neutralization mechanism of HEPN-MNT toxin–antitoxin system

23. Sheldrick, G. M. (2010) Experimental phasing with SHELXC/D/E: combining chain tracing with density modification. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 479–485 CrossRef

24. Terwilliger, T. C. (2000) Maximum-likelihood density modification. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **56**, 965–972 CrossRef

25. Cowtan, K. (2006) The Buccaneer software for automated model building: 1. tracing protein chains. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **62**, 1002–1011 CrossRef

26. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **58**, 1948–1954 CrossRef

27. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 486–501 CrossRef

28. Chen, V. B., Arendall W. B 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 12–21 CrossRef

29. Petoukhov, M. V., Franke, D., Shkumatov, A. V., Tria, G., Kikhney, A. G., Gajda, M., Gorba, C., Mertens, H. D., Konarev, P. V., and Svergun, D. I. (2012) New developments in the ATSAS program package for small-angle scattering data analysis. *J. Appl. Crystallogr.* **45**, 342–350 CrossRef Medline

30. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1277–1282 CrossRef

31. Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr.* **25**, 495–503 CrossRef

32. Svergun, D. I., Petoukhov, M. V., and Koch, M. H. (2001) Determination of domain structure of proteins from X-ray solution scattering. *Biophys. J.* **80**, 2946–2953 CrossRef Medline

33. Kozin, M. B., and Svergun, D. I. (2001) Automated matching of high- and low-resolution structural models. *J. Appl. Crystallogr.* **34**, 33–41 CrossRef

34. Volkov, V. V., and Svergun, D. I. (2003) Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Crystallogr.* **36**, 860–864 CrossRef

35. Svergun, D., Barberato, C., and Koch, M. H. J. (1995) CRYSOl: a program to evaluate X-ray solution scattering of biological macromolecules from atomic coordinates. *J. Appl. Crystallogr.* **28**, 768–773 CrossRef

36. Lees, J. G., Smith, B. R., Wien, F., Miles, A. J., and Wallace, B. A. (2004) CDTool: an integrated software package for circular dichroism spectroscopic data processing, analysis, and archiving. *Anal. Biochem.* **332**, 285–289 CrossRef Medline