The first structure of Polarity Suppression protein, Psu from Enterobacteria phage P4, reveals a novel fold and a knotted dimer

Ramanuj Banerjee, Seema Nath, Amitabh Ranjan, Susmita Khamrui, Bibhusita Pani, Ranjan Sen and Udayaditya Sen

1 Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, 1/AF, Bidhannagar, Kolkata-700064, India
2 Laboratory of Transcription Biology, Center for DNA Fingerprinting and Diagnostics, Tujguda Complex, 4-1-714 Mojamjahi Road, Nampally, Hyderabad, India

To whom correspondence should be addressed: Udayaditya Sen, PhD, Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, 1/AF, Bidhannagar, Kolkata-700064, India, phone: 91-33-233-0379, fax: 91-33-2337-4637, email: udayaditya.sen@saha.ac.in

CAPSULE

Background: Phage P4 Psu protein is a capsid decoration protein with unknown structure.

Results: The first structure of Psu reveals a novel fold and a knotted dimer.

Conclusion: The V-shaped molecular architecture is important for capsid binding.

Significance: The structure of Psu will help to design peptide fragments which can be used as drugs against the bacterial transcription machinery.

SUMMARY

Psu is a capsid decoration protein of bacteriophage P4 and acts as an antiterminator of Rho-dependent transcription termination in bacteria. So far, no structures have been reported for the Psu protein or its homologues. Here we report the first structure of Psu solved by Hg-SAD method, which reveals that Psu exists as a knotted homodimer and is first of its kind in nature. Each monomer of Psu attains a novel fold around a tight coiled-coil motif. CD spectroscopy and the structure of an engineered disulphide-bridged Psu derivative reveal that the protein folds reversibly and re-assembles by itself into the knotted dimeric conformation without the requirement of any chaperone. This structure would help to explain the functional properties of the protein and can be used as a template to design a minimal peptide fragment that can be used as a drug against Rho dependent transcription termination in bacteria.

INTRODUCTION

Factor dependent transcription termination in bacteria is mediated by a hexameric RNA-dependent ATPase protein, Rho (1). This mode of transcription termination causes polarity in gene expression pattern. Psu, a polarity suppression protein, is the first reported antiterminator of Rho specific transcription termination in bacteria which also acts as a capsid decoration protein in bacteriophage P4 (2, 3). Protein moonlighting is a phenomenon in which a protein can perform more than one function. So, Psu can be categorized as a moonlighting protein due to its dual activities. Upon binding to the Rho hexamer, Psu reduces the ATPase activity of Rho without affecting its primary RNA binding activities (4). Furthermore, Psu interacts with P4 phage capsid protein and stabilizes P4 against heat treatment (5). However, the relation between the heat stabilization by Psu and its polarity suppression, if any, is yet to be established.

Circular dichroism studies demonstrate that Psu is predominantly an α-helical protein and size exclusion chromatography along with in vitro cross-linking experiments reveal that Psu exists as a dimer in solution (4). Extensive mutational and cross-linking studies suggests that the interaction of Psu with Rho is direct and specific, mediated by the C-terminal tail of Psu, whereas the N-terminal domain of Psu maintains the conformational integrity of the C-terminal tail (6). However, the atomic structure of Psu, its domain organization and assembly remains unknown.

Here we report the first structure of Psu (21 kDa) consisting of a symmetric ‘V’ shaped homodimer. The striking feature of the dimeric interface is that it incorporates a significant portion of the polypeptide chain in a knotted conformation which is extensive and exclusively hydrophobic in nature. Each monomer of Psu attains a novel protein fold with a long N-terminal coiled-coil motif wrapped by the C-terminal helices, while the central region self assembles into the knot with another monomer to form the dimer. We have validated the existence of
this knotted dimeric conformation of Psu in solution by solving the structure of T123C mutant on no-cys construct of Psu (T123C-ΔCys-Psu). In this mutant structure, 123C from each monomer faces each other at the dimeric interface and forms a disulphide bridge under oxidizing condition. We have also demonstrated that the denatured Psu can self assemble into the knot spontaneously in solution. Furthermore, the knotted ‘V’ shaped architecture of the Psu dimer, with its inherent tensile properties makes Psu suitable to interact with the hexameric capsomere on phage capsid to block DNA leakage.

