

The moonlighting function of bacteriophage P4 capsid protein, Psu, as a transcription antiterminator

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Psu, a 20-kD bacteriophage P4 capsid decorating protein moonlights as a transcription antiterminator of the Rho-dependent termination. Psu forms specific complex with *E. coli* Rho protein, and affects the latter's ATP-dependent translocase activity along the nascent RNA. It forms a unique knotted dimer to take a V-shaped structure. The C-terminal helix of Psu makes specific contacts with a disordered region of Rho, encompassing the residues 139–153. An energy minimized structural model of the Rho–Psu complex reveals that the V-shaped Psu dimer forms a lid over the central channel of the Rho hexamer. This configuration of Psu causes a mechanical impediment to the translocase activity of Rho. The knowledge of structural and mechanistic basis of inhibition of Rho action by Psu may help to design peptide inhibitors for the conserved Rho-dependent transcription termination process of bacteria.

Introduction

Transcription termination of about 50% of the operons in bacteria is performed by a conserved protein called Rho. Rho is a homo-hexameric RNA/DNA helicase or translocase, capable of binding to and translocating along the single stranded RNA, by utilizing its RNA-dependent ATPase activity. It recognizes the *rho* utilization (*rut*) sites on the exiting mRNA through its N-terminal primary RNA binding domain (PBS). Binding of RNA to the PBS of

Rho, guides the 3'-side of the RNA into the central channel of the hexamer that constitutes the secondary RNA binding domain (SBS). The interaction of mRNA with the SBS activates its ATPase activity that provides energy for its translocation activity along the RNA, and enables Rho to reach at the vicinity of the elongating RNA polymerase (RNAP).^{1,2} The mechanism of Rho-mediated RNA release from the elongation complex (EC) is not fully understood. According to the classical model, the kinetic energy associated with the translocase activity helps Rho to dislodge the RNA polymerase.² More radical views suggest that Rho remains associated with the RNAP throughout the transcription cycle,³ and allosterically inactivates the EC in the termination zone.⁴ The essentiality of the Rho protein for bacterial survival makes it a potent target for the bactericidal agents..

Bacteriophage P4, isolated from *E. coli* K-235, lacks the genes for its head and tail formation, and lysis functions. Proteins required for these purposes are encoded by a helper bacteriophage, P2. The lysogenic or lytic life cycle of the phage P4 depends on P2-encoded factors. In the absence of P2, P4 may enter into an immune-integrated condition that is similar to the lysogenic state, or it is maintained as a multi-copy plasmid.⁵ Likewise, the de-repression function of P2 is dependent on the replication genes of P4.

The rightward operon of the P4 genome codes the capsid proteins Sid and Psu, the only two morphogenic proteins made by this phage. The hexameric Sid

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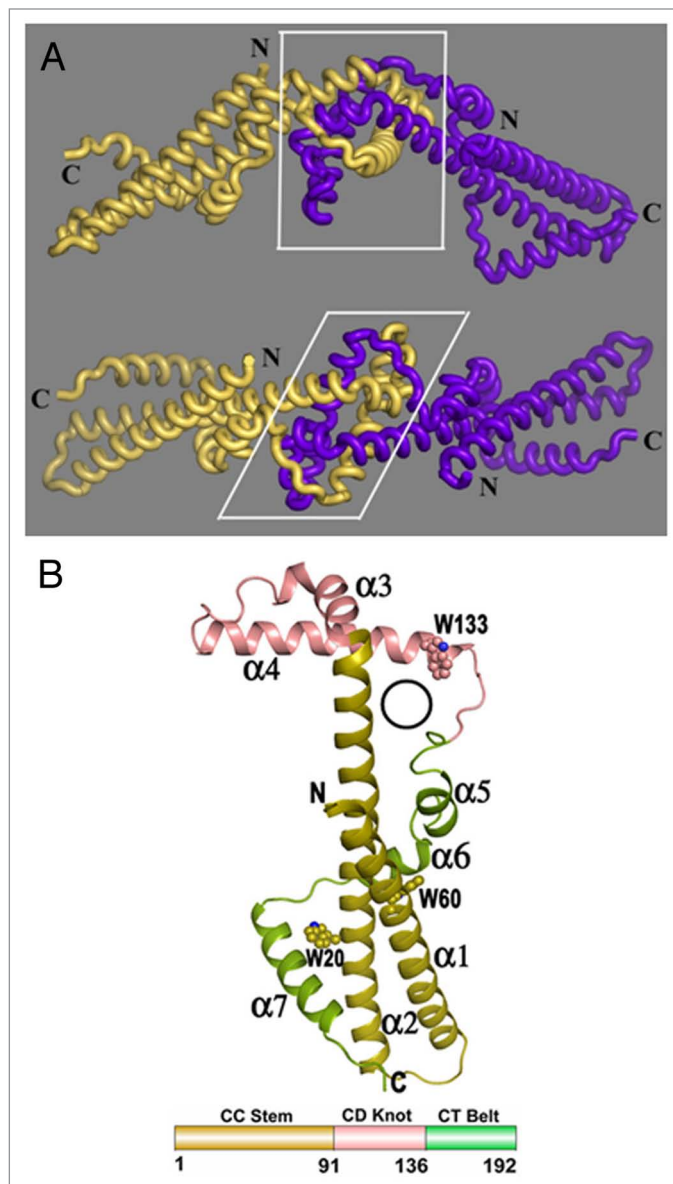


