The mechanism of inhibition of protein synthesis by the proline-rich peptide oncocin

Raktim N Roy1,4, Ivan B Lomakin2,4, Matthieu G Gagnon2–4 & Thomas A Steitz1–3

Antibiotic-resistant bacteria are a global health issue necessitating the development of new effective therapeutics. Proline-rich antimicrobial peptides (PrAMPs), which include oncocins, are an extensively studied class of AMPs that counteract bacterial infection at submicromolar concentrations. Oncocins enter and kill bacteria by inhibiting certain targets rather than by acting through membrane lysis. Although they have recently been reported to bind DnaK and the bacterial ribosome, their mode of inhibition has remained elusive. Here we report the crystal structure of the oncocin derivative Onc112 bound to the Thermus thermophilus 70S ribosome. Strikingly, this 19-residue proline-rich peptide manifests the features of several known classes of ribosome inhibitors by simultaneously blocking the peptidyl transferase center and the peptid-exit tunnel of the ribosome. This high-resolution structure thus reveals the mechanism by which oncocins inhibit protein synthesis, providing an opportunity for structure-based design of new-generation therapeutics.

Throughout the course of evolution, the innate immune system in plants and animals has developed efficient ways to counter infections. One of the lines of defense is through the synthesis of antimicrobial peptides (AMPs) that kill bacterial and fungal pathogens1–3. These AMPs are an effective weapon against the ability of microorganisms to develop resistance4. Most AMPs are small peptides of 15–70 amino acid residues generated from post-translational processing of larger precursors, whose synthesis is induced through the Toll and Toll-like receptors4,5. Although AMPs are extremely diverse, they can be categorized into several groups on the basis of their structures or sequences as α-helical, cysteine-rich, glycine-rich and proline-rich peptides1.

The PrAMPs, expressed in mammals and insects, have attracted particular attention because of their wide distribution and unique mechanism of killing bacteria without cell-membrane disruption6. The PrAMPs oncocin, apidaecin, drosocin and pyrrhocoricin interact with the substrate-binding domain of the chaperone DnaK with dissociation constants in the micromolar range, thus resulting in protein misfolding and aggregation, and subsequent bacterial death7–11. This mechanism is bacteria specific because PrAMPs are only slightly toxic toward mammalian cells, owing to their inability to penetrate the mammalian cell membrane12. Structural studies of bacterial DnaK complexes with PrAMPs have revealed two modes for PrAMP binding to the conventional binding cleft of DnaK13,14, indicating that DnaK is rather promiscuous in peptide binding.

Recent biochemical data have demonstrated that an apidaecin derivative, ApI88, and its truncated mutants are equally efficient in entering Escherichia coli cells and binding to DnaK. However, only the full-length ApI88 is functional15. This, combined with the susceptibility of a DnaK-null mutant to PrAMPs, suggests that DnaK is not the main target for this group of peptides16. Cross-linking, cell-free translation and binding assays have shown that apidaecin derivatives (ApI88 and ApI137) and oncocin derivatives (Onc72 and Onc112) inhibit protein synthesis by binding the 70S ribosome with dissociation constants in the nanomolar range16. Remarkably, Onc112 binds to the 70S ribosome approximately 50-fold more strongly than it does to DnaK16, thus making the ribosome the preferred target.

To gain insights into the mode of its interaction, we have determined the 2.9-Å-resolution crystal structure of Onc112 bound to the T. thermophilus 70S ribosome in complex with mRNA and a tRNA\textsuperscript{Met} in the peptidyl (P) site (Online Methods and Table 1). Remarkably, unlike most known antibiotics, a single Onc112 molecule interacts with not just one but three adjacent functional sites of the ribosome. Its N terminus binds near the peptidyl transferase center (PTC) of the 50S subunit, where it interferes with the aminoacyl (A)-site tRNA and the peptidyl-tRNA in the P site. The rest of Onc112 binds inside the peptide-exit tunnel of the 50S subunit and blocks it completely (Fig. 1). Our study provides insights into the mechanism by which PrAMPs inhibit translation and builds a foundation for further structure-based design of a new generation of antimicrobial therapeutics, which may be more effective against the development of bacterial drug resistance. In addition, the visualization of a peptide in the 50S-subunit exit tunnel at high resolution sheds light on the function of the tunnel during translation.

