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Control of Substrate Specificity by a Single Active Site Residue of the KsgA Methyltransferase

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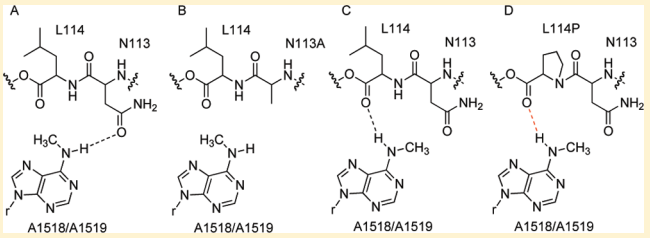
Supporting Information

ABSTRACT: The KsgA methyltransferase is universally conserved and plays a key role in regulating ribosome biogenesis. KsgA has a complex reaction mechanism, transferring a total of four methyl groups onto two separate adenosine residues, A1518 and A1519, in the small subunit rRNA. This means that the active site pocket must accept both adenosine and N^6-methyladenosine as substrates to catalyze formation of the final product N^6,N^6-dimethyladenosine. KsgA is related to DNA adenosine methyltransferases, which transfer only a single methyl group to their target adenosine residue. We demonstrate that part of the discrimination between mono- and dimethyltransferase activity lies in a single residue in the active site, L114; this residue is part of a conserved motif, known as motif IV, which is common to a large group of *S*-adenosyl-L-methionine-dependent methyltransferases. Mutation of the leucine to a proline mimics the sequence found in DNA methyltransferases. The L114P mutant of KsgA shows diminished overall activity, and its ability to methylate the N^6-methyladenosine intermediate to produce N^6,N^6-dimethyladenosine is impaired; this is in contrast to a second active site mutation, N113A, which diminishes activity to a level comparable to L114P without affecting the methylation of N^6-methyladenosine. We discuss the implications of this work for understanding the mechanism of KsgA's multiple catalytic steps.



Nucleotide methyltransferases are ubiquitously found, and their resultant modifications play important roles in DNA and RNA function. Numerous locations on ribonucleotide and deoxynucleotide scaffolds are available for methylation including the exocyclic amines present in adenosine, cytidine, and guanosine, and their deoxy counterparts. Exocyclic amines have the potential for the transfer of two methyl groups, thus providing a greater opportunity for diversification. Numerous examples of dimethylation exist, with one example being the universally conserved N^6,N^6-dimethyladenosine ($m^6{}_2A$) nucleotides present at positions 1518 and 1519 (*Escherichia coli* numbering) in small subunit rRNA. The enzyme responsible for these modifications is named KsgA in bacteria[1,2] and Dim1 in eukarya[3] and archaea.[4]

The KsgA family belongs to a well-characterized group of *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases, known as Class I MTases, that includes RNA methyltransferases, DNA methyltransferases, protein methyltransferases, and small molecule methyltransferases.[5] Many of these methyltransferases have been well characterized structurally and biochemically, and their mechanisms have been explored. KsgA is unique among this class of enzymes in that it must catalyze the transfer of four methyl groups onto two adjacent bases. This requires that KsgA must accommodate two adenosines separately in the active site pocket and that KsgA must be able to utilize both adenosine and N^6-monomethyladenosine (m^6A) as substrates. Unlike other known N^6-adenosine methyltransferases, KsgA recognizes a complex ribonucleoprotein particle that closely resembles the fully formed 30S subunit.[6,7]

KsgA is closely related to the clinically important Erm methyltransferases, which also belong to the Class I MTase family. The majority of Erm enzymes, including the commonly found ErmC′, confer high levels of resistance to the MLS_B antibiotics by dimethylating A2058 (*E. coli* numbering) of 23S rRNA; this is known as type II resistance. A smaller subset of Erm enzymes monomethylate the same nucleotide, leading to type I resistance to the MLS_B antibiotics, which is characterized by high lincosamide resistance but low to moderate macrolide and streptogramin B resistance.[8,9] Some mechanistic details are known for the Erm enzymes, which overall are less complex than for KsgA. The Erm enzymes mono- or dimethylate a single nucleoside target; dimethylation occurs as two separate random bi-bi reactions.[10] This mechanism is termed distributive, as the enzyme releases the monomethylated intermediate and must rebind to the substrate to catalyze transfer of the second methyl group.

In contrast to the distributive mechanism of the Erm enzymes, data suggest that KsgA works in a processive

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manner,[4,11] which means that all four reaction steps are carried out during a single binding event without the release of intermediates. This mechanism may be an integral part of KsgA's broader function as a checkpoint in ribosome biogenesis,[12,13] whereby KsgA monitors nascent 30S subunits for proper assembly; methylation by KsgA and subsequent product release is a signal that the subunit is ready for further maturation. Premature release of submethylated species could be detrimental to this monitoring function.

Nucleic acid methyltransferases face challenges of substrate access; in many cases, the target base is involved in secondary or tertiary structure and must be "flipped out" of its native conformation in order to be methylated. This process has been extensively studied in DNA methyltransferases, whose targets reside within the double helix. Unlike RNA methyltransferases, which can add one or two methyl groups to their target base, the DNA methyltransferases catalyze a single methyl transfer per nucleotide. In the case of DNA adenosine methyltransferases, monomethylation presumably is all that can be tolerated at these DNA loci because dimethylation would create nucleotides incapable of forming Watson−Crick base pairs.

