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Crystallization and preliminary X-ray diffraction analysis of KsgA, a universally conserved RNA adenine dimethyltransferase in *Escherichia coli*

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The bacterial enzyme KsgA catalyzes the transfer of a total of four methyl groups from *S*-adenosylmethionine (SAM) to two adjacent adenosines in 16S rRNA. These modified adenosines are universally conserved in all species of eubacteria, eukaryotes and archaebacteria studied. Recombinant KsgA from *Escherichia coli* was overexpressed as a His-tagged fusion protein and purified. The recombinant protein was crystallized using PEG 4000 as a precipitant. The crystals belong to space group *C*2 and diffract X-rays to a resolution of 1.9 Å. The unit-cell parameters are $a = 173.9$, $b = 38.4$, $c = 83.0$ Å, $\beta = 90.0^\circ$. Structure determination using the molecular-replacement method is at the early stages of refinement.

1. Introduction

KsgA is a bacterial adenosine dimethyltransferase that converts two adjacent adenosines in the apical loop of helix 45 of 16S rRNA to N^6,N^6-dimethyladenosines, using *S*-adenosylmethionine (SAM) as the methyl donor (Fig. 1). KsgA orthologues appear to be ubiquitous throughout all kingdoms and dimethylation of the two adenosines is the only ribosome modification conserved in all species analyzed to date (van Knippenberg *et al.*, 1984), with the interesting exception of the archaebacterium *Sulfolobus solfataricus*, which may have only a single dimethylated adenosine (Noon *et al.*, 1998). The same dimethylated adenosines are also found in the mitochondria and chloroplasts of many eukaryotic species (van Knippenberg *et al.*, 1984).

The methylating activity of KsgA is not essential in *Escherichia coli* and loss of enzymatic function is the dominant mechanism of resistance to the antibiotic kasugamycin (Helser *et al.*, 1971). However, knockout of the yeast orthologue Dim1p is lethal (Lafontaine *et al.*, 1995). Dim1p performs a second and vital role in rRNA processing that is not performed by the bacterial enzyme. Dim1p binds to 33S pre-rRNA in the nucleolus and allows cleavage at sites A_1 and A_2. In the absence of Dim1p this cleavage is inhibited and the pre-rRNA cannot be properly processed (Lafontaine *et al.*, 1998). In addition, a human mitochondrial transcription factor has recently been identified that shares a remarkable sequence similarity with the KsgA family of dimethyltransferases (McCulloch *et al.*, 2002). This transcription factor, h-mtTFB1, methylates bacterial ribosomes *in vitro*, in the same way as KsgA, and the 12S rRNA of human mitochondrial ribosomes contains an analogous modification (Seidel-Rogol *et al.*, 2003). Crystal structures of Dim1p and h-mtTFB have not been reported and their structural features and mechanisms have not yet been characterized. A crystal structure of KsgA could be used to construct homology models of Dim1p and h-mtTFB1 and help to explain the functional differences between these closely related proteins.

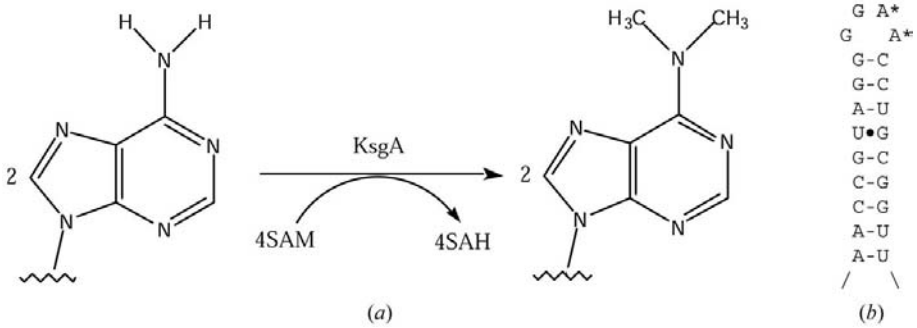

Figure 1
(*a*) The reaction mechanism catalyzed by KsgA. A total of four molecules of SAM are used to convert two adenosines into N^6,N^6-dimethyladenosines. Four molecules of *S*-adenosyl-homocysteine (SAH) are also produced. (*b*) Sequence of helix 45 of *E. coli* 16S rRNA. The dimethylated adenosines are indicated with stars.