**EXPERIMENTAL PROCEDURES**

**Preparation of WT Psu and T123C-ΔCys-Psu mutant**- The wild type Psu protein was expressed and purified by a three step process involving a 25% ammonium sulphate precipitation in buffer containing 10 mM Tris–HCl pH 7.9, 0.1 mM EDTA, 0.1 mM DTT and 5% (v/v) glycerol followed by purification through Q-sepharose (GE Healthcare) and CM sepharose columns (GE Healthcare). Psu was eluted between 50 and 150 mM NaCl and the eluted fractions were stored in 20 mM Tris–HCl pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol and 100 mM NaCl. This procedure yielded about 95% pure protein. For the preparation of the T123C-ΔCys-Psu mutant, a zero-cys Psu (ΔCys-Psu) was prepared by mutating all the cysteines (C13 and C117) to serine. The T123C change was made by site-directed mutagenesis on the ΔCys-Psu and was purified in a similar way as WT.

**Protein crystallization, data collection and structure determination of wild type Psu and Psu T123C**- The crystallization trials of Psu was carried out at 277 and 293 K. Typically, 3 ml protein solution (5 mg/ml) was mixed with 2 ml reservoir solution containing 7.5%(w/v) PEG 6000, 5% (v/v) glycerol in 0.1 M MES pH 6.0 at 277 K. Cube shaped crystals appeared within a couple of days. Data was collected upto 3 Å in conditions similar to the WT Psu. For phasing, the coordinates of WT Psu was used for molecular replacement with Phaser (13) in CCP4 (14). Model building was done with Phenix refine. The data processing and refinement statistics are summarized in **Table 1**.

**Structural analysis**- Average B-factors for each residue were calculated using Baverage in CCP4 (14). The oligomeric state of the protein was analysed using the PISA webserver (15). Figures were prepared using Pymol (16). Flexible regions of the molecule were identified by Normal mode analysis with NOMAD-Ref server (17) using the elastic network model and default settings for the other parameters. Briefly, all atoms were taken for the analysis of total 16 modes with a cut-off value of 10Å for mode calculation and 5Å as elastic constant.

**Circular Dichroism Spectroscopy**- Far-UV CD spectra were acquired for protein concentrations of 1 mg/ml (0.047 mM) in 10 mM sodium phosphate (pH 7.4) at 25°C using a BioLogic Science Instruments (France) spectropolarimeter and cuvettes of 1 mm path length. For denaturation studies, the protein was incubated in varying concentrations of GdmCl (1, 1.5, 2, 2.5 and 3 M) at 25°C. The protein was initially standardized for the minimum GdmCl concentration at which denaturation reaches saturation. Initial denaturation with 6 M, 5 M, 4 M and 3 M gave the same CD profiles. So, maximum of...
The N-terminal motif consists of two long antiparallel helices (‘CC stem’ having α1 and α2), followed by two helices involved in the dimerisation (‘DR knot’; α3 and α4) and finally a flexible C-terminal tail comprising three helices (‘CT belt’; α5–α7) which folds back and interacts with the N-terminal helices. Polypeptide chain (135–147) that connects the helix α4 and the ‘CT belt’ is oriented in a fashion such that it leaves a hole in the monomeric structure (Fig. 1a). A search of the Protein Data Bank (PDB) using the DALI server (19) and PDBeFold (20) did not produce any significant match with the Psu structure designating it to be a new fold.

**RESULTS**

**Psu has a novel structural fold** - The structure of Psu was solved by Hg-SAD method at 2.04 Å (Table 1). Psu crystallizes as a dimer where each asymmetric unit accommodates one Psu monomer. The refined model of Psu includes 187 of 190 residues with unambiguous electron density (Data not shown) and excellent stereochemistry (Table 1). Psu monomer adopts a ‘golf stick’ like structure and is characterized by seven helices (α1–α7) (Fig. 1a). The N-terminal motif consists of two long antiparallel helices (‘CC stem’ having α1 and α2), followed by two helices involved in the dimerisation (‘DR knot’; α3 and α4) and finally a flexible C-terminal tail comprising three helices (‘CT belt’; α5–α7) which folds back and interacts with the N-terminal helices. Polypeptide chain (135–147) that connects the helix α4 and the ‘CT belt’ is oriented in a fashion such that it leaves a hole in the monomeric structure (Fig. 1a). A search of the Protein Data Bank (PDB) using the DALI server (19) and PDBeFold (20) did not produce any significant match with the Psu structure designating it to be a new fold.