Figure 1. (A) The 'V' shaped knotted structure of Psu. Top view of the Psu knot is shown below (knotted region is shown in white box). (B) Different structural elements described in the text are indicated on the Psu monomer.

protein forms the capsid scaffold together with the major P2-encoded capsid protein N.⁶ Psu appears to function as a capsid decoration protein. The cryo-electron micrographs of the P4 particles show that Psu protein is docked on the top of the Sid hexamers in a fixed orientation.⁷ The presence of Psu on the hexamers may help to prevent P4 DNA from escaping through the capsid shell during heat inactivation.⁷

The Psu (polarity suppression) protein, in addition to its structural roles, is also capable of suppressing the Rho-dependent

polarity in the late genes of phage P2.⁸ In vivo, Psu inhibits the Rho-dependent transcription termination in phage, plasmid and bacterial transcriptional units, and this activity does not depend on any other genes of either P2 or P4 phages.^{8,9} Unlike, the transcription antiterminator, N, from the lambdoid phages, Psu function does not depend on the specific sequences of the mRNA.¹⁰ Thus Psu, primarily a phage-capsid protein, moonlights as an antiterminator of transcription termination in *E. coli*.

The Psu Protein

Earlier we have cloned and purified the recombinant Psu protein from *E. coli*.¹¹ Psu can be overexpressed from pET vectors and purified as His-tagged or non-His-tagged protein. Its solubility is not very high, and upon overexpression a significant amount of the protein is always found in the inclusion bodies. The purified protein is highly active in in vitro assays. However, the activity tends to decline within a month, even though it is structurally very stable (see below).

Psu is a 21-kD protein that migrates in a non-reducing SDS-PAGE as a dimer upon cross-linking with glutaraldehyde, but elutes as a trimer from a size-exclusion column.¹¹ Its existence as a dimer was also revealed from its crystal structure (see below). A specific Rho–Psu complex was obtained in vivo, upon co-overexpression of both the proteins. So far, we have failed to form a stable WT Rho–Psu complex in vitro, even after trying several solution conditions. And hence, quantitative binding parameters of Rho–Psu complex formation remained undetermined.

Mutational analyses of the Psu protein showed that its C-terminal 20 amino acids are important for anti-terminating the Rho-dependent termination, and other parts of the protein are required for structural integrity.^{11,12} The binding of Psu to Rho affects the RNA-dependent ATPase activity of the latter that in turn slows down its speed of RNA release from the EC.¹¹ In vitro transcription antitermination assays revealed that binding of Psu to Rho is sufficient for the antitermination function of the former, and no other factors are involved.^{11,12} However, both in the in vivo and in vitro assays, it was observed that concentrations of Psu required to exert the effect have to be well excess over Rho.

The Uniquely Folded Structure of the Psu Dimer

Circular dichroism studies demonstrated that Psu is predominantly an α -helical protein, and in vitro cross-linking experiments suggested that it exists as a dimer in solution.¹¹ We have recently solved a high resolution crystal structure

of Psu.¹³ The structure reveals Psu to be a symmetric “V”-shaped homodimer (Fig. 1). Each monomer of Psu has a novel protein fold consisting of seven helices. The N-terminal, $\alpha 1$ and $\alpha 2$ helices form a tight coiled-coil structure (‘CC-stem’) which is wrapped by the three C-terminal helices, $\alpha 5$ – $\alpha 7$, like a belt (‘CT-belt’), whereas the central region self assembles into the knot with another monomer to form the dimer (‘DR-knot’)¹³ (Fig. 1B). Though proteins are known to form self-tying knots, where the polypeptide backbone folds over itself to reach a state of lowest potential energy,¹⁴ our results show for the first time an architecture of a deeply buried knotted dimer with an exclusive pattern of hydrophobic interactions over a large interface. Interestingly, this knot formation occurs spontaneously in in vitro folding assays.

