Structural basis for the methylation of A1408 in 16S rRNA by a panaminoglycoside resistance methyltransferase NpmA from a clinical isolate and analysis of the NpmA interactions with the 30S ribosomal subunit

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ABSTRACT

NpmA, a methyltransferase that confers resistance to aminoglycosides was identified in an Escherichia coli clinical isolate. It belongs to the kanamycin–apramycin methyltransferase (Kam) family and specifically methylates the 16S rRNA at the N1 position of A1408. We determined the structures of apo-NpmA and its complexes with S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) at 2.4, 2.7 and 1.68 Å, respectively. We generated a number of NpmA variants with alanine substitutions and studied their ability to bind the cofactor, to methylate A1408 in the 30S subunit, and to confer resistance to kanamycin in vivo. Residues D30, W107 and W197 were found to be essential. We have also analyzed the interactions between NpmA and the 30S subunit by footprinting experiments and computational docking. Helices 24, 42 and 44 were found to be the main NpmA-binding site. Both experimental and theoretical analyses suggest that NpmA flips out the target nucleotide A1408 to carry out the methylation. NpmA is plasmid-encoded and can be transferred between pathogenic bacteria; therefore it poses a threat to the successful use of aminoglycoside antibiotics. The results presented here will assist in the development of specific NpmA inhibitors that could restore the potential of aminoglycoside antibiotics.

INTRODUCTION

Aminoglycosides are bactericidal antibiotics that are widely used in treatment against severe infectious diseases caused by Gram-negative and Gram-positive bacteria. They bind to the 30S subunit of the ribosome at the A-site and induce a conformational change that is responsible for the loss of fidelity of the protein translation process (1). Due to the indiscriminate and excessive use of these antibiotics, pathogenic bacteria have acquired from antibiotic producers and further evolved several mechanisms of resistance to these antibiotics such as: alteration of the antibiotic to inactivate it, mutation on the small ribosomal subunit, which modifies the target site of the antibiotic, or methylation at specific sites on the 16S rRNA that prevent the binding of the antibiotic to the ribosome (2). Mutation of A to G at position 1408 in the 16S rRNA has been found in clinical isolates of mycobacteria (3). However, methylation provides high-level resistance to most aminoglycosides used in the clinical practice. It is carried out by specific methytransferases that are found in two classes of bacteria: the antibiotic producers and the pathogens. The results presented here will assist in the development of specific NpmA inhibitors that could restore the potential of aminoglycoside antibiotics.
pathogens. In antibiotic producers, the gene encoding the methyltransferase is usually found on the chromosome, whereas in pathogens it is typically present on an extrachromosomal plasmid (4).

There are two families of methyltransferases that are responsible for the resistance against aminoglycosides. These are the Arm (aminoglycoside resistance methyltransferase) family (5) and the Kam (kanamycin–apramycin methyltransferase) family (6), which specifically methylate the 16S rRNA at the N7 position of G1405, and the N1 position of A1408, respectively. Kam enzymes confer high-level resistance to broad spectrum aminoglycosides. KamA and KamB have been found in antibiotic producers, while NpmA from *Escherichia coli* clinical strain ARS3 is the first methyltransferase found in a clinical isolate that can methylate 16S rRNA at the N7 position of G1405, apramycin methyltransferase family (6), which specifically methylates the AdoMet (S-adenosyl-L-methionine) and AdoHcy (S-adenosyl-L-homocysteine) in the functional NpmA structure and the knowledge of its interaction with the small ribosomal subunit by footprinting analyses and computational docking. These studies provide insight into the mechanism of substrate recognition and methylation of A1408 by NpmA. The NpmA structure and the knowledge of its interaction with the substrate will serve as a starting point for the development of specific inhibitors of the Kam family of methyltransferases that could reestablish aminoglycoside sensitivity in aminoglycoside-resistant pathogens.

**MATERIALS AND METHODS**

**Cloning and site-directed mutagenesis**

The *npmA* gene synthesis was ordered from the Epoch Biolabs Inc. following the sequence published in ref. (7). The *npmA* gene was subsequently recloned into NdeI and EcoRI sites of the pET-25b(+) vector with the addition of a C-terminal non-cleavable (His)_6-tag. The amino acid sequence of NpmA is devoid of methionine. In order to facilitate the structure determination through SelMet-labeled protein, we mutated four codons for leucine residues (L31, L90, L128 and L196) to methionine residues (L31, L90, L128 and L196) to methionine codons by inverse PCR (8) using Phusion® high-fidelity DNA polymerase (Finnzymes). Mutations leading to alanine substitutions of selected conserved amino acids (D30, D55, E88, P106, W107, W109, F177, S195, W197, K199, R200 or R205) were introduced either by using Platinum® Pfx DNA polymerase (Invitrogen) in the PCR-overlapping method (9) or by inverse PCR using Phusion® high-fidelity DNA polymerase (Finnzymes). All introduced mutations were verified by DNA sequencing. Deoxyligonucleotides used in this work are listed in Supplementary Table S1.

**Expression and purification**

*E. coli* BL21 (DE3) was transformed with recombinant vector pET-25b(+) carrying the *npmA* gene for protein expression. *E. coli* cells were cultured in 11 of LB medium supplemented with ampicillin (100 mg/l) at 37°C until the OD₆₀₀ reached 0.5–0.6 AU. The expression was induced with 150 μM IPTG and cells continued to grow at 20°C overnight. Cells were harvested by centrifugation (9000g, 30 min, 4°C), and the pellet was resuspended in 40 ml of lysis buffer [50 mM HEPES sodium pH 7.5, 250 mM NaCl, 10% glycerol, 0.5% Triton-X 100, 5 mM β-mercaptoethanol (BME) and 1 protease inhibitor tablet (Roche)].

