Solution Structure of YaeO, a Rho-specific Inhibitor of Transcription Termination

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Rho-dependent transcription termination is an essential process for the regulation of bacterial gene expression. Thus far, only two Rho-specific inhibitors of bacterial transcription termination have been described, the psu protein from the satellite bacteriophage P4 and YaeO from Escherichia coli. Here, we report the solution structure of YaeO, the first of a Rho-specific inhibitor of transcription termination. YaeO is an acidic protein composed of an N-terminal helix and a seven-stranded β sandwich. NMR chemical shift perturbation experiments revealed that YaeO binds proximal to the primary nucleic acid binding site of Rho. Based on the NMR titrations, a docked model of the YaeO-Rho complex was calculated. These results suggest that YaeO binds outside the Rho hexamer, acting as a competitive inhibitor of RNA binding. In vitro gel shift assays confirmed the inhibition of nucleic acid binding to Rho. Site-directed mutagenesis showed that the negative character of YaeO is essential for its function in vivo.

Transcription termination is the process by which a nascent RNA is released from its complex with RNA polymerase and DNA template. In bacteria, two main mechanisms of transcription termination have been described. These mechanisms, commonly referred to as Rho-independent and Rho-dependent termination, are essential for the regulation of bacterial gene expression (1). Rho-independent termination occurs at a GC-rich self-complementarity region that forms a stem-loop structure believed to cause the RNA polymerase to pause, allowing the release of the RNA (2, 3). Rho-dependent termination, on the other hand, requires the presence of a hexameric helicase, Rho (4, 5). Rho is an essential transcription factor that binds nucleic acids at specific termination sites (rut) and translocates along the RNA until it reaches the transcription complex (6–8). There, it facilitates termination by unwinding RNA/DNA heteroduplexes upon hydrolysis of ATP (9).

Currently, only two Rho-specific inhibitors of transcription termination have been reported. The first to be described is a 21.3-kDa protein encoded by gene psu of the satellite bacteriophage P4 (10). Psu interferes with transcription in phage, plasmid, and bacterial operons, and its activity does not depend on sequences in the transcript. In vitro, protein Psu causes efficient readthrough of Rho-dependent terminators A terminator 1 and TIS2 in a manner that seems to be insensitive to NusG (11). Whether Psu inhibits an enzymatic activity of Rho or the interaction of Rho with RNA, ATP, NusG, or RNA polymerase is unknown. NusG is a transcriptional elongation factor that interacts with both Rho and RNA polymerase (12, 13). The second inhibitor is the product of gene yaeO from Escherichia coli, which has been shown to reduce termination in the Rho-dependent bacteriophage terminator T1 and upstream the autonomously regulated gene rho (14). Overexpression of YaeO can cause the pleiotropic suppression of conditional lethal mutations in division and heat shock genes such as ftsQ, ftsA, gprE, groEL, and groES (14). YaeO is a 9-kDa acidic protein that binds tightly to Rho, but the exact nature of this interaction was unknown (14). Here, the solution structure of YaeO is reported, the first of a Rho-specific inhibitor of transcription termination. Additionally, the binding surface of the Rho-YaeO complex was mapped for both proteins and a docked model calculated. Finally, the effect of some YaeO mutants on Rho-dependent transcription termination was tested in vivo and a mechanism for YaeO-mediated regulation proposed.

EXPERIMENTAL PROCEDURES

Sample Preparation—The gene yaeO from E. coli K12 was subcloned into pET15b (Novagen, Inc., Madison, WI) and expressed in E. coli BL21 as an oligo-histidine (His tag) fusion protein of 106 residues. Cells were grown at 37 °C to an A600 of 0.8 and induced with 1 mm isopropyl-1-thio-β-D-galactopyranoside. Afterward, the temperature was reduced to 30 °C and the cells were allowed to express the protein for 3 h before harvesting. The media used were either Luria Bertani or M9 minimal medium containing [15N] ammonium chloride and/or [13C]glucose (Cambridge Isotopes Laboratory, Andover, MA). YaeO was purified by affinity chromatography on Ni2+-loaded chelating Sepharose (Amersham Biosciences). NMR samples were −2 mm protein in 50 mm phosphate buffer 1 mm NaN3, 2 mm dithiothreitol, pH 7.0. Full-length Rho and the amino-terminal domain of Rho, residues 1–130 (Rho130), were cloned, expressed, and purified in a similar fashion. For simplicity in the

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The atomic coordinates and structure factors (code 1SG5) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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text, residues in YaeO and Rho are numbered according to the wild-type protein sequences.