The KsgA family of proteins is also closely related to another family of dimethyltransferases, the Erm family (van Buul & van Knippenberg, 1985). The two protein families show high sequence similarity in the N-terminal catalytic domains, but relatively poor similarity in the C-terminal domains. The Erm enzymes dimethylate an adenosine in the peptidyl transferase center of the 50S ribosomal subunit, conferring resistance to the macrolide-lincosamide-streptogramin B family of antibiotics (Skinner *et al.*, 1983). Minimal substrates consisting of as few as 32 nucleotides of 23S rRNA have been found for ErmC′ from *Bacillus subtilis* (Schluckebier *et al.*, 1999). In contrast, KsgA requires the full 16S rRNA and a subset of the 30S ribosomal proteins for enzymatic activity (Thammana & Held, 1974), although it can bind naked 16S rRNA fragments as small as 49 nucleotides (van Gemen *et al.*, 1989). The crystal structure of ErmC′ has been solved (Schluckebier *et al.*, 1999) and has the Rossmann-fold motif (Rao & Rossmann, 1973) characteristic of many methyltransferases (Fauman *et al.*, 1998). Based on sequence and functional similarity, we expect KsgA to also display the Rossmann fold.

KsgA's existence, function and some details of its biological roles in ribosomal maturation were reported many years ago (van Knippenberg, 1986), but little structural probing of the enzyme has been performed. In this paper, we report the crystallization and preliminary data analysis of *E. coli* KsgA.

2. Methods

2.1. Cloning

The 819 base-pair KsgA gene was amplified by PCR of genomic DNA from *E. coli* XL1-Blue cells and cloned into the pET15b vector (Novagen) as an *Nde*I/*Xho*I fragment for expression as a His-tagged fusion construct. The recombinant plasmid was sequenced (Nucleic Acids Research Facilities, Virginia Commonwealth University) to confirm the presence and correct sequence of the insert.

2.2. Expression and purification

LB medium containing 50 µg ml^{-1} ampicillin was inoculated with an overnight culture of *E. coli* HMS174 cells containing the recombinant pET15b-KsgA-His construct. Cells were incubated with shaking at 300 K to an OD_{600} of 0.6. Expression was induced with 1 m*M* IPTG and cells were harvested after 4 h.

The cell pellet was resuspended in lysis buffer (50 m*M* sodium phosphate, 300 m*M* NaCl, 10 m*M* imidazole pH 8.0), cells were disrupted by two passages through a French press at 83–103 MPa and cell debris was removed by centrifugation. The supernatant was loaded onto a HiTrap Desalting column (Amersham Pharmacia) equilibrated with 0.1 *M* $NiSO_4$, washed twice with increasing amounts of imidazole and the protein eluted with elution buffer (50 m*M* sodium phosphate, 300 m*M* NaCl, 250 m*M* imidazole pH 8.0). Protein purity and size were confirmed by SDS–PAGE and activity was tested according to Poldermans *et al.* (1979). Briefly, purified protein was incubated with non-methylated 30S ribosomal subunits and tritiated SAM and incorporation of the radiolabel was detected in a scintillation counter.

Purified protein was dialyzed into buffer *A* (50 m*M* Tris pH 7.4, 400 m*M* NH_4Cl, 6 m*M* β-mercaptoethanol) and stored in 10% glycerol at 253 K. Prior to crystallization, protein was simultaneously concentrated and exchanged into buffer *B* (50 m*M* Tris pH 7.4, 50 m*M* NH_4Cl, 6 m*M* β-mercaptoethanol) using Microcon YM-10 spin columns (Millipore).