After sonication the cell lysate was centrifuged at 39191g for 30 min at 4°C. The supernatant was allowed to bind to the Ni-NTA agarose beads (Qiagen) for 1 h at 4°C and was subsequently washed with wash buffer I ([50 mM HEPES sodium, pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM BME and 5 mM imidazole, pH 7.5]), wash buffer II ([50 mM HEPES sodium, pH 7.5, 1.0 M NaCl, 10% glycerol, 5 mM BME and 10 mM imidazole, pH 7.5]) and wash buffer III ([50 mM HEPES sodium, pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM BME and 15 mM imidazole, pH 7.5]). Finally, the protein was eluted in three steps using buffer A ([50 mM HEPES sodium, pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM BME, 200 mM imidazole, pH 7.5]), buffer B ([50 mM HEPES sodium, pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM BME and 250 mM imidazole, pH 7.5]), and buffer C ([50 mM HEPES sodium, pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM BME and 300 mM imidazole, pH 7.5]). The eluted protein was loaded onto a size exclusion column (Superdex 200, GE Healthcare) equilibrated with the buffer containing 20 mM HEPES sodium, pH 7.5, 250 mM NaCl, 5% glycerol, 5 mM BME and 10 mM MgCl₂. The eluted NpmA was concentrated up to 11 mg/ml. A similar protocol was adopted to purify the selenomethionine (SelMet) labeled NpmA using the M9 medium (10).

NpmA variants with alanine substitutions used for analysis in functional assays were expressed and purified following a simplified procedure. *E. coli* BL21(DE3)pLysS cells harboring the expression vector pET-25b(+) with different *npmA* variants were grown in LB medium supplemented with ampicillin (100 mg/l) and chloramphenicol (34 mg/l) at 37°C until the OD₆₀₀ of 0.6–0.8. The expression was induced with 1 mM IPTG and cells continued to grow at 37°C for 3 h with the addition of 3% ethanol. Cells were pelleted by centrifugation (3500 g, 10 min, 4°C), frozen at −80°C and the pellet was resuspended in 40 ml of lysis buffer [50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole and a cocktail of protease inhibitors (Roche)]. Following sonication, the debris was removed by centrifugation at 20 000 g for 20 min at 4°C. Clarified supernatant was incubated for an hour at 4°C with the Ni-NTA Agarose (Qiagen) equilibrated with the lysis buffer.
buffer. The resin with the bound NpmA variant was subsequently washed with the wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole). The protein was eluted with the elution buffer (50 mM NaH₂PO₄, pH 8.0, 250 mM NaCl, 5% glycerol, 5 mM BME, 200 mM imidazole) and dialyzed against 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 5% glycerol, 5 mM BME, 10% glycerol. All NpmA variants were purified to 95% homogeneity judged by the SDS-polyacrylamide gel electrophoresis.

Crystallization and data collection

Purified NpmA was complexed with the cofactor S-adenosylmethionine (AdoMet) and reaction by-product S-adenosylhomocysteine (AdoHcy) at 1:12 (NpmA:AdoMet/AdoHcy) molar ratio. Crystallization trials were carried out at room temperature by hanging-drop vapor-diffusion method using crystallization screens from Hampton Research (Aliso Viejo, CA, USA) and Jena Bioscience screens (Jena, Germany). Initially crystals of NpmA and NpmA–AdoMet/AdoHcy complexes were obtained from the Jena Bioscience screens. Subsequently these conditions were optimized to obtain the diffraction quality crystals. The best crystals of NpmA alone or with AdoMet/AdoHcy were obtained by mixing 1 μl of concentrated protein with 1 μl crystallization solution [0.1 M citrate pH 5.5, 20% (v/v) PEG 4000 and 10% (v/v) 2-propanol for NpmA and NpmA-AdoMet complex; 30% (v/v) PEG 4000, 0.1 M sodium acetate, pH 4.6 and 0.2 M ammonium sulfate for NpmA-AdoHcy complex] and were grown up to 8 days.

Prior to data collection, crystals were briefly soaked in a cryoprotectant consisting of the paratone and mineral oil in a 1:1 ratio, and flash cooled in a N₂ cold stream (100 K). A complete single wavelength anomalous dispersion (SAD) data set was collected for the NpmA–AdoHcy complex at the beamline X8C, NSLS, Brookhaven National Laboratory using a Quantum4-CCD detector. All the data sets were processed and scaled with HKL2000 (11). The crystallographic and refinement statics are provided in Table 1.

Structure solution and refinement

The expected eight selenium sites in one asymmetric unit of the NpmA–AdoHcy complex crystal were located using the program Solve (12). The overall figure of merit was 0.77. The resultant electron density map was of good quality to automatically build over 80% of the model using wARP (13). The remaining part of the model was manually built using the program COOT (14). Alternating cycles of model building and refinement using the programs CNS and REFMAC were carried out (15, 16) with appropriate entries made in the dictionary for AdoHcy. The final R-factor is 0.21 (R_free is 0.24) up to 1.9 Å resolution. The structure of apo-NpmA, NpmA–AdoMet complex and the high-resolution NpmA–AdoHcy complex (1.68 Å) was solved by molecular replacement method using MOLREP (17) with the NpmA coordinates of the NpmA–AdoHcy complex as a search model. The overall geometry of the final models was analyzed by PROCHECK (18).

Isothermal titration calorimetry (ITC)

NpmA in a buffer consisting of 20 mM HEPES sodium, pH 7.5, 250 mM NaCl, 5% glycerol, 5 mM BME and 10 mM MgCl₂ was titrated against S-adenosyl methionine (AdoMet/AdoHcy, MP Biomedicals) solution prepared by dissolving the AdoMet in the same buffer as the protein to a final concentration of 100 mM. The ITC experiments were carried out using VP-ITC calorimeter (Microcal, LLC) at 20°C using 0.02–0.07 mM protein in the sample cell and 2–5 mM AdoMet/AdoHcy in the injector. All samples were thoroughly degassed and centrifuged. Injection volumes of 8–10 μl per injection were used for different experiments and for each experiment the heat of dilution for the ligand was measured. To restore the baseline, successive injections were separated by at least 4 min. The ITC data was analyzed by a single site-fitting model using Origin 7.0 (OriginLab Corp.) software.