RESULTS

We determined the structure of the 70S ribosome complex with Onc112 by molecular replacement, with a high-resolution model of the 70S ribosome with its ligands removed17 as a search model
Table 1 Data collection and refinement statistics

|                | T. thermophilus 70S complex |
|----------------|-----------------------------|
| **Data collection** |                             |
| Space group     | P2₁2₁2₁                     |
| Cell dimensions | a, b, c (Å) 210.1, 450.7, 623.5 |
| a, β, γ (°)     | 90, 90, 90                  |
| Resolution (Å)  | 20.3 / 24.8                 |
| Completeness (%)| 97.7 (99.9)                 |
| Redundancy      | 4.6 (4.7)                   |
| **Refinement**  |                             |
| Resolution (Å)  | 50.0 – 2.9                  |
| No. reflections | 1,271,909                   |
| No. atoms       | 191,321                     |
| RNA             | 58.3                        |
| Protein         | 64.0                        |
| Ions            | 53.3                        |
| Waters          | 45.4                        |
| **B factors**   |                             |
| RNA             | 190,537                     |
| Protein         | 90,537                      |
| Ions            | 2,366                       |
| Waters          | 3,990                       |
| **r.m.s. deviations** |                          |
| Bond lengths (Å) | 0.004                       |
| Bond angles (°)  | 0.717                       |

(Online Methods). Clear unbiased electron density for the mRNA, P-site tRNA<sup>Met</sup> and Onc112 inside the peptide tunnel of the 50S subunit with its N terminus near the acceptor stem of the tRNA<sup>Met</sup> appeared after calculation of the difference Fourier map from initially phased diffraction data (Fig. 1a,b and Supplementary Fig. 1).

**Interactions of the Onc112 N terminus with the ribosome**

The universally conserved PTC of the ribosome, which is made exclusively of RNA, catalyzes peptide-bond formation between the incoming aminoaacyl-tRNA and the peptidyl-tRNA, both of which must be properly aligned in the A and P sites, respectively<sup>17</sup>. Although most antibiotics target only certain parts of the PTC or the peptide-exit tunnel, Onc112 overlaps with all of those binding sites located in the 50S exit tunnel (Figs. 1a,b, 2c and 3c). Surprisingly, the orientation of Onc112 inside the peptide tunnel is opposite to that of a natural nascent peptide chain, for which incoming amino acids are added C terminally in the PTC. The N terminus of Onc112 (residues 1 to 5) follows the path of the CCA end of the acceptor stem and of the aminoaacyl moiety of an A-site tRNA (Fig. 2a and Supplementary Fig. 2a). The first three residues of Onc112 form multiple interactions with the 23S rRNA. For instance, the main chain peptide backbone of Val1 and the side chain of Asp2 form hydrogen-bonding interactions with nucleotides C2573 (2584) and G2553 (2564), respectively (Fig. 1c and Supplementary Fig. 3a). The latter nucleotide is part of the A loop of the 23S rRNA, and it normally forms a Watson-Crick base pair with G75 of the A-site tRNA<sup>17</sup>. This interaction is partially replaced by the γ-carboxyl group of the Asp2 side chain, which superposes with the Watson-Crick edge of nucleotide C75 of an accommodated tRNA in the A site<sup>17</sup> (Supplementary Fig. 3b). The side chain of Lys3 is fully extended, and it forms nonspecific electrostatic interactions with 23S rRNA (Fig. 1c). These interactions ensure that the binding of Onc112 to the ribosome and of the tRNA to the A site of the 50S subunit are mutually exclusive. Moreover, the binding of Onc112 reorients nucleotide U2585 (2596) so that it would collide with the amino acid moiety attached to the peptidyl-tRNA in the P site (Supplementary Fig. 2b). By interfering directly with the binding of the CCA end of the aminoaacyl-tRNA in the PTC and indirectly with

Figure 1 The structure of Onc112 bound to the ribosome. (a) Overview of Onc112 (brown) and the P-site tRNA<sup>Met</sup> (blue) bound to the 70S ribosome. The 50S and 30S subunits are shown in gray and yellow, respectively. Portions of the ribosome are omitted for clarity. (b) Close-up view of the positioning of Onc112 in the peptide-exit tunnel. For reference, landmark features of the ribosome PTC are indicated. (c-e) Interactions of Onc112 with the ribosome. Putative hydrogen bonds between oxygen (red) and nitrogen (blue) atoms are shown as black dashes. (c) Interactions of the N terminus of Onc112 with the 23S rRNA. (d) Interactions of Onc112 within the PTC. The middle part of Onc112 occupies the A-site cleft in the PTC. Residues Leu7 and Tyr6 form a three-layer stack with the nucleotide base of C2452 (2463). Additional stabilization of Onc112 is provided by interactions with nucleotides U2506 (2517) and G2061 (2082). (e) Interactions of the C terminus of Onc112 with the peptide-exit tunnel. Arg9 and Arg11 form a stacking interaction with the nucleotide bases of C2610 (2621) and A2062 (2083), respectively.