The N-terminal ‘CC-stem’ followed by the dimerization region together provide a stable architecture, around which the ‘CT-belt’ region is woven efficiently. Normal mode analyses demonstrates that the Psu dimer behaves like an arm and the knotted region acts like an elbow, allowing the dimer to swing inward and outward. During this process, the knotted region experiences minimum fluctuations. Therefore, dimerization of Psu not only reduces the degrees of freedom of the ‘CT-belt’ but disposes the interaction face of Psu in the correct conformation space to bind Rho (see below).

The Binding Interface of the Rho–Psu Complex

In our most recent study,¹⁵ we delineated the interaction surface required for the complex formation between Rho and Psu. From the mutational analyses,¹² and the crystal structure¹³ of Psu, we identified several point mutants, defective for binding to Rho, located at its C-terminal helices 6 and 7 (Fig. 2A). And therefore, in order to identify the Psu-interaction site on Rho, we screened for suppressors of these Psu mutants in Rho. The suppressors N151D and P167L of Rho thus obtained rectify the defects of the C-terminal Psu mutants, but not the N-terminal ones. P167L Rho appeared to have a higher affinity for Psu, and was capable of

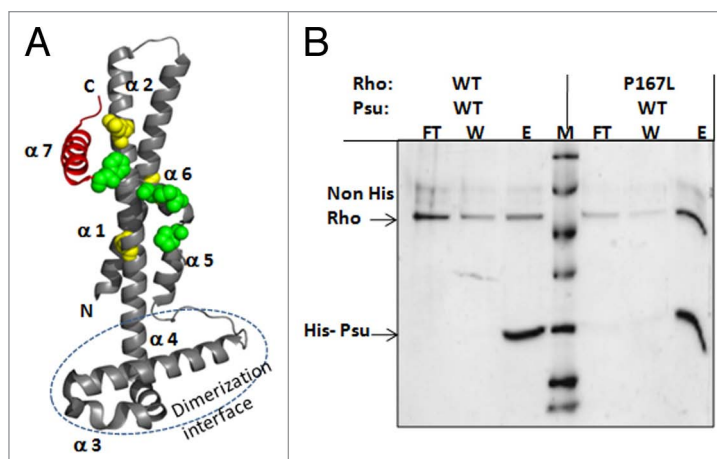


Figure 2. (A) The seven α -helices and the dimerization interface on the crystal structure of Psu monomer are shown (PDB code: 3RX6). The locations of Psu mutants defective for inhibiting Rho are indicated. C-terminal tail region (helix 7; 170–190) is in red. The C-terminal point mutations at 169, 166 and 157 positions are indicated in green spheres. The N-terminal mutations at 21, 56 and 72 are shown in yellow spheres. (B) In vitro pull-down assays of WT and P167L Rho with his-tagged WT Psu in the presence of 1 mM ATP. Rho-Psu mixtures were passed through Ni-NTA beads and flow-through (FT), wash (W) and elute (E) fractions were collected. Stable Rho–Psu complexes were observed for P167L Rho. Proteins were run on a 10%-SDS PAGE. Stable Rho–Psu complexes were observed for P167L Rho. Proteins were run on a 10%-SDS PAGE. Seven μ g of Rho was mixed with 20 μ g of Psu in phosphate buffer before loading onto the gel.