Determination of the minimum inhibitory concentration of kanamycin

Kanamycin minimum inhibitory concentration (MIC) was determined in E. coli BL21(DE3)pLysS cells carrying recombinant pET-25b(+) vector with different npmA variants using the MIC dilution assay with small modifications of a procedure described previously. In brief, overnight cultures of BL21 (DE3) pLysS cells carrying mutant genes in pET-25b (+) vector were diluted 100-fold in fresh LB medium supplemented with ampicillin (100 mg/l) and chloramphenicol (34 mg/l) and grown until the OD₆₀₀ of 1. Two hundred microliter of LB medium containing ampicillin, chloramphenicol and various concentration of kanamycin (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 mg/l) was inoculated with 10⁴ cells and grown in microtiter plates in total volume for 18 h at 37°C. MIC was determined as a minimal concentration of kanamycin that prevents the visible growth of bacteria.

Isolation of 30S ribosomal subunits

Tetra-(Hist)₉-tagged 70S ribosomes were isolated from E. coli JE28 cells (obtained from Dr Suparna Sanyal from Uppsala University, Sweden) using nickel-affinity chromatography, as a modification of a method described in ref. (19). Cells were harvested at OD₆₀₀ of 1, cooled slowly to 4°C, pelleted and resuspended in the lysis buffer [20 mM Tris–HCl, pH 7.6, 10 mM MgCl₂, 150 mM KC1, 30 mM NH₄Cl, 0.5 g/l lysozyme, 10 mg/l DNaseI, protein inhibitor cocktail (Roche)]. The suspension was lysed by sonication and cell debris was removed by two centrifugation cycles at 20 000g for 10 min. Lysate was applied to a HiTrap™ Chelating HP column (1 ml, GE Healthcare) equilibrated with the lysis buffer. Column
was washed with the lysis buffer until A260 reached the baseline. Tetra-(His)6-tagged 70S ribosomes were eluted with the elution buffer (20 mM Tris–HCl, pH 7.6, 10 mM MgCl2, 150 mM KCl, 30 mM NH4Cl, 150 mM imidazole) and dialyzed four times for 10 min against the low-Mg2+ buffer (20 mM Tris–Cl, pH 6.5, 100 mM NH4Cl, 10 mM MgCl2, 6 mM BME with 10 % glycerol). Reactions were stopped by the addition of 1 ml of 0.5 M EDTA. Twenty-five microliter of reaction mixtures were then transferred to the microtiter plate wells coated with immobilized AdoHcy-BSA conjugate and incubated 30 min with 200 ml of mouse monoclonal anti-AdoHcy antibodies. Reaction mixture was initiated with the addition of 100 ml of sulphuric acid and absorbance was measured on 450 nm using a microplate reader. Concentration of AdoHcy was determined using AdoHcy calibration curve that was prepared using control DNA samples provided in the kit. Duplicate samples were measured in three independent experiments in all cases.

### Methylation assay

The activity of NpmA and its variants was determined using a modification of a procedure described in Axis Homocysteine Enzyme Immuno Assay kit (Axis-Shield Diagnostics Ltd.). The concentration of AdoHcy produced in the NpmA-catalyzed methyl transfer is proportional to the concentration of methylated RNA and production of AdoHcy (or indirectly m1A1408) and is monitored in a competitive ELISA assay using anti-AdoHcy antibodies. Reactions for in vitro methylation contained 4 pmol of 30S subunits, 100 pmol of AdoMet (Sigma) and 8 pmol of NpmA. All reactions were carried out for 45 min at 37°C in 50 ml of 20 mM Tris–Cl, pH 6.5, 100 mM NH4Cl, 10 mM MgCl2, 6 mM BME with 10 % glycerol. Reactions were stopped by the addition of 1 ml of 0.5 M EDTA. Twenty-five microliter of reaction mixtures were then transferred to the microtiter plate wells coated with immobilized AdoHcy-BSA conjugate and incubated 20 min with 200 ml of rabbit anti-mouse antibodies conjugated with the horseradish peroxidase. Following the wash step, the detection was initiated with the addition of 100 ml of the peroxidase substrate. Reaction was terminated after 20 min by the addition of 100 ml of sulphuric acid and absorbance was measured on 450 nm using a microplate reader.

### Table 1. Crystallographic data and refinement statistics

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<th>High resolutionb</th>
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<td>12.7</td>
<td>11.6</td>
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</table>

### Additional notes

a Rsym = Σ|Ii| – <I>/Σ|Ii|, where Ii is the intensity of the i-th measurement, and <I> is the mean intensity for that reflection.

b For all models, reflections with I<σ(I) were used in the refinement.

c Rwork = Σ|Fobs|–|Fc|/Σ|Fobs|, where Fobs and Fcalc are the calculated and observed structure factor amplitudes, respectively.

d Rfree = as for Rwork, but for 10% (5% for NpmA–AdoHcy SAD data) of the total reflections chosen at random and omitted from refinement.

f Individual B-factor refinements were calculated.

i Four molecules (chains A–D) in the asymmetric unit. D chains shows high B-factor values and it is not included in the averaging. However, it has continuous well-defined electron density map.
Analysis of rRNA by primer extension

Total rRNA was extracted from *E. coli* BL21(DE3)pLysS cells carrying the expression vector pET-25b(+) with and without the variants of the *npmA* gene using GenElute Mammalian Total RNA Miniprep Kit (Sigma). Briefly, cells were harvested in early log phase (OD₄₅₀ of 0.4–0.5), lysed by sonication and cell debris was removed by two centrifugation cycles at 20 000g for 10 min. Total RNA was then prepared from the lysates using the RNA cleanup protocol. A 5'-Cy5-modified deoxynucleotide primer, complementary to 16S rRNA from nucleotides 1459 to 1479, was hybridized to the rRNA and extended with AMV reverse transcriptase (Finnzymes) (20). Sequencing reactions were performed on rRNA isolated from *E. coli* cells lacking NpmA. Extension products were separated on 6% denaturing polyacrylamide gels and were visualized on Typhoon 9210 imager (GE Healthcare).