**NMR Spectroscopy**—NMR experiments were recorded at 303 K on a Bruker Avance 600 MHz spectrometer. Backbone and side-chain assignments of YaeO were determined using HNCA, CBCA(CO)NH, 15N-edited TOCSY, and 13C-edited TOCSY. Nuclear Overhauser effect (NOE)2 data for the structure determination were obtained from homonuclear NOESY, 15N-edited or 13C-edited three-dimensional NOESY experiments. Evaluation of spectra and manual assignments was completed with XEASY (15). IPAP-HSQC experiments for measuring 15N-1H dipolar couplings were recorded on an isotropic medium and a sample containing 18 mg/ml Pf1 phage (16, 17). 15N(1H)-heteronuclear NOE data were measured by taking the ratio of peak intensities from experiments performed with and without 1H presaturation. Hydrogen bond constraints were introduced to secondary structure regions as determined by chemical shift analysis and characteristic NOE patterns. φ and ψ dihedral restraints were obtained using the TALOS program (18). All NMR spectra were processed using either XWINNMR version 2.5 or 3.1 (Bruker Biospin) or GIFA (19). Evaluation of spectra and manual assignments was completed with XEASY (15).

**Analysis and Structure Calculations**—CNS 1.1 software (20) was used to generate an initial fold of YaeO with a basic set of manually assigned NOEs obtained from three-dimensional 15N-edited NOESY and two-dimensional homonuclear NOE spectra and dihedral angle and hydrogen bond constraints (21). These calculations generated a fold that was used as a model template for automated assignments by ARIA 1.1 (22). The final structure of YaeO was calculated using the constraints in Table 1 and collected from the experiments described above. In the final round of calculations, CNS 1.1 was extended to incorporate residual dipolar coupling restraints (RDCs) for further refinement. The axial and rhombic components of the alignment tensor were defined from a histogram of measured RDCs (23) and optimized by a grid search method (24). Twenty structures were selected based on the lowest overall energy and least violations to represent final structures. PROCHECK-NMR was used to generate Ramachandran plots to check the stereochemical geometry of the protein (25).

**Ligand Titration**—Chemical shift perturbation analyses were performed by recording a series of 15N-HSQC spectra at 298 K on uniformly 15N-labeled YaeO or Rho130 (~2 mM) in the presence of different amounts of ligand. The protein sample and stock solutions of the ligands were all prepared for NMR as stock solutions of the ligands were all prepared for NMR as

2 The abbreviations used are: NOE, nuclear Overhauser effect; HSQC, heteronuclear single quantum correlation; r.m.s., root mean square.

**Docking**—Docking of YaeO and Rho130 was done using HADDOCK (26). Ambiguous interaction restraints were defined for residues at least 50% solvent-exposed and with chemical shift perturbations above the average. Mobile regions were determined based on heteronuclear NOE data. 200 structures were calculated during the first step of rigid body energy minimization followed by 50 structures of semirigid simulated annealing in torsion angle space. The best model was selected based on the convergence of the structures at the interface and intermolecular energy.

**Site-directed Mutagenesis**—For site-directed mutagenesis, plasmid pSEB41 was used as a template. Acidic residues from YaeO thought to be involved in the interaction with Rho were mutated to lysine using the QuikChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing.

**β-Galactosidase Assays**—Strains JS219/pJPB314 (lacZp) and JS219/pJPB314/pSEB41 (lacZp-yaeO) were grown at 37 °C in Luria broth supplemented with 2% glucose. Cultures were maintained below 400 = 1 by dilution. Strain JS219/pJPB314 was also transformed with plasmid pSEB41 containing D14K, E40K, or E73K mutations. β-Galactosidase assays were performed as described by Miller (27). Strains and plasmids were kindly provided by Dr. J-P. Bouché (CNRS, Toulouse Cédex, France).