**A coiled-coil conformation at the N-terminal** - The ‘CC stem’ is composed of two long antiparallel helices, α1 (residues 3–39) and α2 (residues 45–91) connected by an inverse γ turn (residues 43–45) (Fig. 1a). Helices α1 and α2 form a two stranded antiparallel coiled-coil motif with five consecutive heptad repeats of amino acid residues, with the heptad positions ‘abcdefg’ (Fig. 1b) bearing the signature of coiled-coil motifs (hxxhcxc; where ‘h’ designates hydrophobic residues and ‘c’ charged residues). Heptad repeat of hydrophobic residues at position ‘a’, hallmark of a coiled-coil-motif, are observed at L6, C13, W20, L27, Y34 positions of the helix α1 and at L48, I55, V62, Y69, V76 positions of the helix α2 (Fig. 1b and 1c). At the interface of the two helices, hydrophobic residues at position ‘a’ of one helix line up with the residues at position ‘d’ of the other helix to form a hydrophobic core that stabilizes the coiled-coil structure (Fig. 1b and 1c). Some variations are observed with respect to the proper positioning of the hydrophobic amino acids at ‘a’ or ‘d’ and the polar amino acids at ‘e’ or ‘g’. Interestingly, these variations are used to further stabilize the coiled-coil either through formation of polar contacts or formation of salt bridge interactions. Polar/charged residues at ‘a’ or ‘d’ packs their hydrophobic portion in the core of the coiled-coil (Fig. 1d). For example, polar side chain of N16 (‘d’) forms hydrogen bond with S72 (‘d’) (Fig. 1d) at one end of the coiled-coil. At the middle of the motif, two positively charged residues, R23 (‘d’) and R58 (‘d’) come close to each other and this destabilization is converted to a favorable one through salt bridge interactions by E26 (‘e’) and Q61 (‘e’) (Fig. 1d). Near the inverse γ turn connector between α1 and α2, R47 (e) and R36 (c) makes strong salt bridge interactions with E33 (e) (Fig. 1d). Apart from these, there are additional violations in amino acid type and these are engaged to make specific interactions with the ‘CT belt’ residues during wrapping the coiled-coil (Fig. 1e and 1f).

**Fluorescence Spectroscopy** - Steady state fluorescence studies were performed using a Varian Cary Eclipse fluorescence spectrophotometer for protein concentrations of 1mg/ml (0.047) in 10 mM sodium phosphate (pH 7.4) at 25°C and cuvettes of 4mm pathlength. For denaturation studies, the protein was incubated in varying concentrations of GdmCl (1, 1.5, 2, 2.5 and 3M) at 25°C for 20 minutes each. Fluorescence emission from Psu tryptophans were measured using an excitation at 295 nm and the emitted intensity was recorded at 340 nm. In all experiments, contributions of the buffer to the spectra were subtracted before plotting the spectra.

**Cross Linking and SDS-PAGE** - For the cross-linking experiments, T123C-∆Cys-Psu was incubated with the Cu-P (18) before crystallization to induce disulphide bond between the two cysteines. The disulphide bridge can be formed in the presence of Cu-P only if they are quite close to each other (~6 Å) in the tertiary/quaternary structure and are correctly oriented. The concentrations of the cross-linker and T123C-∆Cys-Psu were 5 mM and 80 µM respectively. T123C-∆Cys-Psu and Cu-P were mixed in phosphate buffer (10 mM NaH₂PO₄, pH 7.4) devoid of any reducing agents and incubation was continued for 30 min at 25°C. Non-reducing SDS-sample buffer was added to the cross-linking reactions, which were subjected to SDS-PAGE (15% polyacrylamide gel).
The amino acids from the ‘CT-belt’ makes several ionic and ‘knob/hole’ type hydrophobic interactions with the amino acids from ‘CC-stem’. D146 of helix \( \alpha 5 \) strongly interacts with R84 and S80 of \( \alpha 2 \) whereas residues I149, T152 and I153 makes a hydrophobic hole to pack F10 of \( \alpha 1 \) (Fig. 1e). L150 and I153 of \( \alpha 5 \) form a proper hole to accommodate the side chain of V76. P157 is nicely packed with the amino acids from both helix \( \alpha 1 \) (C13) and helix \( \alpha 2 \) (V76, I70 and Y69) (Fig. 1e). D164 of \( \alpha 6 \) makes strong interactions with K24 and W20 of \( \alpha 1 \) (Fig. 1e). F177 and F169 of \( \alpha 7 \) produce an environment suitable to pack W60 (‘f’) of \( \alpha 2 \) (Fig. 1f) whereas N174 of \( \alpha 7 \) forms H-bonds with W60 and Q64 (Fig. 1f). R187 near the end of helix \( \alpha 7 \) makes a salt bridge with E50 (c) of \( \alpha 2 \) (Fig. 1f). All these interactions, therefore not only reinforce the C-terminal belt in the stabilization of the coiled-coil domain but also clamp the ‘CT belt’ tightly with the ‘CC stem’.