The crystal and solution structures of KsgA/Dim1 enzymes (refs 14−18; Dong, A., et al., structure published in the RCSB Protein Data Bank), Erm enzymes,[19−21] and N^6-deoxyadenosine methyltransferases[22−26] have been reported, thus allowing for direct structural comparison. These three enzyme families share the same structural fold as well as core structural motifs.[27] Several key active site residues are conserved, including residues in catalytic motif IV, despite lower overall sequence and structural similarity between the RNA and DNA methyltransferases in regions outside the active site. It has been proposed that a particular motif IV residue is an important discriminator for mono- and dimethyltransferase activity,[21] but this hypothesis has not been tested. In this paper we have performed mutational analysis of active site residues of *E. coli* KsgA. We discuss implications of this work for understanding a key aspect of KsgA's mechanism: the ability of the enzyme to dimethylate its two target adenosines.

MATERIALS AND METHODS

Cloning and Protein Purification. KsgA cloning into the pET15b expression vector was previously described.[28] Mutagenesis was performed using the Stratagene QuikChange Kit according to the manufacturer's protocol. The resulting plasmids were transformed into *E. coli* XL1-Blue cells, and mutants were confirmed by sequencing. For growth experiments and polysome analysis the wild-type and mutant KsgA genes were subcloned from pET15b into the pBAD-HisA plasmid (Invitrogen) by digesting with NcoI and XhoI and ligating the resulting fragments using T4DNA ligase (New England Biolabs).

Protein expression and purification were carried out as previously described.[4] Proteins were estimated to be >95% pure by SDS-PAGE analysis. Protein concentration was measured using the Bio-Rad Protein Assay.

30S Purification. Construction of an *E. coli* strain lacking functional KsgA was previously described. 30S subunits from this strain and the wild-type strain were prepared as previously described.[4] Purified subunits were dialyzed into reaction buffer (40 mM Tris, pH 7.4; 40 mM NH_4Cl; 4 mM MgOAc; 6 mM 2-mercaptoethanol) and stored at −80 °C in single-use aliquots. 30S concentration was estimated by measuring the absorbance at 260 nm and using a relationship of 67 pmol 30S per 1 unit of optical density.

***In Vitro* Methyltransferase Assay.** The *in vitro* assay was performed essentially as previously described.[4] Time-course reactions contained 0.2 μM each of unmethylated 30S subunits and enzyme and 20 μM ^{3}H-methyl-SAM (780 cpm/pmol) in Buffer K (40 mM Tris, pH 7.4, 40 mM NH_4Cl, 4 mM $MgCl_2$ and 6 mM mercaptoethanol). At each of eight designated time points 150 μL was removed and immediately extracted with an equal volume of phenol:chloroform:isoamyl. 50 μL of the aqueous phase was removed for deposition onto filter paper and subsequent scintillation counting. The remaining 100 μL was extracted again with phenol:chloroform:isoamyl alcohol and precipitated with ethanol for subsequent HPLC analysis.

Fluorescence Anisotropy. KsgA C168A was labeled with fluorescein-5-maleimide (Invitrogen). Five nmol of KsgA was incubated with 10 nmol of fluorescein-5-maleimide in modification buffer (1 M KCl, 80 mM K^+-Hepes (pH 7.6), 0.01% Triton X-100) in 100 μL total volume at 30 °C for 30 min. Excess fluorescein-5-maleimide was removed from derivatized KsgA on a Hi-Trap Desalting column (GE Life Sciences) according to the manufacturer's instructions. Binding experiments were performed at 25 °C in the activity assay buffer using a Tecan Polarion fluorescence microplate reader. Data were fit using the GraphPad Prism software. For k_{off} measurement, 50 nM each 30S subunits and labeled KsgA were allowed to equilibrate. 10 μM unlabeled KsgA was added at zero time and measurements were taken over a 2 h time course. Data were fit using the equation $Y = (Y_0 - \text{plateau})e^{-kX} + \text{plateau}$. In a separate experiment SAM was added at 15 min. These data were fit separately for time points before and after SAM addition, using the same equation.

For IC_{50} experiments 50 nM each 30S subunits and labeled KsgA were mixed with unlabeled wild-type or mutant KsgA in concentrations from 5 nM to 10 μM. Reactions were allowed to equilibrate and then measured. Data were plotted as log(concentration) vs polarization and were fit using the equation $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{X-\log IC50})$.

HPLC Analysis. HPLC analysis was performed as previously described.[4] Briefly, extracted 16S rRNA was digested and dephosphorylated and subjected to nucleoside analysis by reversed-phase HPLC on a Polaris C-18 column (Varian). Buffer A was 20 mM NaH_2PO_4, pH 5.1. Buffer B was 20 mM NaH_2PO_4, pH 5.1:acetonitrile 70:30. Separation was performed at room temperature using a linear gradient from 100% A − 100% B over 20 min, at a flow rate of 1.0 mL/min.