**Electromobility Gel Shift Assays**—Assays of nucleic acid binding to full-length Rho were carried out as described in Ref. 28. Chemically synthesized oligo(dC)34 was 32P-labeled with T4 polynucleotide kinase and mixed with the indicated proteins in 25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and 50 mM KCl, 10% glycerol, and 0.2 mg/ml bovine serum albumin for 5 min before electrophoresis on a 4% polyacrylamide gel with 0.05% Nonidet P-40 in TBE buffer (90 mM Tris base, 44 mM boric acid, 2 mM EDTA) and autoradiography.

**RESULTS**

**Structure Determination of YaeO**—YaeO was produced in *E. coli* as an N-terminal His tag fusion protein and purified by affinity chromatography. The His tag was not removed for structural studies as its presence did not perturb the structure of the protein, as evidenced by comparison of 1H-15N-HSQC spectra of the cleaved and uncleaved protein. The protein was labeled uniformly with 15N or with 15N and 13C for NMR analysis. Backbone resonance assignments were obtained with standard triple resonance NMR experiments. The overall structure of YaeO is well defined by the NMR data, except for residues 1–8 and 84–86, which are not structured. These residues have almost no long-range NOEs and show 1H-15N NOEs close to zero, which is indicative of high mobility in solution (supplemental Fig. S1). From secondary chemical shift analysis, we deduced that YaeO is composed of one α helix and a seven-stranded β-sandwich.

The location of secondary structure elements relative to the primary sequence reveals that helix α1 is the best conserved secondary structure element (Fig. 1). Variations exist in the β3–β4 and β6–β7 loops, possibly reflecting differences in their function. Highly conserved residues cluster at helix α1 or form the hydrophobic core of YaeO. Residues Cys-12, Asp-16, Glu-19, Cys-22, and Glu-52 are completely conserved. The side chains of these residues are located in the exposed surface of YaeO, suggesting that their conservation might reflect their biological function. Fig. 1 shows a stereo superposition of the ten lowest energy NMR structures. The helical region comprises residues 14–23. The two faces of the sandwich are formed by antiparallel β strands between β2/β1/β6/β7 and β3/β4/β5, respectively. β strands 1–7 comprise residues 26–32, 36–42,
the presence of an additional most important difference between the Sm-fold and YaeO is binding domain of small ribonucleoproteins (Sm-fold). The fold of YaeO is topologically similar to that of the RNA ranging from 2.7 to 3.3 Å for matching C atoms. These results reveal that, despite the lack of recognizable sequence similarity, the fold of YaeO is topologically similar to that of the RNA binding domain of small ribonucleoproteins (Sm-fold). The most important difference between the Sm-fold and YaeO is the presence of an additional β-strand (β7) in YaeO. It is important to note that, even though most of the hits are transcriptional regulators or involved in mRNA processing, titrations with single-stranded DNA performed on YaeO did not reveal any observable interaction (data not shown).

**Interactions between YaeO and Rho**—To understand the mechanism of transcription termination inhibition by YaeO, NMR experiments were performed to observe the interaction of YaeO with Rho in vitro. For this, a truncated version of Rho, Rho130, from *E. coli* was constructed. This fragment corresponds to the primary RNA binding site of Rho (residues 1–130) and has been shown to be a good model of Rho-oligonucleotide interactions (29). We performed an NMR titration by recording a series of *H, 15N HSQC spectra of 15N-labeled YaeO as a function of unlabeled Rho130 concentration. Complex formation occurred, as evidenced by fast exchange chemical shift perturbations in the *H, 15N HSQC spectra (Fig. 2A). Comparison of the bound and free spectra allowed us to map the binding site of Rho on YaeO (Fig. 2B and C). The largest chemical shifts occurred in the N and C termini, helix α1 and strands β3, β4, β5, and β7. These regions localize to one edge of the β-sandwich with clustered acidic residues. These results suggest that the unfolded N and C termini of YaeO become structured upon binding to Rho.