**A knotted biological assembly adopted by Psu dimer**- Crystal structure of Psu demonstrates a symmetric ‘V’ shaped homodimer which is depicted in three different orientations (Fig. 2a-c). The striking feature of this dimer is that the two monomers are knotted, which is shown schematically in Fig. 2d. This knotted dimeric self-assembly recruits part of \( \alpha 2 \), helices \( \alpha 3-\alpha 4 \) and the long loop connecting \( \alpha 4 \) to \( \alpha 5 \) from both monomers which are related by a twofold at the dimeric interface. As mentioned earlier, there is a hole in the monomeric Psu structure (Fig. 1a and Fig. 2f) and during dimerisation, \( \alpha 3 \) of one monomer passes through the hole of the other (Fig. 2f). In fact, the helix \( \alpha 2 \) needs to take a sharp turn of \( \sim 80^\circ \) at G93 to project the helix \( \alpha 5 \) (Fig. 2f). At this turning point, G93 faces the bulky side chain of the conserved W133 of its knotting partner. Interestingly, \( \alpha 3 \) bears a stretch of highly conserved residues ‘GXXLAAALAP’ in Psu homologues (Fig. 3). Region beyond P102 is a short loop followed by helix \( \alpha 4 \) which is disposed in an antiparallel fashion to the \( \alpha 4 \) of the other monomer at an intersecting angle of \( \sim 45^\circ \). The loop connecting \( \alpha 4 \) to \( \alpha 5 \) encompasses \( \alpha 3 \) of the knotting partner and finally the ‘CT belt’ wraps its own ‘CC stem’ (Fig. 2f).

During this process, a dimeric knot is formed that incorporates about 25% of the polypeptide chain from each monomer. The knotted interface is devoid of any water molecule and harbors exclusively hydrophobic environment with several residues from \( \alpha 2 \), \( \alpha 3 \) and \( \alpha 4 \) (Fig. 4a). At the interface, T123 lies in close proximity to the T123 of its knotting partner with the two fold symmetry axis present in between (Fig. 2f). Presence of this twofold symmetry is also evident from Fig. 4a. There are only two hydrogen bonds from each monomer within this huge interface. The dimeric self assembly of Psu is also supported by PISA (15) where upon dimer formation each monomer buries about \( \sim 5500 \text{ Å}^2 \) surface area with a \( \Delta G_{\text{diss}} \) value of 39 Kcal/mol. Average B-factors and Normal mode analysis for the residues indicates that the knotted region is more stable than the ‘CT-belt’ (Fig. 4b, 4c).

**Psu also adopts the knotted dimeric conformation in solution**- In order to prove the existence of the knotted dimeric conformation in solution and not as a crystallographic artifact, we prepared a no-cys construct of Psu (\( \Delta \text{Cys-Psu} \)) and then mutated its T123 to Cys (hereafter termed as T123C-\( \Delta \text{Cys-Psu} \)). T123 is located at a concave surface of Psu and just by simple association of the monomers two T123 residues cannot come in close proximity. But at the knotted dimeric interface, T123 of one monomer lies in close proximity to T123 of the other monomer and their side chains face each other (Fig. 2f). We reasoned that, if the same dimer is formed in solution for T123C-\( \Delta \text{Cys-Psu} \), then C123 of the two monomers would come close in the quaternary structure and facilitate the formation of a disulphide bond. H89 also comes close to its knotted counterpart, but His\( \rightarrow \)Cys mutation may disrupt other interactions at the dimeric interface which might be important for the dimer stability. The crystal structure of Cu-P treated T23C-\( \Delta \text{Cys-Psu} \) showed that C123 adopts two conformations of equal population and one of them is able to form the disulphide bridge, validating the formation of the dimer in solution (Fig. 4d). Cu-P treated T123C-\( \Delta \text{Cys-Psu} \) shows the same distribution (50% each) of the disulphide bonded and reduced form in solution (Fig. 4d, lane 2). T123C-\( \Delta \text{Cys-Psu} \) dimer superposes with the Psu dimer with an rmsd of 0.2 Å, implying that engineering a disulphide bond at the knotted interface does not require significant structural changes.