Chemical probing of 30S subunit–NpmA complex

Chemical probing was performed with DEPC and CMCT as described in (21). Briefly, 10 pmol of 30S subunits were incubated with a 2-fold molar excess of NpmA and 1 mM AdoHcy in either modification buffer A (50 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂, 100 mM KCl, 5 mM dithiothreitol) or B (200 mM HEPES-KOH, pH 7.8, 10 mM MgCl₂, 100 mM KCl) for 30 min at 37°C in 50 μl reaction mixtures. Control reactions contained no AdoHcy. The complexes were modified with either 50 μl of CMCT (42 mg/ml in modification buffer A) for 10 min at 37°C, or 1 μl of DEPC for 30 min at room temperature. RNA was purified immediately with a GenElute Mammalian Total RNA Miniprep Kit (Sigma), RNA cleanup protocol. Chemical modifications were detected by primer extension analysis using AMV reverse transcriptase (Finnzymes) and 5'-Cy5 modified deoxyoligonucleotides complementary to the 16S rRNA sequences 1459–1479, 939–955 and 817–833. The cDNA products of primer extension reactions were separated on 6% polyacrylamide sequencing gel and visualized on Typhoon 9210 imager (GE Healthcare).

Bioinformatics analyses

To identify orthologs of NpmA, we used its sequence as a query to search the non-redundant protein database with PSI-BLAST (22). Sequences with significant similarity to NpmA were retrieved and aligned using MUMMALS (23). The alignment was refined based on the results of structure predictions obtained from the GeneSilico MetaServer (24). Based on structural superpositions onto NpmA, the sequences of TrmB, TrmK, TrmI and Sgm proteins were added to the alignment for comparison.

A model of the wt NpmA protein (with SelMet reverted to Leu) was constructed using SwissModel (25). GRAMM 1.03 (26) was used to generate 10 000 alternative docking models (poses) between the NpmA–AdoMet complex and the crystal structure of the small ribosomal subunit (Protein Data Bank code 2I2P). The search was restricted to the area within 50 Å radius from the A1408 nucleotide residue. The following parameters were used: energy scores for repulsion and attraction were 30 and 0, respectively, grid size 64 Å, grid step 2.8 Å. The NpmA structure was rotated with 10° angle intervals. Subsequently, all 10 000 poses were filtered using the Filtrest3D server (27), to rank them according to the proximity of the N1 atom of A1408 and the methyl group of AdoMet bound to NpmA. Top 100 variants have been clustered with MAXCLUSTER (http://www.sbg.bio.ic.ac.uk/~maxcluster/index.html), and a member of the largest cluster that exhibited best shape complementarity and minimal number of clashes between NpmA and the 30S subunit was chosen as the representative model.

**RESULTS**

Overall structure

The structure of NpmA–AdoHcy complex was solved by the SAD experiment, while the structures of the apo-NpmA and NpmA–AdoMet complex were solved by molecular replacement using NpmA–AdoHcy coordinates as a search model. The electron density for the first and the last residue, loop region (aa 144–157) and the C-terminal His-tag are not well defined and these residues are not included in the model (Figure 1). The models were refined to low R-values with good stereo-chemical parameters (Table 1). In apo-NpmA and NpmA–AdoHcy structures the asymmetric unit consists of two molecules, whereas in the NpmA–AdoMet complex, the asymmetric unit has four complex molecules. However, crystal contacts are not very extensive;
suggesting that they are not biologically relevant and the gel filtration experiment showed that NpmA exists as a monomer in solution (Supplementary Figure S1).

NpmA adopts a Class I methyltransferase fold consisting of a seven-stranded β sheet (β5→β4→β3→β6→β7→β9→β8). A search for structurally similar proteins within the PDB database was performed with DALI (28). NpmA shows the highest structural similarity to TrmB from *Bacillus subtilis*, PDB code 2FCA (29), with rmsd of 3.1Å for 179 Cα atoms (Z-score = 16.5; 13% sequence identity) and to a number of its bacterial and eukaryotic orthologs from the TrmB/Trm8 family. TrmB is a guanosine- N7 methyltransferase that specifically methylates the G46 nucleotide in tRNAs. A common feature of NpmA and TrmB that distinguishes them from other Class I MTases identified so far is that the N-terminal helix (corresponding to motif X) folds onto the opposite part of the central β-sheet (Figure 1) compared to other enzymes of this fold (30). The space vacated by the N-terminal helix is instead occupied by a TrmB/NpmA-specific insertion between strands β8 and β9, which in NpmA comprises two short helices, and in TrmB one helix and a loop. Thus, NpmA and TrmB should be considered closely homologous enzymes sharing the same idiosyncrasies within the RFM superfamily. According to DALI searches, NpmA shares structural similarities with other RFM-fold proteins with predicted or determined methyltransferase activity, but they are all more distant than members of the TrmB family (Supplementary Table S2).