As the structure of Rho130 has been solved by NMR (29), we decided to map the interaction with YaeO on Rho130 (Fig. 2D). Rho130 was uniformly 15N-labeled and the 1H-15N HSQC peaks were assigned using the chemical shifts for the previously determined NMR structure, kindly provided by Dr. G. Rule (30). Rho130 is composed of an α-helical (residues 1–47) and a β-sheet subdomain (residues 48–130). Chemical shift pertur-
TABLE 1

Structural statistics for 20 selected conformers for YaeO

| Constraint used for structure calculation | Constraint value |
|------------------------------------------|-----------------|
| Intraresidue NOEs (n = 0)                | 270             |
| Sequential range NOEs (n = 1)            | 253             |
| Medium range NOEs (n = 2,3,4)           | 68              |
| Long range NOEs (n > 4)                  | 159             |
| Hydrogen bonds                           | 107             |
| 15N-1H residual dipolar couplings       | 33              |
| Average r.m.s. deviation from idealized covalent geometry | 0.0025 ± 0.00001 |
| Average r.m.s. difference to mean structure (Å) | 0.5081 ± 0.0072 |
| Average Ramachandran statistics (%)     |                 |
| Residues in most favored regions        | 87.1            |
| Residues in additionally allowed regions| 10.8            |
| Residues in generously allowed regions  | 2.1             |
| Residues in disallowed regions          | 0.0             |
| Analysis of residual dipolar couplings  |                 |
| r.m.s. deviation (Hz)                    | 1.390 ± 0.035   |
| Q-factor                                 | 0.138 ± 0.003   |
| Final energies (kcal/mol)               |                 |
| $E_{total}$                              | 265.67 ± 4.57   |
| $E_{c}$                                  | 8.76 ± 0.55     |
| $E_{angle}$                              | 96.70 ± 2.65    |
| $E_{improper}$                           | 19.52 ± 1.02    |
| $E_{cart}$                               | 80.94 ± 3.39    |
| $E_{van}$                                | 30.95 ± 1.68    |
| $E_{dihedral}$                           | 9.02 ± 0.61     |
| $E_{ani}$                                | 19.75 ± 1.53    |
| Total number of constraints              | 951             |

Solution Structure of YaeO

Bation analysis showed that YaeO binds primarily to strands $\beta_3$, $\beta_4$, $\beta_5$ and helix 4 of the $\beta$-sheet subdomain (Fig. 2E). Minor shifts were also observed for helix 3 and strands 1 and 2. Structural evidence has shown that RNA binds to the N-terminal domain of Rho mainly by contacts with Phe-64, Arg-66, Glu-78, Tyr-80, Arg-88, Phe-89, Glu-108, and Tyr-110 (31–33). These residues are neighbors of the YaeO-interacting region of Rho, suggesting a mechanism of transcription termination inhibition by blocking the RNA binding site on Rho (Fig. 2F).

Model of the Rho-YaeO Complex—As NMR titration data for the YaeO-Rho interaction were available for both proteins, a model of the complex was built using high ambiguity-driven protein docking (HADDOCK) (26). Ambiguous interaction restraints were derived from the NMR titration data by selecting residues with both the biggest chemical shifts and solvent accessibility. Unfolded residues were allowed to move freely during the docking protocol. The model with the lowest intermolecular energy after the last stage of refinement in Cartesian space with explicit solvent was selected. The interaction between YaeO and Rho seems to be facilitated by charge complementarity and burial of $\sim$2558 Å$^2$ of surface area. This is consistent with in vitro binding results that show the YaeO-Rho interaction is salt-dependent and can be disrupted at high ionic strengths (0.4 M KCl) (14). The docking model suggests six potential salt bridges: Asp-5:Arg-87, Asp-44:Lys-100, Asp-44:Lys-115, Glu-52:Arg-88, Glu-57:Arg-102, and Glu-64:Lys-105. The model is compatible with the hexameric structure Rho as there are no clashes when the YaeO-Rho130 model is superposed to the hexameric, open ring structure of Rho (Fig. 3).
**Solution Structure of YaeO**

![Diagram A](image1)

**Figure 3. Model of the YaeO-Rho complex.** A, lowest energy YaeO-Rho130 complex calculated with the program HADDOCK. Chemical shift perturbation data shown in Fig. 2 were used for the docking. YaeO is colored green, and Rho130 is magenta. YaeO blocks the path of RNA bound to Rho (dashed yellow line). B, model of YaeO bound to the open ring, hexameric form of Rho (31), showing the steric clash (dotted yellow circles) between YaeO and bound RNA.