Growth Curves. The pBAD-HisA-KsgA constructs were transformed into strain JW0050 (Δ*ksgA*) from the Keio Collection. Cells were grown in LB media and ampicillin (50 μg/mL) at 37 °C overnight. Saturated culture was subcultured to an OD_{600} of 0.02 in LB media containing arabinose (0.5%) and ampicillin (50 μg/mL). Cells were incubated with shaking (200 rpm) at 37 or 25 °C, and the OD_{600} was monitored.

Polysome Analysis. Cells containing the pBAD-HisA-KsgA constructs were grown in LB media containing arabinose (0.5%) and ampicillin (50 μg/mL) to mid-log phase. Cells were harvested, and the cell pellet was resuspended in Buffer PA (20 mM Tris, pH 7.8, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM mercaptoethanol). Glass beads (0.090−0.135 mm, Thomas Scientific) were added to a final concentration of 1 mg/μL, and the suspension was vortexed for 10 min. The lysates were cleared by centrifugation at 4 °C and loaded onto a 10−40% sucrose gradient in Buffer PA. The gradients were spun in an

SW-28 rotor at 19 000 rpm for 17 h at 4 °C. Gradients were analyzed at 254 nm using a Biocomp piston gradient fractionator with a BIORAD Econo UV monitor with a full scale of 1.0. Data were recorded using DataQ DI-158-UP data acquisition software, and the 70S peaks were then normalized to 1.

Crystallography. Crystallization was performed at room temperature by the hanging drop vapor diffusion technique. Crystals grew in screens containing either $MgSO_4$ or PEG monomethyl ether 5000 as a precipitant. Crystals growing from PEG monomethyl ether 5000 solution had better diffraction quality. After optimization of the conditions, the best crystals were obtained from drops containing the mixture of 2 μL of protein solution (10 mg/mL in 50 mM Tris, pH7.4, 50 mM NH_4Cl, 6 mM β-ME) and 2 μL of reservoir solution (80 mM Na MES, pH 6.5, 25% PEG monomethyl ether 5000, and 0.15 M $(NH_4)_2SO_4$).

Prior to data collection, crystals were washed with a solution of 14% PEG monomethyl ether 5000, 70 mM $(NH_4)_2SO_4$, 25 mM NH_4Cl, 3 mM β-ME, and 40 mM Na MES, pH 6.5. The wash was followed by a quick transfer to the same solution with the addition of 17% ethylene glycol. The crystals were then rapidly frozen in liquid N_2 stream maintained at 100 K. The crystals diffracted to resolution limits of 2.1 Å. Crystals belonged to the C_2 space group with unit cell dimensions: a = 239.52, b = 38.65, c = 64.93 Å, and β = 96.4°. Oscillation frames were measured using a Molecular Structure Corp. (MSC) X-Stream cryogenic crystal cooler system and R-Axis IV ++ image plate detector with a Rigaku MicroMax-007 X-ray source equipped with MSC Varimax confocal optics operating at 40 kv and 20 mA. Data were processed and scaled with d*TREK. Data collection and refinement statistics are compiled in Table S1.

Initial models for the L114P structure were obtained by molecular replacement using phaser with the coordinates of a monomer from Protein Data Bank entry 1QYR as a search model.[14] The initial model was subjected to automated rebuilding and one cycle of Cartesian simulated annealing in PHENIX version 1.3 followed by alternating cycles of manual rebuilding in COOT using $2mF_o - dF_c$ and $mF_o - dF_c$ maps and computational maximum likelihood refinement with REFMAC v5.5.0109. Figures were generated using the software package Pymol.

RESULTS

Construction of Mutants. Structures of a variety of N^6-adenosine methyltransferases were used to construct a structure based sequence alignment using the Expresso software;[29] Figure 1 shows the region of the alignment that contains residues considered in this study. Many structural motifs were present in all of the structures considered. One such conserved motif is known as motif IV;[30] residues of this motif form the core of the catalyic site and help form both SAM and the target adenosine binding pockets. Structural, biochemical, and theoretical reports support the importance of these residues on catalytic efficiency.[21,26,31−36] The core of this motif is a sequence of four amino acids, conserved as N-L/I-P-Y in KsgA/Dim1 and N/D-P-P-Y in DNA adenosine methyltransferases. In ternary structures of DNA MTases complexed with DNA and SAM the N^6 of the adenosine makes hydrogen bond contacts with the side chain of the Asp/Asn residue and with the backbone of the first Pro residue (Figure 2a). When KsgA is superimposed on the T4 Dam ternary structure, these two