**NpmA–AdoMet/AdoHcy interactions**

The ligands in NpmA–AdoMet and NpmA–AdoHcy complexes display a similar mode of binding (Figure 2; Supplementary Figure S3). The highly conserved consensus motif G×G×G (G32×G×G36) in the loop connecting the β3 to its successive α helix forms the bottom of the cofactor-binding site, while residues D30, N38, P56,
T109, E88, A87, D55, G32, L104 and S195 delineate the cofactor-binding site (Figure 2). The residues D30, G32 and D55 are highly conserved in Class I AdoMet dependent MTases and are involved in cofactor binding. D30 coordinates the carboxypropyl moiety via two water molecules and D55 coordinates both hydroxyls of the ribose moiety. The residues equivalent to E88, A87 and L104 of NpmA have also been found to interact with the cofactor in other MTases (31). In particular the side chain of E88 (like its equivalents in other related enzymes) coordinates the exocyclic amino group of the AdoMet adenine moiety, while L104 and A87 are engaged in hydrogen bonds with the cofactor via their backbone. The adenine moiety is stacked between the side-chains of P56 and L110, and its N7 atom is additionally coordinated by the side chain of T109. Besides, residues N38, G32, L104 and S195 interact with the carboxypropyl moiety of AdoMet/AdoHcy. The cofactor binding site in apo-NpmA is highly similar to that of the NpmA–AdoMet/AdoHcy complex, with the exception of the D55, whose side chain exhibits a slightly different orientation than in the cofactor-bound complexes.

In order to study the predicted cofactor-binding role of residues D30, D55, E88, T109 and S195, we used site-directed mutagenesis to generate alanine substitutions in the respective positions. We analyzed the cofactor binding ability and methylation activity of the corresponding protein variants in vitro by a methylation assay (Figure 3) and in vivo by comparing them with the wt enzyme in their ability to render bacteria resistant to kanamycin (Table 2). We also identified the presence of methylated A1408 in cells expressing different NpmA variants by primer extension on 16S rRNA (Figure 4). Interestingly, the only residue we found critical for the NpmA activity is D30. The D30A variant was almost completely unable to render bacteria resistant to kanamycin, its methylation activity in vitro was reduced to nearly 20% activity of the native enzyme and primer extension analysis revealed that m1A1408 was present in minimal amounts in 16S rRNA isolated from cells expressing D30A. Surprisingly, D55 that coordinates the ribose moiety of the cofactor via two hydrogen bonds was found to be important, but not crucial for the NpmA methyltransferase activity. The kanamycin MIC and enzyme activity of D55A were reduced to 50% compared to the wt NpmA, but the reverse transcriptase stop due to the m1A modification on nucleotide A1408 in the primer extension analysis was clearly visible. Furthermore, E88A, T109A and S195A variants showed no substantial difference from the wt enzyme in their methyltransferase activity, as shown by all in vivo and in vitro activity tests.

To gain more insight into AdoMet/AdoHcy binding ability of unexpectedly active D55A, E88A, T109A, S195A and D30A protein variants versus the wt enzyme, we analyzed these proteins by ITC (Figure 5, Supplementary Figure S5 and Table 3). The dissociation constant (K_d) for AdoMet and AdoHcy of the wt NpmA was determined to be 20 μM with N = 1.1 and 0.6 μM with N = 1.0, respectively. The K_d for AdoMet (18 μM) is comparable to that of the Sgm methyltransferase, whereas the K_d for AdoHcy is 500 times higher than that of Sgm (21).

### Table 2. Effects of NpmA single mutations on kanamycin resistance

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<th>NpmA variant</th>
<th>Target</th>
<th>Kanamycin MIC (mg/l)</th>
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<tbody>
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<td>Negative control (empty vector)</td>
<td>–</td>
<td>4–8</td>
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<tr>
<td>NpmA-wt</td>
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<tr>
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<td>A1408 binding/catalysis</td>
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<tr>
<td>W107A</td>
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<td>T109A</td>
<td>AdoMet binding</td>
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<tr>
<td>F177A</td>
<td>RNA binding</td>
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<tr>
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Figure 3. Relative activities of NpmA variants expressed with respect to the wild-type enzyme.
The T109A variant retained its binding ability, although weaker, with $K_d$ values of 0.4 mM and 2 $\mu$M for AdoMet and AdoHcy, respectively. Interestingly, the $K_d$ of S195A for AdoHcy was found to be 33 $\mu$M, but we observed a complete loss of AdoMet binding, while the NpmA variants D30A, D55A and E88A completely lost their ability to interact with both AdoMet and AdoHcy. It is surprising that S195A, E88A and D55A variants, for which we could not detect cofactor binding in vitro, showed remarkable activity in vivo. However, the ITC experiments were performed in the absence of the substrate, the 30S subunit.

**NpmA-target adenine interactions**

Spatially adjacent and conserved residues P106 and W107 from motif IV, F177 from motif VII and W197 from motif VIII were predicted to form the adenosine binding pocket. Notably W107 side chain adopts different conformation in NpmA–AdoMet and NpmA–AdoHcy complexes. In AdoMet complex, the indole ring of W107 moves closer to the methyl group of the cofactor to enhance the interactions, whereas the lack of methyl group in AdoHcy keeps the side chain of W107 away from AdoHcy (Supplementary Figure S2). The results of our functional tests confirmed the importance of the two Trp residues, as the in vitro methylation activity of W107A and W197A variants was significantly reduced (to ~15% of the wt enzyme activity). Interestingly, m1A1408 could not be detected in 16S rRNA from the cells expressing the altered proteins and they failed to cause any kanamycin resistance in bacterial cells under the conditions tested. P106A and F177A on the other hand, were as active in vivo as the wt enzyme according to the amount of the methylated 16S rRNA (Figure 4) and kanamycin resistance (Table 2). We observed a decrease of activity in the in vitro methylation assay, where P106A and F177A showed ~70 and 60% activity, respectively, compared to the wt NpmA. This effect might be a consequence of the protein instability outside the living cell, especially in the case of F177A, where we observed a lower yield of the purified protein (data not shown). W107 and W197 are positioned similarly to aromatic residues in the active sites of many other nucleic acid MTases that are known to stabilize the target base via stacking interactions e.g. DNA:m5A MTase M.TaqI (32) or 23S rRNA:m6A2058 MTase Ermc (33). Our structural and functional analyses suggest that these two Trp residues function in a similar manner in NpmA, by stabilizing the target adenosine in a favorable position for the catalytic step. On the other hand, the side-chains of P106 and F177 seem to have a secondary and non-essential role in this process.