**Dimeric assembly of Psu on the phage capsid is similar to the crystal structure**- A 45 Å electron micrograph (EM) reconstruction of the P4 phage capsid demonstrated that 12 pentameric and 30 hexameric capsomers are arranged on a T=4 lattice (Fig. 4e, top) (3). A P4+Psu reconstruction is very similar to P4 with an additional mass on top of the hexamers (Fig. 4e, bottom). This extra mass on the P4 capsid bridges across the hexamers (3) and has an extended V shaped structure similar to the knotted dimeric assembly of Psu observed in our crystal structure (Fig. 2b). The largest holes on the P4 capsid hexamer have a diameter 50-70 Å and Psu dimer essentially covers its entire width. The shortest distance measured between the two \( \alpha 7 \) helices of our
Psu dimer (Fig. 2c) is 60 Å suggesting that Psu, particularly the two α7 helices of its dimer, are involved in the interactions with the hexameric subunits and covers up the hole to prevent DNA leakage from the capsid core.

The ‘CT belt’ is flexible compared to the relatively rigid ‘CC stem’- We performed Circular Dichroism (CD) spectroscopy in presence of guanidine chloride (GdmCl) to judge the stability of different regions of Psu, especially its coiled-coil motif. The native protein gives characteristics signature of an all-α protein in CD giving minima at 208 nm and 222 nm respectively (Fig. 5a) corroborating our crystal structure. Tryptophan fluorescence studies of wild type Psu shows a maxima at 333 nm, characteristics of buried tryptophan residues (Fig. 5b). Psu dimer also demonstrates that all the tryptophan residues (W20, W60 and W133) are buried (Fig. 1a, 2f). W20 and W60 residing at ‘CC stem’ are obscured by the ‘CT belt’ (Fig. 1a) while W133 becomes buried during dimer formation (Fig. 2f). Unfolding of the protein with increasing concentrations of GdmCl resulted in the gradual loss of helical signature in the CD spectra (Fig. 5a; Table 2) and Psu practically loses its secondary structure at ~2.0 M of GdmCl (Fig. 3a, 3c; Table 2). Interestingly, the signature of the coiled-coil motif, indicated by the ratio of ellipticity at 222 nm to 208 nm, is maintained even up to ~2.0 M of GdmCl (Table 2) indicating that the coiled-coil part is stable till this point but unfolds as the concentration of GdmCl is increased further. A gradual decrease in the fluorescence intensity and a red-shift of the emission maxima of the protein was observed as the concentration of GdmCl is increased similar to CD experiments, indicating that the Trp residues are getting exposed to the solvent as the molecule unfolds (Fig. 5b). The major shift at 333 nm in the fluorescence spectra observed is due to the change from dimeric state to its monomer (Fig. 5b). CD experiments demonstrate that coiled-coil part of Psu is highly stable and with its unfolding whole structure disrupts. However, the relative stability of the ‘CT belt’ and ‘DR knot’ is not available from our CD experiments. The way the dimeric knot is formed and considering its highly hydrophobic nature and huge surface area, it is hard to believe that unfolding of the knotted region would occur before the ‘CT belt’ unfolds. High b-factors for the ‘CT belt’ region and normal mode perturbation data (17) (Fig. 4b and 4c) also supports the flexibility of the ‘CT belt’. Moreover, unfolding of the ‘CT belt’ would render four out of six Trp (W20, W60 from each monomer) residues solvent accessible while only two Tryptophans (W133 from each monomer) would be solvent accessible when the ‘DR knot’ unfolds. Considering the initial huge decrease of fluorescent intensity we suggest that with gradual increment of denaturant ‘CT belt’ of Psu unfolds first and then the ‘DR knot’ which corresponds to a transition from dimeric to monomeric form.