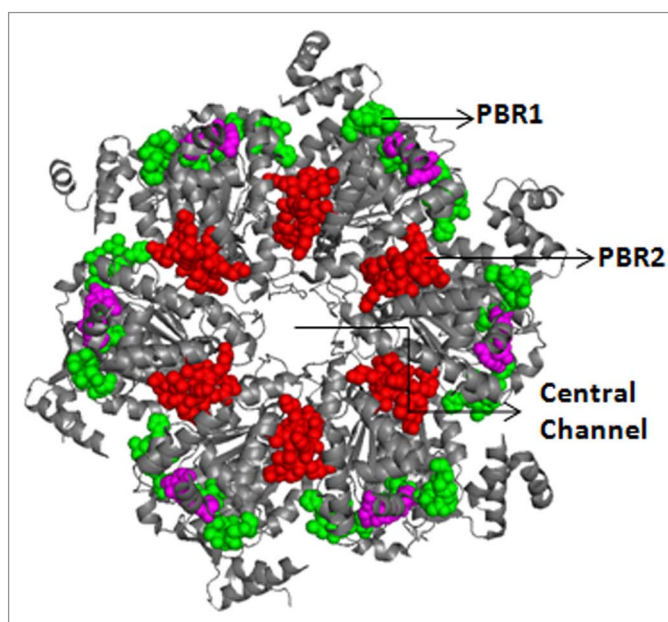


Figure 3. The Psu interaction sites on Rho. A top view of the two PBRs on the hexameric structure of Rho (PDB code: 3ICE). The PBR1 and PBR2 are showing by green and red spheres, respectively. The point mutants in the PBR1 region, defective for Psu binding, are shown by magenta spheres. (This figure was originally published in ref. 15).

forming stable Rho–Psu complex in vitro (Fig. 2B). We used this “super” Rho for forming the in vitro Rho–Psu complexes and to probe the interaction surfaces.

We probed the interacting surfaces using site-specific cross-linking, Fe-BABE ([iron (bromoacetamidobenzyl)-ethylene-

diaminetetraacetate]) cleavage and FRET analyses, and concluded that a disordered region encompassing the amino acids 139–153 of Rho acts as the primary interaction site (PBR1; Psu binding region) for Psu. Protein foot-printing assays also indicated the existence of a nearby secondary