Guiding of the target base into the catalytic center of MTases that modify nucleic acids is often accompanied by interactions of positively charged residues exposed on the enzyme surface with either DNA/RNA backbone or with the nucleotide residues located next to the target (34–36). We therefore analyzed three conserved residues K199, K200 and R205 that are in the vicinity of the adenosine binding pocket. Surprisingly, all three residues turned out to be completely dispensable and no difference could be found between the variants with individual alanine substitutions and the wt NpmA. Our results differ from those obtained for KamB enzyme, where the corresponding R195 was found to be very important and R196 and R201 were indispensable for the MTase activity in vivo (37).

**RNA footprinting of NpmA interaction with the 30S ribosomal subunit**

To obtain information about interactions between NpmA and the 30S subunit, which was found to be the only...
Figure 5. ITC experiments. (A) NpmA–AdoMet titration. (B) NpmA–AdoHcy titration. (C) D30A–AdoMet titration. (D) D30A–AdoHcy titration. The upper panels show the injection profile after baseline correction and the bottom panels show the integration (heat release) for each injection (except the first one). The ITC profile for other variants such as D55A, E88A that are similar to D30A (no binding) are provided in the Supplementary section. Similarly the remaining variants mentioned in the main text (e.g. T109A and S195A) are given in the Supplementary section (Supplementary Figure S5).
substrate methylatable in vitro (7), we performed DEPC (specific for A) and CMCT (specific for G and U) probing of 16S rRNA. The summary of the results is shown in Figures 6 and 7. Chemical modifications in 16S rRNA isolated from the MTase-treated 30S subunits were detected by primer extension. We used primers complementary to the 16S RNA nucleotides 1459–1479, 939–955 and 817–833. This enabled us to examine the regions that are proximal to the target A1408 in the fully assembled 30S subunit and thus could form the putative binding site for NpmA. Chemical probing allowed us to monitor the changes in the reactivity of nucleotides upon NpmA binding, where nucleotides became either less reactive (potentially protected from the water-soluble reagent) or more reactive (potentially more exposed to the water-soluble reagent). In addition, we also observed a different pattern of reverse transcriptase stops upon NpmA binding at nucleotides other than those expected to react with either DEPC or CMCT. This effect is often observed within regions with A and C residues and may be caused e.g. by structural changes in the RNA after its isolation from the 30S particles that were incubated with NpmA, resulting from chemical modifications.

A distinct section containing the majority of nucleotides that become protected upon NpmA binding was found in helix 24 (between nucleotides 810 and 760). In addition, a few protected nucleotides were also found in helices 22 and 23 (nucleotides 754, 706, 682, 665) and in helix 25 (nucleotide 880). In helices 26 and 26a, we observed a few nucleotides that become exposed upon NpmA binding. Binding of NpmA results in protection of nucleotides in helices 42 and 43 (residues 1299–1362) and exposure of residues in helix 41. It is remarkable that the target nucleotide residue A1408 was not found to be protected by NpmA. Instead, two distinct regions upstream of A1408 (including nucleotides 1388–1390, 1392, 1393, 1395, 1397 in helix 28 and 1404, 1406 and 1407 in the top part of helix 44) showed enhanced reactivity as a result of NpmA-induced structural changes.

Model of the NpmA-30S subunit complex
To predict the three-dimensional mode of protein–substrate interactions, we docked the NpmA–AdoMet complex to the 30S subunit structure, with a restraint imposed as to minimize the distance between the methyl group of AdoMet and the target nucleoside A1408.
Figure 7. Panel I. Schematic representation of chemical footprinting of NpmA methyltransferase on the 30S ribosomal subunit. Samples were modified with CMCT and DEPC. RNA was purified from 30S subunits and analysed by primer extension from primer 817–833 (A), 939–955 (B) and 1459–1479 (C). Nucleotides found protected upon NpmA binding are marked with a black triangle (CMCT) or a black dot (DEPC). Nucleotides found exposed upon NpmA binding are marked with a grey triangle (CMCT) or a grey dot (DEPC). Regions showing enhanced non-specific reactivity with chemicals (indicating local structural changes caused by NpmA binding) are circled. Helix numbering is indicated in boxes. Panel II. Mapping of footprinting data onto the 30S structure and the model of NpmA-30S complex. Components of the 30S subunit are shown in the surface representation. NpmA is shown as a solid ribbon in blue. Ribosomal proteins and regions of 16S rRNA not analyzed by footprinting are indicated in light grey. Results of the footprinting experiments are color coded as follows: residues with unchanged reactivity are shown in white, residues relatively more reactive in the footprinting experiment are shown in red, residues less reactive in the footprinting experiment are shown in green. The target A1408 is shown in orange.
DISCUSSION

The nucleotide residue A1408 in 16S rRNA of the 30S ribosomal subunit is a crucial ‘hot spot’ of aminoglycoside resistance. The universally conserved A1408 has a critical role in binding of aminoglycoside antibiotics to the A site of 16S rRNA. Structural studies have shown that the hydroxyl group on sugar ring I of 4,6- and 4,5-disubstituted 2-deoxystreptamines forms hydrogen bonds with the N1 and N6 atoms of A1408 (38–40). The N1 methylation of A1408 by NpmA apparently disrupts the interaction of the 16S rRNA with the drug, as indicated in footprinting experiments (7) thereby conferring resistance. The recent detection of the Kam family member NpmA in a clinical isolate (7) indicates an imminent threat to the utility of aminoglycosides as antibacterial drugs and warrants attention. The structure of NpmA and its complex with the cofactors and substrate holds key to understanding its mode of action and potentially to develop specific inhibitors of the A1408 methylation activity that could reinstate the activity of aminoglycoside antibiotics.