**Knotted conformation of Psu is reversible and sequence directed**- To understand the renaturation ability of Psu into the crystallographic knotted dimer we used WT and T123C-ΔCys-Psu, denatured them using GdmCl, removed the denaturant gradually and compared the spectroscopic properties of these proteins with the native proteins by CD spectroscopy. The CD spectra of the renatured WT and T123C-ΔCys-Psu proteins overlapped almost perfectly with their native forms (Fig. 5d and 5e) indicating that the denatured proteins refolds to a structural state quite similar to their native folded forms. However, from these experiments it is difficult to say whether the refolded proteins form the knotted structure or not. Therefore, we treated the refolded T123C-ΔCys-Psu protein with Cu-P and analyzed the cross-linked products through non-reducing 15% SDS-PAGE (Fig. 5e). The gel picture shows the same profile of the protein band distribution (i.e. 50% crosslinked product) for both the renatured T123C-ΔCys-Psu and T123C-ΔCys-Psu treated with Cu-P confirming the fact that the denatured form of T123C-ΔCys-Psu upon refolding culminates to the knotted dimeric conformation. Interestingly Psu does not require any chaperone to refold into such a complicated knotted dimer. An analysis of the primary sequence of Psu shows several patches of highly conserved sequences which might be important in performing definite role in the overall folded structure. In particular, the conservation of coiled-coil region, sequence of α3, crucial knotting residues and the clamping residues at the CT belt (Fig. 3) are highly conserved. So, it can be proposed that the refolding of Psu to the native conformation is feasible as the sequence of the protein itself contains the necessary information for the formation of the knotted dimeric state.

**DISCUSSION**

The most striking feature of Psu structure is its novel fold and the knotted dimeric conformation where considerable portions of the polypeptide chain of one Psu monomer pass through the other to form the knot. Though proteins are known to form self-tying knots where the polypeptide backbone folds over itself to reach a state of lowest potential energy (21), our results show for the first time an architecture of a deeply buried knotted dimer in nature with an exclusive pattern of hydrophobic interactions over a large interface. The dimeric knot of Psu is distinctly different from domain swapping...
that involves the exchange of domains or structural elements between two monomers and is characterized by the presence of a hinge loop where a segment of polypeptide chain links the swapped domain to the rest of its subunit, a C-interface which occurs between domains in a monomeric subunit and an O-interface which occurs between monomers in a domain swapped dimer (22).

Why does Psu dimerise through such a complicated knot? Recently, knotted structures have gained popularity among structural biologists and material scientists due to their tensile strength and their effect on mechanical properties of polymers and biomolecules (23). Psu binds to the hexameric capsomere on P4 capsid to prevent DNA leakage. Dimerisation of Psu provides two equivalent binding sites capable of interacting with the hexameric capsomeres whereas the knot formation can be envisaged to provide the necessary stability to withstand the shearing stress exerted by the DNA while coming out of the capsid. Interaction surface of Psu with the capsid is not yet known, but a V45F mutation was reported to abolish the antitermination activity of Psu while its capsid binding ability remains unaltered (3). This observation suggests that the interaction surface of Psu with Rho and capsid are distinct. Therefore, Psu by virtue of its knotted dimerisation is capable to bind both the capsid and Rho efficiently and specifically, providing the basis for its moonlighting activity.

For normal mode analysis, the first six modes were not considered as they were zero-frequency modes and represented only rigid body motions. Normal mode perturbation data corresponding to mode 7 (Fig. 4c) demonstrates that Psu dimer behaves like an arm and the knotted region acts like the ‘elbow’; allowing the dimer to swing inward and outward. During this process, the knotted region experiences minimum movements whereas the C-terminal helix and the \( \alpha_1-\alpha_2 \) connector regions exhibit larger motions (Fig. 4c). Binding of Psu to the gpN hexameric capsomeres induces a conformational change, as the capsomeres, in their Psu bound form, show smaller hole compared to their Psu unbound version (5), further supporting our normal mode data.

Multiple sequence alignment of Psu like proteins, found in strains of different species as part of cryptic prophages in their genome (6), reveals that residues involved in the packing of coiled-coil (‘a’ and ‘d’ positions) are highly conserved (Fig. 3). Also, the conservation of residues forming the knot, especially the residues at \( \alpha_3 \), indicates similar dimer formation for the other Psu proteins as well. The coiled-coil region followed by the dimerisation region provides an architecture around which the functional CT region is woven efficiently. Residues responsible for clamping the ‘CT belt’ with the ‘CC stem’ are highly conserved which not only reduce the degrees of freedom of the ‘CT belt’ but offers an interaction face of Psu oriented in the correct conformation space to bind Rho. A significant question is how this knotted dimeric conformation is attained? There may be two distinct paths to reach such a conformation. In the first mechanism, one monomer is formed and the second monomer is assembled around the first monomer. In the second mechanism, two monomers simultaneously become knotted before the folding process is complete.