2.3. Crystallization

Crystals were obtained by the hanging-drop vapor-diffusion method using a variety of conditions. Initial screening was performed using Crystal Screens 1 and 2 from Hampton Research (Laguna Niguel, CA, USA). While none of these conditions produced crystals, we noted a strong solubility dependence on the presence of calcium acetate or magnesium acetate. This prompted us to investigate other divalent salts in a screen which closely resembled the Hampton Research PEG/Ion Screen. Showers of needles were obtained with 0.2 *M* calcium acetate and 20%(*w*/*v*) PEG 4000. Conditions were refined by optimizing the amount of PEG 4000 with a constant concentration of 0.2 *M* calcium acetate; diffraction-quality crystals were obtained with 18–20% PEG 4000. The best crystals were obtained by using 4 µl of protein sample in buffer *B* mixed with 4 µl of reservoir solution.

A single poor-quality crystal was also obtained with 0.2 *M* magnesium acetate and 20%(*w*/*v*) PEG 4000 and showers of needles occurred with 30 or 40% ammonium sulfate and 0.1 *M* sodium phosphate pH 8.0. These conditions were not optimized as we had already obtained several high-quality crystals using PEG 4000 and calcium acetate.

Table 1
Data-collection and processing statistics.

Values in parentheses refer to the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 173.9, b = 38.4, c = 83.0, β = 90.0
Resolution limits (Å)	32–2.1 (2.14–2.10)
No. of unique reflections	31547 (1148)
Completeness (%)	95.7 (76.6)
$R_{\rm merge}$† (%)	8.1 (32.8)
$I/\sigma(I)$	17.2 (2.7)
Solvent content‡ (%)	43
Matthews coefficient (V_M) (Å^3 Da^{-1})	2.17

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h. ‡ Assuming two molecules per asymmetric unit.

2.4. X-ray diffraction data collection

A crystal was cryoprotected by gradually increasing the amount of PEG 4000 to a final concentration of 30%(*w*/*v*). The crystal was mounted in a nylon loop and cooled to 98 K in a stream of N_2 gas. A diffraction data set containing 115 images with 2° oscillations was collected on an R-AXIS IIc image-plate area detector using Cu $K\alpha$ radiation from a rotating-anode X-ray generator operating at 50 kV and 100 mA. The X-ray beam was focused using Confocal Optics (Molecular Structure Corporation). Data integration, scaling and merging were performed with the *HKL* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

High-quality crystals appeared 3–4 d following crystallization setup (Fig. 2). The crystals belong to space group *C*2 and diffract X-rays to 1.9 Å resolution. The 1.9 Å spacings occurred at the corners of the square image plate, which resulted in very low completeness for this highest resolution

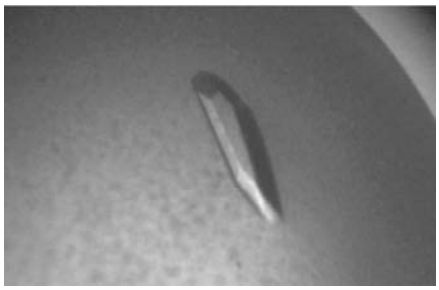

Figure 2
A crystal of the KsgA adenosine dimethyltransferase from *E. coli*. Its approximate dimensions are 0.5 × 0.1 × 0.05 mm.

range, so data were truncated at 2.1 Å. The unit-cell parameters are a = 173.9, b = 38.4, c = 83.0 Å, β = 90.0°. Higher symmetry space groups that require $\alpha = \beta = \gamma = 90°$ were excluded because the data failed to merge. There are two protein molecules per asymmetric unit, corresponding to a solvent content of 43% (Matthews coefficient = 2.17 Å^3 Da^{-1}; Matthews, 1968). Table 1 summarizes the data-collection statistics. Structure determination is being performed using molecular replacement with a search model consisting of the N-terminal domain of ErmC′ (Schluckebier *et al.*, 1999; PDB code 1qan), which exhibits a relatively high sequence homology to KsgA. This molecular-replacement solution has resulted in interpretable electron density within the region encompassed by the search model and also for the expected location of the C-terminal domain.

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