Based on structural analyses we identified residues in NpmA potentially important for cofactor-binding and catalysis and carried out site-directed mutagenesis to obtain protein variants with alanine substitutions in the corresponding positions. As predicted, the ITC experiments revealed that NpmA variants with cofactor-binding residues substituted by Ala have completely lost or exhibited significantly reduced ability to bind AdoMet or AdoHcy (Figure 5, Supplementary Figure S5 and Table 3). We were, however, greatly surprised by the fact that the variant D55A retained the ability to carry out m'1A1408 methylation in vitro, as well as to render the host-cell resistant to aminoglycosides, because substitutions of this conserved residue in other RNA methyltransferases, e.g. D55 in KamB (37) or D156 in Sgm (41) has lead to drastic reduction of these enzymes’ activity both in vivo and in vitro. We speculate that while the D55A variant of NpmA cannot form a binary complex with the cofactor, it may bind AdoMet and methylate the target when it is already engaged in interactions with the substrate. For some related methyltransferases, e.g. Dam enzyme from phage T4 phage, it has been demonstrated that the binding of the methyl group donor or the nucleic acid substrate reciprocally improve the affinity for each other in the ternary complex (42). This suggests that NpmA may also exhibit significantly increased affinity for AdoMet when pre-bound to the 30S subunit. Other enzymes, however, e.g. Dam from E. coli (which is very closely related to T4 Dam), exhibit similar affinity for AdoMet in the presence or in the absence of the substrate (43). Apparently, large variations of parameters concerning the binding of the ligands and their mutual interactions are not uncommon among related methyltransferases. We speculate that in NpmA, binding of the 30S subunit increases the affinity for AdoMet to such extent that it can rescue the ligand binding ability of the D55A variant, while the similar effect has not been observed for KamB. Our results show that the relative importance of ‘the same’ residues in closely related proteins may vary. This study highlights the importance of comparative biochemical and functional analyses of proteins that belong to the same family and suggests that structural and functional characterization of just single members of each protein family (as in the structural genomics approach) may be insufficient to understand sequence-structure–function relationships in proteins. The finding that the side chain of D55 residue is dispensable for the in vivo activity of NpmA, and hence that mutations altering that residue may appear in the nature, has to be taken into consideration in analyses aiming at structure-based design of inhibitors.

NpmA binding to the 30S subunit

NpmA was found to successfully methylate only fully assembled 30S subunits, while the entire 70S ribosome
or naked 16S rRNA could not be methylated (7). This reactivity towards 30S subunits is analogous to aminoglycoside resistance MTases from the Arm family, ArmA and Sgm, which methylate G1405 (44,45). Here we present the analysis of interactions between a member of the Kam family and small ribosomal subunit as its substrate. Using DEPC and CMCT chemical probing and subsequent primer extension analysis we were able to identify helices 24 and 42 with associated loop as primary regions that interact with NpmA. In addition, two short sections of 16S rRNA 5' from the target nucleotide residue A1408 showed increased non-specific reactivity to chemicals when isolated from 30S subunits incubated with the NpmA. This might be the result of a local structural change in the rRNA caused by NpmA binding, suggesting that NpmA-30S interaction could be associated with the top of helices 44 and 28 as well.

We have recently reported a similar footprinting and docking analysis for Sgm MTase from the Arm family that modifies G1405 (21). While G1405 and A1408 are positioned very closely in rRNA, suggesting a similar mode of binding for Sgm and NpmA, we could not clearly define the region(s) of 16S rRNA that form distinctive interactions with Sgm. Instead, protected nucleotides were scattered over a wide area, together with a large number of nucleotides with the activity enhanced upon Sgm binding, indicating complex structural changes of a rather global character that enable the otherwise buried G1405 base to become available for methylation by Sgm. Unlike Sgm, NpmA binding to the 30S subunit does not lead to global structural rearrangements; instead we observe only local changes. Remarkably, the target base A1408 was not found to be protected upon NpmA binding, which suggests that it may be flipped-out and more solvent accessible in the complex with the enzyme than in the 30S subunit before modification. Its binding may be stabilized in the NpmA active site by residues W107 and W197, found here to be essential for the methylation to occur in vivo. The conformation of W107 side chain is different in complexes with AdoMet and AdoHeCy, which suggests that limited conformational changes upon binding may occur also on the protein side. In the crystal structures of NpmA, the side-chains of these Trp residues are too close to each other to enable binding of the target base, but a simple change to alternative rotamers allows the protein to form a binding pocket to accommodate the adenine moiety (data not shown). The necessity of minor conformational adjustments has to be taken into consideration in the future analyses aiming at structure-based design of inhibitors.

The results of footprinting experiments with Sgm and NpmA are in perfect agreement with the docking studies. In our previous work (21) we found that the Sgm structure cannot be docked to its target G1405 without very severe steric clashes with the 30S subunit, which implies that significant conformational changes upon its binding must occur. On the other hand, NpmA can be easily docked to its target A1408, to form a relatively close-fitting complex with the 30S subunit. Therefore we postulate that Sgm and NpmA use very different mechanisms to access their target bases.