![Diagram B](image2)

**Figure 4. YaeO inhibits single-stranded DNA binding to Rho and Rho function.** A, electromobility gel shift assay. Addition of Rho (5 μM) retards migration of d(C34) through formation of a Rho complex. Complex formation is inhibited by addition of increasing amounts of YaeO (5 and 50 μM). At 50 μM, the YaeO charge-reversal mutants D14K, E19K, and E52K are unable to block complex formation. Control lanes show that YaeO alone does not interact with single-stranded DNA. B, effect of YaeO mutants on ρ gene transcription. When YaeO is expressed, levels of β-galactosidase regulated by an upstream Rho binding sequence increase 7-fold relative to the negative control (-). YaeO mutants D14K, E19K, and E52K showed β-galactosidase levels similar to the negative control. These results support the notion that charge complementarity is an important factor for formation of the YaeO-Rho complex and that in vivo YaeO acts by decreasing the affinity of Rho for nucleic acids.

YaeO binding overlaps with the predicted path of RNA when bound to Rho and is expected to inhibit binding.

**YaeO Inhibition of Nucleic Acid Binding by Rho**—To test this prediction, we carried out electromobility gel shift assays with oligo(dC)34 and Rho in the presence and absence of YaeO. The binding of poly(dC) by hexameric Rho is competitive with the binding of poly(rC) (34). Addition of 5 μM YaeO blocked approximately half of complex formation at two different concentrations of Rho (supplemental Fig. S3). At a 10-fold excess of YaeO to Rho, single-stranded DNA binding was completely blocked.

**Mutagenesis of the Rho Binding Site**—The model of YaeO bound to Rho reflects the electrostatic complementarity of the two protein surfaces. To test the importance of the expected salt bridges, three YaeO mutants, D14K, E19K, and E52K, were constructed. The effects of these charge-reversal mutations were tested in the gel shift assay. All three mutations prevented the inhibition of nucleic acid binding to Rho (Fig. 4A). The structural integrity and proper folding of the mutant proteins were verified by NMR (supplemental Fig. S4).

The mutants were also tested in vivo using a β-galactosidase reporter system developed by Pichoff et al. (14). In this system, the expression of β-galactosidase is under the control of a Rho binding sequence upstream of the coding region. Expression of YaeO inhibits Rho, allowing the production of high levels of β-galactosidase. The three mutants tested showed β-galactosidase levels similar to the negative control, indicating that the acidic residues are required for YaeO inhibition of Rho activity.

**DISCUSSION**

Our data demonstrate that YaeO binds to the transcription factor Rho and provide a structural basis for its inhibition of transcription termination. It is likely that YaeO binds to the Rho hexamer in a 1:1 monomer-to-monomer ratio. Gel shift assays show that YaeO reduces the affinity of Rho for nucleic acids with an approximate in vitro $K_{d}$ for YaeO of 5 μM. Previous studies have suggested that the pathway of RNA binding to hexameric Rho consists of four steps: PR1, PR2, PR3, and PR4 (35). State PR1 is formed when the RNA binds to the primary binding site of Rho. Bound RNA then fills the continuous binding sites in the crown to form PR2. The third step consists of the opening of the ring, leading to passage of the RNA through the central channel to form PR3. Finally, PR4 is formed when the ring closes, rendering Rho competent in ATPase and translocation activities. Our data suggest that the YaeO acts by inhibiting the formation of the PR1 and/or PR2 states.

Rho is unique to prokaryotes and essential for the viability of many bacterial species; as a consequence it is an attractive target for drug development. The use of antibiotics targeting Rho dates from the isolation of bicyclomycin (Bicozymycin, BCM) in 1972 from Streptomyces sapporosensis and Streptomyces aizumenses (36, 37). BCM possess antimicrobial activity against Gram-negative bacteria such as E. coli, Shigella, and Salmonella and Gram-positives such as Micrococcus luteus (38).

The inhibitory mechanism of YaeO is different from that of BCM, as the latter binds to the C-terminal domain of Rho (39). The knowledge of the structure of YaeO and its binding site on
Rho opens a new possibility for the rational design of antibiotics targeting Rho. YaeO mimetics offer the possibility of a novel class of antibacterial compounds that block the initial steps in the association of Rho with RNA.

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