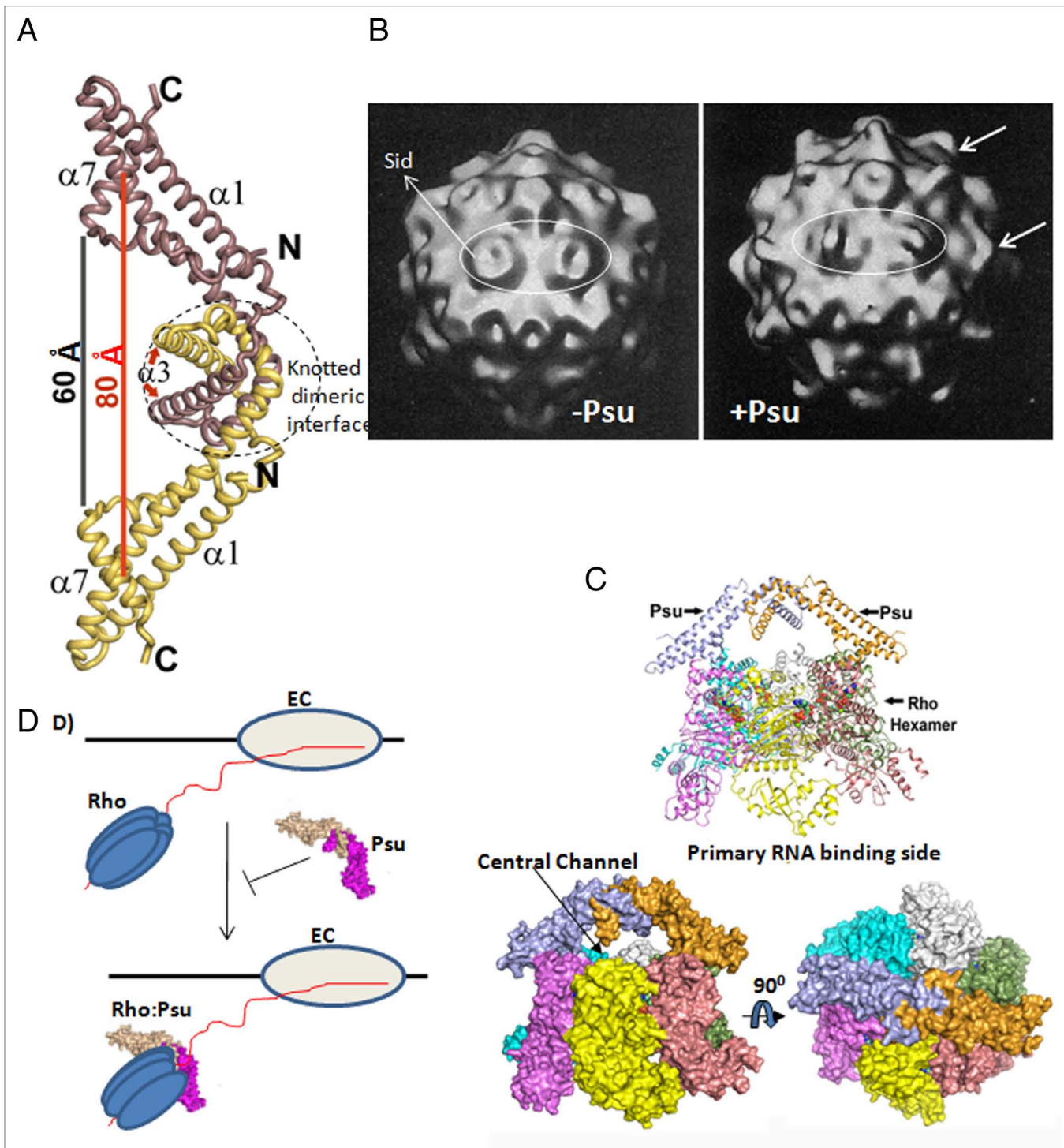


Figure 4. (A) The V shaped dimeric crystal structure of Psi.¹⁵ Each of the subunits are shown in violet and gold. Different structural elements are indicated. Distances between the two Rho-binding $\alpha 7$ helices are indicated. (B) The cryo-electron microgram of P4 capsid. The hexameric Sid proteins are indicated. The V-shaped Psi proteins covering the central channel of Sid are also visible in “+” Psi panel.⁷ (C) Above: A cartoon representation of the docked model showing the orientation of Psi (gold and sky), when bound to the Rho hexamer (magenta, yellow, salmon, green, gray and cyan in the anticlockwise manner). Below: Surface representation of the same in two orientations. Psi dimer occupies the regions near the two diagonally opposite ATP binding sites of Rho, and blocks its central channel. A 90° rotated view shows that the Psi dimer covers the central channel. (D) A cartoon showing the action of Psi as a polarity suppressor. The EC carrying a hexameric Rho on its nascent RNA is indicated. A Psi dimer binds with the closed ring state of the Rho, interacts near the ATP binding site and inhibits the translocation of the latter on the nascent RNA. (This figure was originally published in ref. 15).