The folding and unfolding of the knotted dimer by GdmCl does not exhibit any hysteresis. 123C-\( \Delta \)Cys-Psu refolds like WT Psu and the formation of a disulphide bond in the refolded protein indicates the attainment of the same knotted structure. It would be interesting to decipher the folding energy landscape of Psu to attain the reversible knotted dimeric conformation and the role of amino acid sequence signature in this process.

Another feature of the molecule is the presence of the coiled-coil motif which is generally seen to be involved in protein-protein interactions. So, apart from providing structural rigidity to Psu, it may mediate atleast in part the interaction of Psu with the Rho hexamer and the hexameric holes on the phage P4 capsid. Our structure may provide the starting point for designing a minimal fragment from the CT belt which can be used a potential drug against the bacterial transcription machinery and would add to the existing knowledge of protein knots.
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Accession codes

Protein Data Bank: Coordinates and structure factor files have been deposited with the accession code 3RX6 and 4DVD.

FOOTNOTES

The abbreviations used are: Cu-P, Copper Phenanthroline; T123C-ΔCys-Psu, T123C mutant on no-cys variant of Psu; GdmCl, Guanidine Hydrochloride; SAD, Single wavelength anomalous dispersion.
### Table 1
**Data collection, phasing and refinement statistics**

#### Data collection

| Protein name | Psu-WT | T123C-ΔCys-Psu |
|--------------|--------|----------------|
| Space group  | I422   | I422           |
| Cell dimension | a, b, c | 148.75, 148.75, 63.37 | 149.61, 149.61, 62.7 |
| α, β, γ (°)  | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å) * | 47.04-2.04 (2.11-2.04) | 47.31-3.00 (3.78-3.00) |
| Rmerge       | 0.057 (0.343) | 0.116 (0.463) |
| I / σ(I)     | 13.4 (3.7) | 6.2 (2.0) |
| Completeness (%) | 98.5 (92.0) | 95.5 (96.4) |
| Redundancy   | 4.8 (4.6) | 3.0 (3.0) |

#### Refinement

| Protein name | Psu-WT | T123C-ΔCys-Psu |
|--------------|--------|----------------|
| Resolution (Å) | 47.04-2.04 | 47.31-3.00 |
| Rcryst / Rfree | 19.2 / 21.9 | 23.11/27.66 |
| No. Atoms    | 1705   | 1480           |
| Protein      | 1479   | 1480           |
| Ligand       | 10     |                |
| Water        | 216    |                |
| B-factor (Å²) |        |                |
| Average      | 36.53  | 58.13          |
| Protein      | 35.79  | 58.13          |
| Ligand       | 31.42  |                |
| Water        | 40.52  |                |
| R.m.s. deviations |    |                |
| Bond length  | 0.007  | 0.011          |
| Bond angles (°) |    |                |
| Most favoured | 97.7   | 97.3           |
| Ramachandran statistics (%) | | |
| Additionally allowed | 2.3 | 2.7 |
| Disallowed   | 0      | 0              |

*Numbers in parentheses refer to the highest resolution shell with all data collected from a single crystal.*
Table 2
Helical content and ratio of ellipticity at $\theta_{222}$ and $\theta_{208}$ of Psu for different concentrations of GdmCl

| [GdmCl] in M | 0   | 1   | 1.5 | 2.0 |
|-------------|-----|-----|-----|-----|
| % Helicity  | 0.901 | 0.87 | 0.55 | 0.29 |
| $\Theta_{222} / \theta_{208}$ | 1.03 | 1.12 | 1.69 | 1.72 |
FIGURE LEGENDS

Figure 1: Novel fold of Psu and stabilization of its ‘coiled coil’ region. (a) Cartoon representation of Psu monomer resembling the structure of a ‘golf stick’, seven helices and three Trp residues are labeled. A black circle represents the hole in one monomer through which α3 of the second monomer can pass (top), topological diagram of Psu showing different regions (bottom) (same colour scheme is used in c-f). (b) Schematic representation of antiparallel two stranded ‘coiled-coil’ in Psu. Amino acid sequence of α1 and α2 are shown vertically and the arrows indicate the chain direction. Residues at position ‘a’ of each heptad repeat are numbered while residues at position ‘d’ are indicated as black dot. (c) Hydrophobic core of the ‘CC stem’ formed by residues ‘a’ of one helix with residues ‘d’ of the other. (d) View 180° rotated of ‘c’ along the coiled coil axis showing the polar/charged interactions that stabilize the ‘CC stem’ (e) Clamping of α5 and α6 with coiled coil. P157, shown as dotted surface, packs with C13, Y69, I57, H73. (f) Interactions of α7 with α2.