Throughout the text, we use E. coli nucleotide numbering, with the T. thermophilus numbers following in parentheses.
Figure 2 Peptide binding to the A-site cleft in the peptidyl transferase center. (a) Residues 1 to 5 of Onc112 (brown) follow the path of the CCA end of an accommodated tRNA in the A site (gray) (PDB 1VY4)\textsuperscript{17}. (b) Stacking interaction between the aromatic ring of Tyr6 of Onc112 (brown) and the nucleotide base of C2452 (2463) of the 23S rRNA, analogous to attachment of the phenylalanine residue to a Phe-tRNA\textsuperscript{Phe} bound in the A site (gray) (PDB 1VY4)\textsuperscript{17}. Nucleotides of the 23S rRNA (light blue) forming the A-site cleft are labeled. (c) Structures of antibiotics chloramphenicol (magenta, CAM; gray, 23S rRNA) (PDB 4V7W)\textsuperscript{18} and homoharringtonine (pink, HHT; dark cyan, 26S rRNA) (PDB 4U4Q)\textsuperscript{20} bound in the A-site cleft. Nucleotide C2452 (2463) of the 23S rRNA in the complex of Onc112 with the ribosome is light blue.

the peptidyl-tRNA via the displacement of nucleotide U2585 (2596), Onc112 presumably blocks the peptidyl transferase function of the ribosome, thereby accounting for its antimicrobial properties.

Interactions of Onc112 within the PTC
The middle part of Onc112 (residues 6 to 8) occupies the A-site cleft in the PTC, which forms one side of the peptide-exit-tunnel wall, where the aminoacyl moiety of an A-site tRNA would normally bind (Fig. 1d). The Tyr6 residue, located right after a consecutive pair of prolines, is rotationally confined such that its phenyl ring forms a stacking interaction with the nucleotide base of C2452 (2463), in a manner analogous to binding of the phenyl moiety of a Phe-tRNA\textsuperscript{Phe} in the A site (Fig. 2b), while its hydroxyl group forms a hydrogen bond with a water molecule (Supplementary Fig. 4b). This binding mode is also similar to that of the antibiotics chloramphenicol (CAM) and homoharringtonine (HHT)\textsuperscript{18–21} (Fig. 2c). Like Onc112, CAM also displays broad-spectrum activity in bacteria but does not inhibit eucaytolic translation. However, in contrast to the structure of the CAM complex, nucleotide U2585 (2596) in this Onc112–70S complex is rotated by about 55°, and the base of A2062 (2083) is rotated by about 45° to accommodate Pro5 and Pro8 of the peptide, respectively (Fig. 3a,b). In addition, the interactions of Onc112 with the A-site cleft in the PTC are further stabilized by the Leu7 side chain, which caps a three-layered stacked unit, and by the N3 and O2 of U2506 (2517), which interact with the main chain of residues Tyr6 and Leu7 (Fig. 1d and Supplementary Fig. 4a). The high conservation of this region of the ribosome suggests that Onc112 will probably inhibit eucaytolic protein synthesis, provided that it passes through the cell membrane.

Interactions of Onc112 in the exit tunnel
In the upper chamber of the peptide-exit tunnel, the nucleotide base of A2062 (2083) adopts a conformation allowing the side chain of Arg11 to form a favorable stacking interaction with its nucleotide base (Figs. 1e and 3b). We also observed a similar interaction between Arg9 and C2610 (2621) (Figs. 1e and 3b). These extended arginine side chains span about 16 Å across the ribosome peptide-exit tunnel, thereby completely plugging the upper chamber (Figs. 1e and 3b). Interestingly, nucleotide A2062 (2083) adopts different conformations when interacting with erythromycin on the stalled ribosome\textsuperscript{22,23}, its mutation, as well as mutation of C2610 (2621), substantially reduces nascent peptide–dependent and antibiotic-dependent ribosome stalling\textsuperscript{24,25}. The location of Onc112 overlaps with the macrolide antibiotic erythromycin and azithromycin\textsuperscript{18} (Fig. 3c), thus explaining the antimicrobial effects of Onc112, which occupies the binding sites of several classes of antibiotics. Our data explain the previous observation that alinate substitutions of Lys3, Tyr6, Leu7 and Arg11 severely reduce the antimicrobial activity of oncocin\textsuperscript{26} and decrease its binding affinity to the 70S ribosome by more than 30-fold\textsuperscript{16}. The side chains of these residues are all involved in tight interactions with the 23S rRNA, and their mutation will consequently affect the binding of oncocin to the ribosome. We do not see electron density for the last six amino acid residues of Onc112, a result suggesting that they are flexible and probably not essential for the binding to the ribosome.