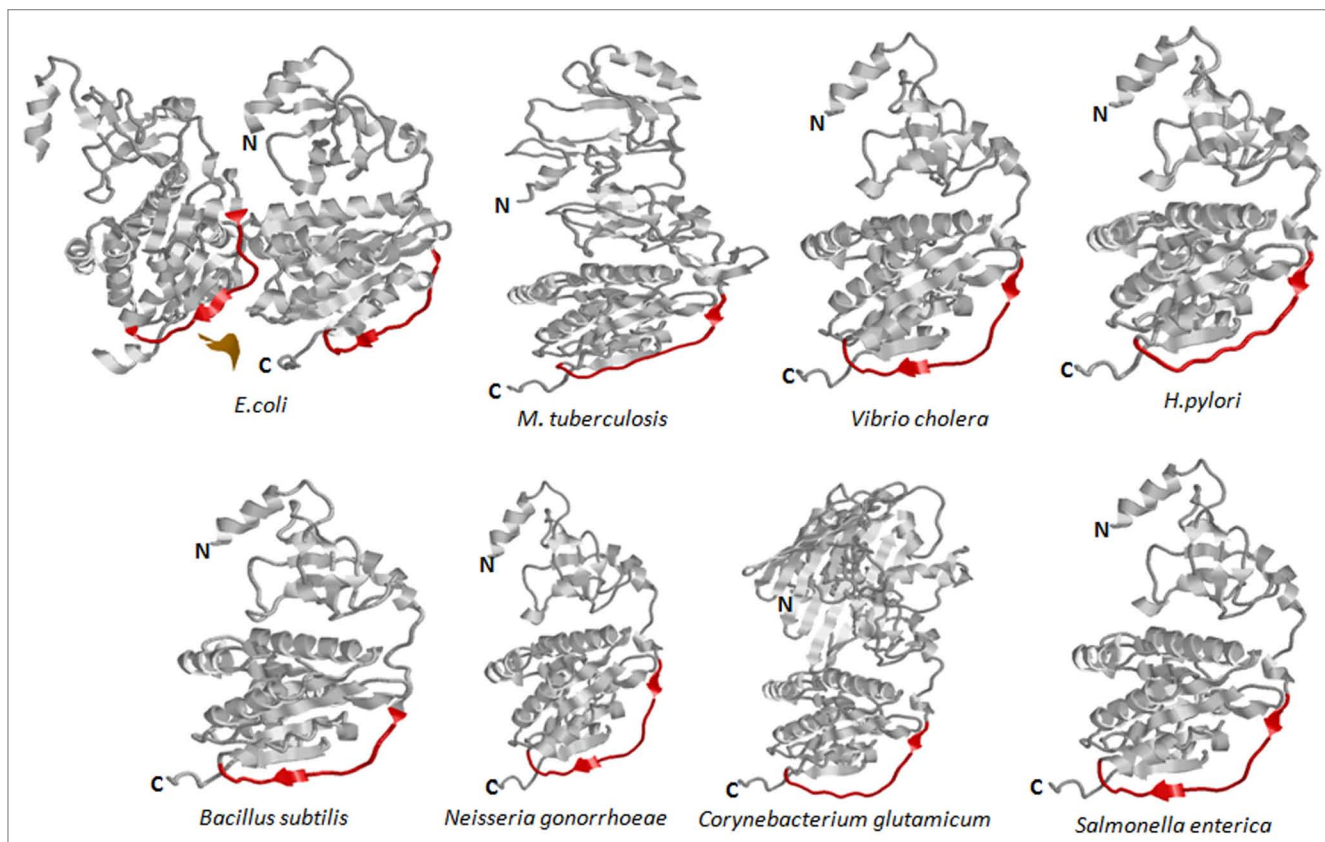


Figure 5. The homology models of Rho monomers from different pathogenic bacteria. The Psu-binding loop is conserved in many Rho proteins (shown in red). (This figure was originally published in ref. 15 as supplementary material).

site spanning the amino acids 347–355 (PBR2). Specific point mutations, R144E, R146E and E148R, in the PBR1 indeed showed severe binding defects for Psu, and thereby validated the functional importance of this region. Phenotypes of the mutations in PBR2 were mild in nature, and we concluded that this region plays a supporting role in the interaction, whereas PBR1 is the major interaction site for Psu. **Figure 3** summarizes the Psu interaction sites of Rho.

A Structural Model of the Rho–Psu Complex

We generated a structural model of Rho–Psu complex based on the following informations. (1) The distance between the two C-terminal tails, located at diametrically opposite direction of the ‘V’-shaped structure of the Psu dimer (**Fig. 4A**). (2) The electron microgram of a Psu dimer covering the central hole of the hexameric capsid protein, Sid, on the surface of the phage P4 capsomere (**Fig. 4B**).⁷

(3) The interacting regions defined by the genetic and biochemical data described in **Figure 3**. (4) The Rho-interacting regions of Psu (**Fig. 2**). (5) The inhibition of ATP binding as well as ATPase activity of Rho by Psu.¹¹

In this docked structure, a Psu dimer bridges the two diagonally opposite intersubunit grooves of the Rho hexamer (PDB code: 3ICE), located near the ATP and secondary RNA binding sites (**Fig. 4C**). There are three pairs of potential interaction sites on the Rho hexamer capable of binding three Psu dimers. However, because of the steric hindrance, only one dimer of Psu can bind to a Rho hexamer at a time. The binding free energy of the docked model is -7Kcal/mol , suggesting an energetically favorable interaction. Similarity in the mode of binding to the two hexameric partners, Rho and Sid proteins, might have conferred the moonlighting function of Psu. We also speculate that the flexible arms of the knotted Psu dimer (**Fig. 1**) is likely to provide the requisite dynamism to interact with the two unrelated partners.

The above configuration of Rho–Psu complex predicts that (1) Psu dimer will block the central hole of the Rho hexamer, and (2) its presence at the RNA exit channel will cause a mechanical impediment to the translocase activity of Rho. These were validated by fluorescence quenching and Rho-translocation assays.¹⁵ This configuration appeared to block the RNA exit path of Rho. It should also be noted that the distance between the two C-terminal helices (helices 6 and 7; see **Fig. 4A**) of the Psu dimer is compatible with the dimension of the central channel of the ‘closed’ state hexamer of Rho.^{16,17} Therefore, it is likely that Psu targets the translocating Rho having the ‘closed’ conformation (**Fig. 4D**).

Will Psu Inhibit Rho Proteins from the Pathogenic Bacteria?

The sequence of the Rho protein is extremely conserved among different bacteria. This led us to build homology models of different Rho proteins from a

diverse set of bacterial pathogens (Fig. 5), and it revealed that the looped out primary docking site of Psu (PBR1) is very well conserved in different species. And hence, it is likely that, Psu will also be able to inhibit Rho proteins from different pathogens. As this conserved looped out region of Rho directly interacts with the helix 7 of Psu, it is possible to design peptide inhibitor(s) of Rho proteins from its C-terminal helices.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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