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                                        -Motiv IV-
EcKsgA   QQDAMTFNFGELAEKMGQPLRVFGNLPYN 117
MjDim1   WGDALKVDLNK-----LDFNKVVANLPYQ 105
ScDim1   LGDFMKTELPY-------FDICISNTPYQ 132
ErmC'    NKDILQFKFPK-----NQSYKIFGNIPYN 105
T4dam    SLHFKDVKIL-------DGDFVYVDPPYL 175
M.TaqI   LADFLLWE-PG-----EAFDLILGNPPYG 109
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Figure 1. Structure-based sequence alignment of selected adenosine methyltransferases. Resisidues in and around Motif IV are shown, with residues discussed in the text boxed; residues mutated in this study are indicated in red, bold type. Sequences and structures used are *E. coli* (Ec) KsgA (accession number P06992; PDB ID 1QYR[14]), *Methanocaldococcus jannaschii* (Mj) Dim1 (accession number NP_248023; PDB ID 3GRR[45]), *Saccharomyces cerevisiae* (Sc) Dim1 (accession number P41819), *Bacillus subtilis* ErmC′ (accession number P13956; PDB ID 1QAM[21]), T4dam (accession number P04392.1; PDB ID 1Q0S[24]), and *Thermus aquaticus* M.*Taq*I (accession number AAA27506.1; PDB ID 1AQJ[22]). *Homo sapiens* (Hs) Dim1 (PDB ID 1ZQ9; Dong, A., et al., structure published in the RCSB Protein Data Bank) was used as the structural template for ScDim1.

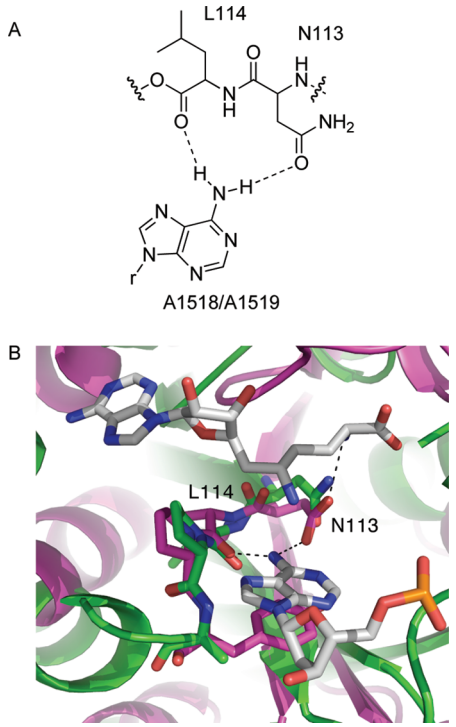

Figure 2. Motif IV hydrogen bonding interactions. (A) Interaction of adenosine with N113 and L114. (B) Active site pocket of KsgA (green) and T4 Dam (magenta; PDB ID 1YFL[25]). The substrate adenosine and the substrate analogue sinefungin are shown in white. Residues from Motif IV are shown as sticks; N113 and L114 are labeled.

residues, N113 and L114, are oriented similarly to their counterparts in T4 Dam (Figure 2b), suggesting their importance for catalysis.

In order to test the importance of these hydrogen-bonding interactions for enzyme activity, we constructed mutants of N113 and L114. The N113A mutation removes the asparagine side chain and thus disrupts the ability of the N^6 atom to hydrogen bond with this residue. In the case of the L114 residue the interaction involves the backbone carbonyl oxygen; therefore, there is no mutation that will remove the potential for forming this hydrogen bond. Instead, we mutated L114 to proline, the residue found in this position in DNA adenosine

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methyltransferases. Schluckebier et al. hypothesized that the identity at this position may determine, at least in part, the ability of a given enzyme to transfer one or two methyl groups.[21] A proline at this position will make the local backbone more rigid and possibly alter the backbone trajectory. Therefore, this change could lead to conformational differences that preclude accommodation of the m^6A substrate, for example, by disallowing formation of the backbone-N^6 hydrogen bond.

***In Vitro* Methyltransferase Activity.** *In vitro* analysis was used to assess the overall catalytic activity of each of the KsgA mutant proteins. In this assay, 30S subunits are purified from an *E. coli* strain lacking functional KsgA protein;[4] methylation of these particles is monitored by using ^3H-methyl-SAM as the methyl donor. At each time point an aliquot is removed from the reaction for scintillation counting; a separate aliquot is removed for subsequent nucleoside analysis (see below). The results of the time-course assay are shown in Figure 3. The

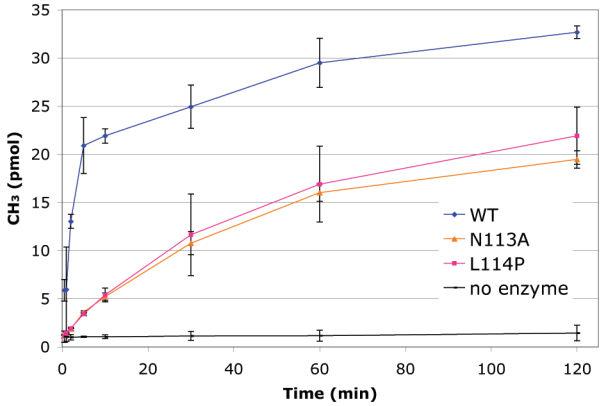

Figure 3. Time course of KsgA activity. Experiments were performed in triplicate, with points representing the mean ± SD.

L114P and N113A mutations both showed 60−65% the catalytic activity of wild-type KsgA after 2 h; moreover, these two mutations showed essentially the same overall activity. This similarity in activity, while coincidental, was fortuitous because it allowed subsequent comparison of the separate methylation steps (see below) between the two mutants.