Figure 2: Knotted dimerisation of Psu (a-c) Knotted dimer of Psu shown in three orientations with one monomer in surface representation and the other in cartoon. Extended ‘V’ shape of the dimer and distance between the α7 helices are evident from Fig. 2b. (d) Schematic representation of the knotted dimer (top) and involvement of secondary structural elements of Psu in knot formation drawn using ‘Topdraw’ (24). (e) Stereo view of the Psu dimer with the Ca backbone, N and C termini are labeled. (f) Close-up view around the knotted dimeric interface showing entrance of α3 through the ‘hole’ of its knotting partner, packing of W133, P102 and close proximity of two T123 is evident.

Figure 3: Sequence alignment of Psu with its homologues. Important conserved residues are represented with color codes similar to those used in Fig. 1a. Secondary structural elements are overlaid on top and numbered. Yellow=Coiled coil, Pink=Dimerisation region, Green=C-terminal belt and Red=Residues passing through the hole in the dimerisation region. The yellow circle with the helix within represents the helix that passes through the knotted interface. E9XHX9=Esherichia coli TW10509, E0T1Q3=Edwardsiella tarda (strain FL6-60), E0T1Q3=Enterobacter sp. (strain 638), A4WDI7=Salmonella paratyphi B (strain ATCC BAA-1250 / SPB7), A9MZ75=Salmonella arizonae (strain ATCC BAA-731 / CDC346-86 / RSK2980), D0ZF48=Edwardsiella tarda (strain EIB202).

Figure 4: Stability of the Psu dimer from B-factor plot and Normal mode analysis and validation of the Psu knot in solution. (a) Hydrophobic interactions at the dimeric interface. Residues interacting at the dimeric interface are shown as ball-and-stick for one monomer and labeled, while its knotting partner is shown as stick and are not labeled. Two monomers are related by a twofold that is passing through two F89 and perpendicular to the paper. Residues of α3 are labeled but not numbered for clarity. Close proximity of G93 and W133 is evident. (b) B-factor differences between residues of the Psu crystal structure. Indicated differences were calculated from the Ca mainchain atoms. The b-factor values of the ‘CT belt’ residues are comparably higher than the rest of the molecule. (c) Normal mode analysis showing the low frequency ‘mode 7’ with large vibrational movements (Å) for regions α1-α2 and the CT-belt regions, while vibrations around the knotted interface are minimum. (d) Formation of disulphide linked knotted dimer of Psu in solution and in crystal structure. (Top) 15% SDS PAGE of T123C-ΔCys-Psu (lane 1), T123C-ΔCys-Psu treated with Cu-Phenanthroline (lane 2) along with MW marker (Lane 3) (Bottom) T123C-ΔCys-Psu crystal structure showing the disulphide bond. (e) EM of phage capsid (5) (without (top) and with Psu (bottom), bulged out regions are the location of Psu (black arrow).

Figure 5: Biophysical characterization of Psu knot. (a) Far-UV CD spectra of Psu at different GdmCl concentration. (b) Fluorescent emission spectra of Psu at 340nm, using an excitation at 295nm with concentrations of GdmCl varied similar to CD experiments. (c) Bar-diagram showing folded fractions of Psu-WT at different GdmCl concentrations. (d)-(e) Comparison of native, denatured (3M GdmCl) and renatured (0.26M GdmCl) Psu-WT (d) and T123C-ΔCys-Psu (e), (f) (1), 15% SDS PAGE of renatured T123C-ΔCys-Psu treated with Cu-Phenanthroline (2), T123C-ΔCys-Psu treated with Cu-Phenanthroline (3) native T123C-ΔCys-Psu.
FIGURE 4.
FIGURE 5.
The first structure of Polarity Suppression protein, Psu from Enterobacteria phage P4, reveals a novel fold and a knotted dimer
Ramanuj Banerjee, Seema Nath, Amitabh Ranjan, Susmita Khamrui, Bibhusita Pani, Ranjan Sen and Udayaditya Sen

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