Regions of 16S rRNA that we found to be involved in interactions with NpmA come in close proximity and form functional sites in fully assembled 30S subunits, including the decoding site and site of association with 50S subunit, which clearly explains why NpmA cannot act on 70S ribosomes. In addition, regions identified as NpmA binding sites partly overlap with the KsgA-30S interaction sites identified by directed and solution hydroxyl radical footprinting that include helices 2, 11, 24, 27, 28, 44 and 45 and loop 790 (46). KsgA is a housekeeping dimethyltransferase that modifies A1518 and A1519 (both to m6,6A) in helix 45 of 16S rRNA. It acts as a regulator of ribosome biogenesis and binds to immature 30S subunits in a translationally inactive conformation (47). NpmA is known to act on mature 30S subunits, but it has not been determined whether it can methylate any of the reconstitution intermediates (RIs) in the 30S subunit assembly. The first intermediate, RI, lacks tertiary ribosomal proteins S2, S3, S10, S14 and S21 in central and 3'-domain and is not competent to form a small ribosomal subunit. rRNA in RI undergoes an extensive conformational rearrangement to form RI*, which can then bind the remaining five ribosomal proteins and form 30S subunit (48). Holmes and Culver have performed an extensive analysis of conformational changes in 16S rRNA during the assembly of small ribosomal subunit (49) and mapped structural differences between the assembly intermediates RI and RI* (50). The majority of conformational changes related to the central and 3'-domain of the 30S subunit occur during transition from RI to RI*. These changes include functional sites on 30S, where NpmA binds and recognizes its target, hence we may propose that the earliest stage that supports the NpmA action could be a nearly assembled RI* of the 30S subunit.

**Relationship of NpmA to other MTases; insight from structural analyses**

The availability of a high-resolution NpmA structure allowed us to establish its relationship to other MTase families. NpmA is a member of the RFM superfamily and exhibits a class I MTase fold with the exceptional topological rearrangement involving the N-terminal helix. The arrangement of the N-terminal helix in NpmA is similar to those found in the structure of tRNA:m'G56 MTase TrmB, a representative of a protein family that appears to be the closest homolog of Kam-family MTases (Supplementary Figure S6). It is very intriguing that an RNA:m'G MTase (NpmA) appears to be more closely related to an RNA:m'G MTase (TrmB) than to other m'A MTases, for which structures have been determined, including tRNA:m'AS8 MTase Trml (51) and tRNA:m'A22 MTase TrmK (52,53). NpmA is also structurally distinct from Arm/RmtB/Sgm enzymes that form m'G1405 in 16S rRNA (Supplementary Figure S6). G1405 is very close to A1408, the site of methylation of NpmA. While NpmA/Kam and Arm/RmtB/Sgm methyltransferases belong to the same RFM superfamily and methylate bases that are very close to each other in the 30S subunit leading to a similar
phenotypic effect, i.e. resistance to aminoglycoside antibiotics, their catalytic domains are not very closely related and presumed substrate-binding domains are completely different.

Based on structural superposition we generated a multiple sequence alignment involving all members of the Kam family (including NpmA studied in this work), as well as individual representatives of m1A MTase families TrmI and TrmK, and m7G MTases TrmB and Sgm (Supplementary Figure S4). Motifs I and II involved in the binding of the common cofactor AdoMet exhibit residues that are universally conserved between these enzymes (in particular carboxylates involved in coordination of the methionine and ribose moieties). On the other hand, motifs IV, VI, and VIII that commonly harbor residues involved in binding of the target base and catalysis are completely different. In the active sites, traces of similarity are observed only between NpmA and TrmB enzymes; Y193 is homologous and most likely functionally equivalent to the base-binding residue W197 of NpmA. Both proteins also share a common Phe residue (F116 in TrmB and F105 in NpmA), which stacks face to edge with the above-mentioned base-binding aromatic residue and thereby stabilizes it. Other than that, however, the active sites of TrmB and NpmA are different, consistent with the activity on a different substrate: the N7 atom of guanosine versus the N1 atom of adenosine.

The absence of common residues in motifs responsible for catalysis suggest that m1A MTases acting on different RNA targets have non-homologous active sites and most likely developed independently from different ancestors within the RFM superfamily. In particular, our analysis suggests that the Kam family evolved from the TrmB/Trm8 family of m7G MTases. This is in agreement with the results of our phylogenetic analysis of the entire RFM superfamily, in which the Kam, TrmI and TrmK families of m1A MTases do not form a single branch on the tree superfamily, in which the Kam, TrmI and TrmK families likely developed independently from different ancestors with different specificities (e.g. RrmJ-like, HEN1 and Trm13) that exhibit different active sites anchored to the common RFM fold (54) or even to a different fold (55). Recently, a family of RNA MTases of the SPOUT fold have been characterized, in which some members exhibit m1G specificity (56), while others form m1A but not m1G or exhibit dual specificity and generate m1A or m3G (57). This variety of protein families and protein folds with different active sites able to carry out RNA:m1A methylation suggests that this type of reaction is probably relatively easy for an enzyme to evolve (e.g. from an MTase of a different specificity). On the other hand, the uniqueness of the adenosine-binding site of MTases from the Kam family suggests that they may be good targets for the development of Kam-specific inhibitors. The structural analyses presented in this work provide a stepping stone for structure-based virtual screening to find candidates for such compounds.

ACCESSION NUMBER
Coordinates of apo-NpmA, NpmA–AdoMet and NpmA–AdoHcy were deposited in the Protein Data Bank (http://www.pdb.org) under accession codes 3P2I, 3P2K and 3P2E, respectively. The PDB code for the NpmA–AdoHcy complex SAD data set is 3PB3.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

NOTE ADDED IN PROOF
During submission of this article the crystal structures of KamB-AdoHcy and NpmA-AdoMet were independently published by another group (Mcmaester et al., Nucleic Acids Res. published electronically 2010 Jul 17, 10.1093/nar/gkq627). The other group’s structures were on hold in PDB, which prevented us from making detailed comparisons.

REFERENCES