Analysis of Modified Nucleosides. We used HPLC analysis to monitor the formation of m^6A and $m^6{}_2A$ over time. An aliquot of each reaction was digested to single nucleosides and separated by HPLC. Figure 4a shows a representative chromatogram, depicting a single 120 min time point of each enzyme. Integration of the resulting peaks allowed us to determine the relative percentage of m^6A and $m^6{}_2A$ at each data point of the methyltransferase assay (Figure 3); we can thus quantitate the amount of each methylated species present (Figure 4b). WT KsgA generates very low levels of m^6A throughout the reaction, in agreement with previously published work. The N113A and L114P mutants, despite having nearly identical rates of overall methylation, showed very different $m^6A/m^6{}_2A$ profiles. The N113A enzyme yielded a product distribution similar to that of WT KsgA, albeit at an overall reduced rate, with little m^6A being formed. The L114P mutant, on the other hand, generated considerably higher levels of m^6A, consistent with this residue being a mono- vs dimethylation determinant. This mutant does produce $m^6{}_2A$,

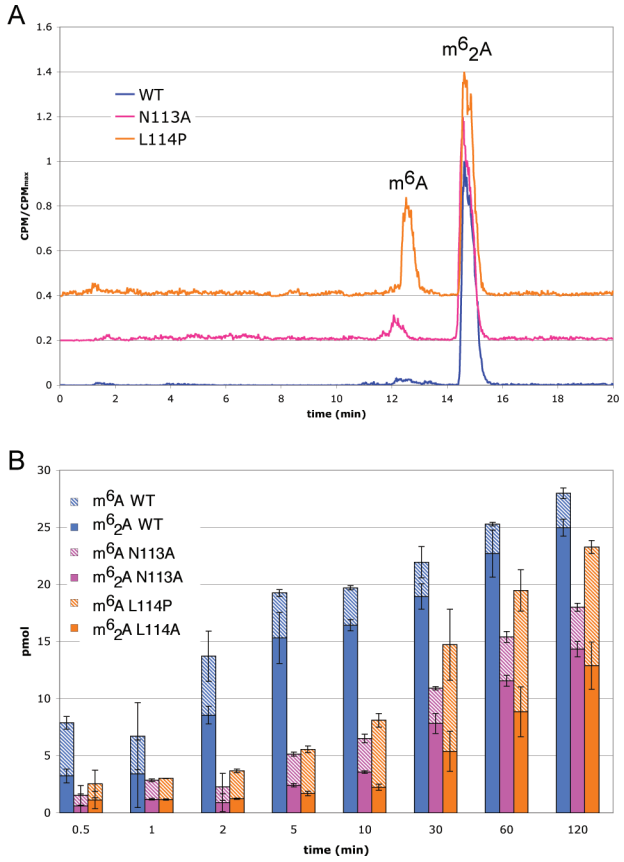

Figure 4. Levels of m^6A and $m^6{}_2A$. (A) HPLC chromatograms of 120 min reactions. Counts per minute (CPM) were normalized to the maximum CPM. Data for N113A and L114P were offset on the *Y*-axis by 0.2 and 0.4, respectively. These data are representative of experiments performed in triplicate. (B) Quantitation of m^6A and $m^6{}_2A$ amounts throughout the time course. Experiments were performed in triplicate, with bars representing the mean ± SD.

but its formation is much slower than with the WT and N113A enzymes.

Substrate Binding. To determine binding of KsgA wild-type and mutant proteins to substrate 30S subunits, we used fluorescence anisotropy. Direct measurements of binding affinity proved difficult to obtain, as preliminary experiments suggested that the K_D of KsgA binding to 30S subunits was too low to measure accurately (not shown). Instead, we measured the off rate of KsgA binding to unmethylated 30S subunits. We constructed a mutant form of KsgA that contained a single cysteine residue, at residue 258, by mutating the only other cysteine (C168) to an alanine; we previously showed that this mutation did not affect KsgA activity.[37] The C168A-KsgA was labeled with fluorescein-5-maleimide. The fluorescently labeled protein was allowed to come to equilibrium with stoichiometric amounts of unmethylated 30S subunits. A 200-fold excess of unlabeled KsgA was added, and the release of labeled protein over time was measured as a change in polarization of the fluorescent label (Figure 5a). The k_{off} measured by this technique is 6.7×10^{-4}/s.

The binding of KsgA to substrate is complex and requires regions of rRNA well beyond helix 45, including regions of the 790 loop (refs 11 and 37; Boeringer, D., Rife, J., and Ban, N., unpublished data). Therefore, it is unlikely that release of the fully methylated 30S or pre-30S particle is solely driven by dimethylation of the adenosine residues. Rather, as suggested