DISCUSSION
The crystal structure of the proline-rich peptide Onc112 bound to the 70S ribosome highlights the network of extensive interactions between the peptide and the 23S rRNA. This peptide forms a 34-Å-long plug that blocks access to the A and P sites, the peptidyl transferase center and the peptide-exit tunnel of the 50S ribosomal subunit. The path of the peptide overlaps with the binding sites of multiple classes of antibiotics, thus suggesting that it inhibits the bacterial ribosome through a concerted mode of action. This would probably prevent the transition to the elongation phase of protein synthesis and result in the accumulation of 70S ribosomal particles. The structure presented...
here sheds light on the mechanism that has evolved in eukaryotic organisms to defeat bacterial resistance to ribosome-targeting compounds. Such peptides, which simultaneously occupy multiple drug-binding sites, limit the probability of the appearance of resistance mutations. Our study builds a platform for structure-based design of the next generation of improved antimicrobial agents.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 4Z8C.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.N.R., I.B.L. and M.G.G. designed and performed experiments, analyzed data and wrote the manuscript; T.A.S. analyzed data, wrote the manuscript and oversaw the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Synthesis of mRNAs, tRNAs and peptides. The mRNA with a Shine-Dalgarno sequence and an initiation codon in the P site was synthesized by integrated DNA technologies with the sequence 5′ GGCAAGAGGAGGUAAAGAUGUUCUA 3′. The fMet-tRNA^{Met} was prepared as previously described27. The oncocin derivative, peptide Onc112, with the sequence VDKPPYLPRLPPPRrINr-NH₂16, where ‘r’ stands for d-arginine, was chemically synthesized by GenScript USA.

Complex formation and crystallization. The *T. thermophilus* 70S ribosomes were purified, crystallized and cryoprotected as previously described28. Essentially, 4 µM ribosomes were incubated with 8 µM mRNA and fMet-tRNA^{fMet} in 50 mM KCl, 5 mM HEPES-KOH, pH 7.6, 10 mM NH₄Cl, 10 mM Mg acetate and 6 mM β-mercaptoethanol at 55 °C for 6 min. The complex was further incubated at room temperature for 10–15 min in the presence of 50 µM Onc112. Crystals were grown in sitting-drop trays in which 3 µl of ribosome complex was mixed with 3.5–4.5 µl reservoir solution containing 0.1 M Tris-HCl, pH 7.6, 2.9% (w/v) PEG 20000, 9% (v/v) MPD, 0.175 M l-arginine and 0.5 mM β-mercaptoethanol, and incubated at 19 °C. The crystals were transferred stepwise to a cryoprotectant solution containing 10 mM NH₄Cl, 0.1 M Tris-HCl, pH 7.6, 10 mM Mg acetate, 50 mM KCl, 6 mM β-mercaptoethanol, 2.9% (w/v) PEG 20000 and 40% (v/v) MPD, with the last stabilization step also containing 50 µM Onc112. The crystals were left to equilibrate for about 24 h at 19 °C and were frozen at 80 °K in a N₂ stream before being plunged in liquid nitrogen.

Data collection, model building and structure refinement. All data collection was carried out at 100 °K. X-ray diffraction data were collected at beamline 24ID-C at the Advanced Photon Source at Argonne National Laboratory, with 0.3° oscillations. We used the XDS package29 to integrate and scale collected data. All crystals belong to the orthorhombic P₂₁2₁2₁ space group and exhibit similar cell dimensions, as previously reported for the wild-type *T. thermophilus* 70S crystals (Table 1).

We used PHASER from the CCP4 suite30 to determine the initial solution for the structure by molecular replacement. The search model was generated from the previously published structure of the *T. thermophilus* 70S ribosome (PDB 1VY4)31 with all its ligands removed. The refinement with two 70S ribosomes in the asymmetric unit was performed by rigid-body refinement, and then by five cycles of position and B-factor refinement with PHENIX31. After initial refinement, unambiguous difference electron density for Onc112, mRNA and P-site tRNA^{Met} became clearly visible in the F₀ – F₁ difference Fourier map. The Onc112 peptide, mRNA and the tRNA^{Met} in the P site were built into the F₀ – F₁ electron density map with Coot32, and the model was further refined with PHENIX31. The electron density for Onc112 allowed us to unambiguously build the first 13 residues. The electron density for the last six residues was not seen, thus indicating their conformational flexibility. The absence of electron density for the fMet moiety of the tRNA^{Met} is probably due to the hydrolysis of the aminoacyl group from the tRNA or its flexibility. The final statistics of refinement are provided in Table 1.

Figures. PyMOL (http://www.pymol.org/) was used to generate all figures.

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