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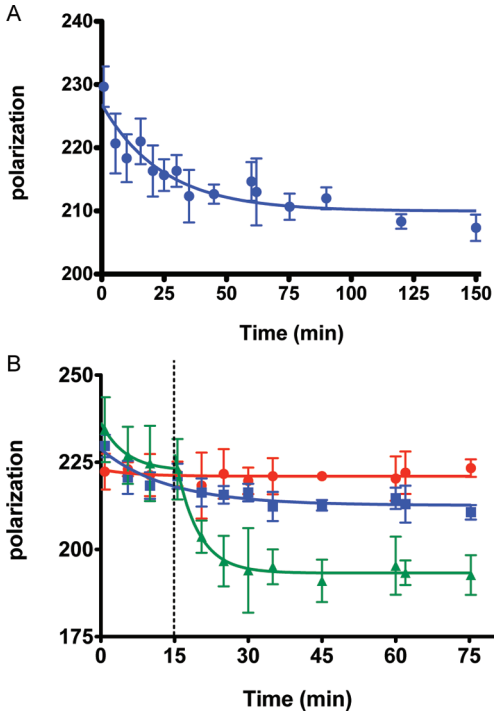

Figure 5. KsgA binding to 30S subunits. (A) Determination of k_{off}. Experiments were performed in triplicate, with points representing the mean ± SD. (B) Dissociation after addition of SAM. Experiments were performed in triplicate, with points representing the mean ± SD. The red line represents KsgA in complex with unmethylated 30S subunits. The blue line represents the same complex, with 200× excess of unlabeled KsgA added at zero time. The green line represents the same complex, plus excess KsgA; in this case SAM was added after 15 min, as indicated by the dashed line. This condition was fit separately for points before and after SAM addition.

by Connolly et al., methylation by KsgA could be linked to a structural change in the maturing pre-30S subunit that is incompatible with KsgA binding.[12] This hypothesis is bolstered by a recent structural comparison of ribosomes from wild-type and *ksgA*$^-$ cells.[38] Methylation by KsgA facilitates small but significant structural changes in mature 30S subunits as compared to subunits unmethylated by KsgA. A corollary to this hypothesis is that formation of the fully methylated product must result in facile dissociation of KsgA. To explore this supposition, we followed KsgA-30S binding in the presence of SAM (Figure 5b). This experiment was performed as above, except that at 15 min SAM was added to the KsgA-30S complex. After the SAM addition, KsgA dissociates rapidly from the subunits.

These binding data demonstrate two important points. First, KsgA binds tightly to unmethylated substrate 30S subunits, with a very slow off rate. Second, enzymatic activity of KsgA leads to rapid release of the methylated product, despite the fact that the overall binding interaction is not directly determined by the target adenosines. This supports the hypothesis that methylation prompts product release in an indirect way, likely by facilitating a conformational change.

In order to compare binding of the wild-type and mutant proteins, we determined the IC_{50} values of unlabeled wild-type or mutant protein competing with fluorescently labeled wild-type (WT) protein for binding to 30S subunits (Figure 6). WT, L114P, and N113A reported similar IC_{50} values, demonstrating that neither of the two catalytic site mutations reduced the

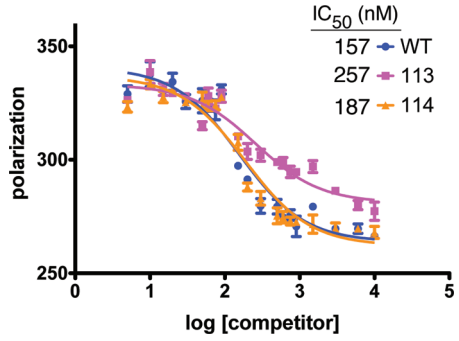

Figure 6. IC_{50} determination. Experiments were performed in triplicate, with points representing the mean ± SD.

affinity of substrate binding. Therefore, reduced activity is not due to disruption of 30S substrate binding.

Ribosome Biogenesis and Growth. We next wanted to assess the ability of the mutant proteins to support proper ribosome biogenesis and assess their impact on bacterial growth. For these experiments we used a strain of *E. coli* containing a deletion of the *ksgA* gene that was obtained from the Keio Knockout Collection.[39] This strain was transformed with an arabinose-inducible plasmid containing either wild-type, N113A, or L114P KsgA. Cells were grown at both 37 and 25 °C, and we performed sucrose gradient analysis of the ribosomal material. These experiments were performed under conditions that favor the association of 30S and 50S subunits to form 70S ribosomes. As expected, the strain expressing wild-type protein showed very little accumulation of free 30S particles as compared to 70S ribosomes (Figure 7). The strain

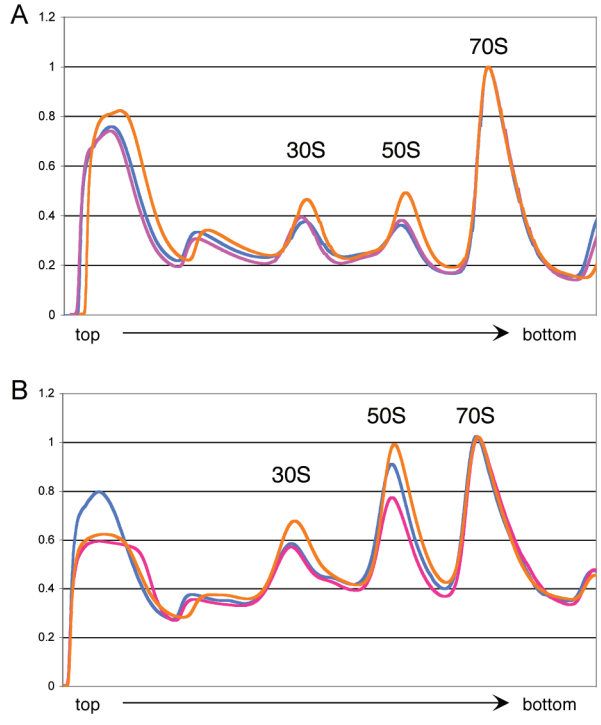

Figure 7. Polysome analysis. Sucrose sedimentation profiles of ribosomes and ribosomal subunits were analyzed by monitoring A254 across a 10−40% gradient. WT enzyme is shown in blue, N113A in magenta, and L114P in orange. (A) Analysis of cells grown at 37 °C. (B) Analysis of cells grown at 25 °C.

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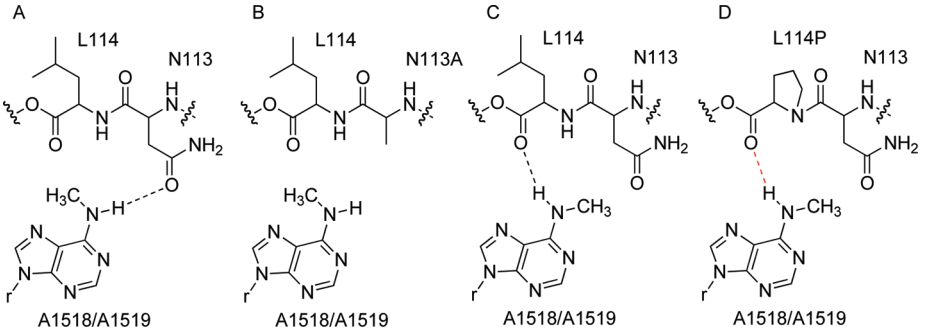

Figure 8. Accommodation of m^6A in the active site. Dashed lines indicate hydrogen-bonding interactions; the red dashed line indicates a suboptimal hydrogen-bonding interaction.

expressing N113A showed a similar result. The L114P strain, however, showed a distinct increase in 30S particles when compared to the 70S peak; this effect was exacerbated at the lower temperature. This defect in ribosome biogenesis was correlated to a small decrease in growth rate at 25 °C; no significant difference in growth rate was seen at 37 °C (data not shown).

Crystallographic Analysis. To better understand the role of L114 in KsgA in directing or "permitting" dimethylation, we crystallized the L114P mutant for comparison with wild-type KsgA and with the structure of the DNA adenosine methyltransferase T4 Dam in a ternary complex with substrate DNA and sinefungin. We superimposed the four core residues of motif IV, using only the backbone atoms for the superposition (Figure S1). This comparison reveals slight but potentially important changes in backbone geometry and side chain orientation between the three structures. Most notable is the relationship between the carboxyl of N113 (D171 in T4 Dam) and the backbone carbonyl of P115/L115 (P172 in T4 Dam). In the T4 Dam ternary complex, these two functional groups form hydrogen bonds to the target N^6 atom of the adenosine substrate. In T4 Dam and KsgA L114P the distance between the two oxygen atoms is 3.8 and 3.9 Å, respectively, while in WT KsgA the distance is 4.5 Å.

DISCUSSION

DNA adenosine methyltransferases are constrained in their function by the structural requirements of the DNA double helix. N^6-Methyldeoxyadenosine is able to make canonical Watson−Crick base pairing interactions with thymidine, whereas N^6,N^6-dimethyldeoxyadenosine could not interact appropriately and would disrupt local DNA structure. Therefore, evolutionary pressure has dictated that DNA adenosine methyltransferases are exclusively monomethyltransferases. RNA adenosine methyltransferases, on the other hand, can either mono- or dimethylate their target base depending on the individual enzyme. The Erm family of antibiotic resistance methyltransferases encompasses both activities, with m^6A and m^6_2A providing overlapping profiles of resistance to the MLS_B antibiotics.[8] In this case, both modifications can be accommodated structurally and both classes of enzyme have persisted.

KsgA, which has been retained throughout evolution, is only found naturally as a dimethyltransferase, suggesting that there is strong evolutionary pressure to maintain the dimethyltransferase activity, but the genesis of this pressure remains unknown. Recent work suggests that dimethylation of A1518 and A1519 facilitates proper structural arrangement of the active site of the mature 30S subunit and thus is important for ribosomal function.[38] The dimethylations are also important for ribosome biogenesis.[12] However, these studies compared full dimethylation with complete lack of methylation. In order to expand on this previous work, we constructed a KsgA mutant, L114P, that we predicted would no longer be able to methylate the m^6A intermediate to produce m^6_2A, as well as a second mutant, N113A, that we predicted would affect catalysis without altering the ability of the enzyme to perform as a dimethyltransferase.

In the present study, the two mutations resulted in similar reductions in overall enzymatic activity as compared to the wild-type enzyme. Additionally, the L114P mutation disrupted the dimethyltransferase mechanism. Since both mutants were able to bind to substrate 30S particles with similar affinity to that of the wild-type protein, differences observed in overall activity and mono- vs dimethylation among these forms of KsgA must be localized exclusively in the catalytic core. These data suggest that the hydrogen bonds between these two residues and the target nitrogen are important for full activity of the enzyme but are not absolutely essential for either of the methylation steps. In the case of the L114P mutation, we can conclude that m^6A is a suboptimal substrate, leading to the observed buildup of this intermediate at the expense of the m^6_2A product. It must be stressed that additional factors beyond this sole residue are critical for monomethyltransferase vs dimethyltransferase ability because some dimethylation does take place, albeit at a greatly reduced rate.

Unlike adenosine monomethyltransferases, in which m^6A is the final product, KsgA must be able to accommodate the methylated species in the active site as a substrate. We hypothesize that the m^6A substrate is presented in the active site with the methyl group oriented toward the N113 side chain, since loss of this side chain does not preclude addition of the second methyl group. m^6A can only form a single hydrogen bond with the enzyme's active site. If the methyl group were oriented toward the backbone carbonyl of L114, then N113 would provide the single hydrogen bond (Figure 8A). We would predict that loss of this hydrogen bond (Figure 8B) would impair the ability of m^6A to serve as a substrate. However, this is not the case, as the N113A mutant is as efficient as the wild-type enzyme in producing m^6_2A from m^6A. Instead, we believe that the methyl group of m^6A is oriented toward N113, leaving the backbone carbonyl of L114 to make the required hydrogen bond (Figure 8C). Mutation of this residue to proline does not remove the potential for hydrogen bonding (Figure 8D); rather, it makes the local backbone more rigid and shifts the relative position of the two functional groups, the side chain of N113 and the carbonyl of L114.

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Goedecke et al. suggested that steric hindrance by these two residues in M.TaqI may precipitate release of the methylated product;[32] by extension, this would prevent an additional methylation of the N^6-methyldeoxyadenosine because it could not be accommodated in the active site as a substrate. In a similar manner, the small but significant changes in the L114P structure as compared to wild-type KsgA may lead to poor accommodation of the m^6A substrate, without completely abolishing its substrate potential.

Comparing our *in vitro* results to mutational analysis of other adenosine methyltransferases demonstrates that KsgA shows less reliance on Motif IV residues. In ErmC′, alanine mutation of N101, equivalent to N113 in KsgA, leads to a severe loss in activity. A wide variety of amino acid substitutions in Motif IV lead to complete loss of DNA methyltransferase activity.[31,35,40−44] This includes relatively conservative mutations at the position corresponding to N113; although this residue is conserved as either an Asp or an Asn, mutation of one to the other generally results in an inactive enzyme. Mutation of the Pro at the second position, equivalent to L114 in KsgA, is not tolerated. It is an intriguing possibility that DNA methyltransferases have evolved to be dependent on having a proline at that residue because any other amino acid that allowed catalytic activity could also allow some formation of dimethyladenosine; this would disrupt DNA structure and thus could not be tolerated.

KsgA's higher tolerance to mutation in the active site may reflect that enzyme's requirement to accommodate two separate adenosine substrates. These target bases are found in the loop of rRNA helix 45 and are likely approaching the binding pocket from slightly different orientations. Looser requirements for active site hydrogen-bonding interactions may be required to accommodate the binding of A1518 and A1519 at different times.

The observed differences in enzymatic activity led to distinct effects *in vivo*. Strikingly, the L114P mutation led to a modest buildup of free 30S particles as compared to the wild-type and N113A enzymes. In a previous study, expression of a catalytically inactive KsgA mutant led to a considerable accumulation of 30S particles, which were shown to contain a higher proportion of immature 30S subunits than seen in wild-type cells.[12] These particles were shown to be associated with the inactive protein; in the absence of catalysis, the protein was unable to release the particles and thus sequestered them from further maturation. It seems likely that expression of the L114P mutant, but not the N113A mutant, has a similar deleterious effect on ribosome biogenesis when compared to expression of the wild-type enzyme. In this case, the diminished rate of dimethyltransferase activity slows completion of the reaction and thus retards particle release. This effect is specific to the disruption of the second methylation reaction that produces the final product $m^6{}_2A$, and not a result of overall slowed catalysis, since the N113A and L114P mutants showed the same overall catalytic rate. Further experiments may clarify this effect.

Many questions remain to be answered about KsgA's mechanism. Molecular details of rRNA recognition and base-flipping are unknown, as are specifics of the required exchange of the product *S*-adenosylhomocysteine for a fresh SAM molecule. The work presented here furthers our understanding of the complex reaction mechanism of the KsgA methyltransferase, exploring active site residues necessary for catalysis and giving insight into the processive nature of the transfer of multiple methyl groups. Further studies will be necessary to fully understand the intricacies of KsgA's enzymatic activity and its role in ribosome biogenesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Information about the crystal structure of the L114P mutation of the MjDim1 enzyme; Figure S1: the active site of the enzyme superimposed with the active sites of related proteins; Table S1: crystal structure collection and refinement data. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Atomic coordinates have been deposited into the Protein Data Bank as entry 3TPZ.

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■ ABBREVIATIONS

rRNA, ribosomal RNA; MTase, methyltransferase; $m^6{}_2A$, N^6,N^6-dimethyladenosine; m^6A, N^6-monomethyladenosine; SAM, S-adenosyl-L-methionine; WT, wild-type.

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