

Ribosome Biogenesis Regulation by Mutant KsgA Modified with a Photocaged Tyrosine

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I. Introduction

As bacteria that were once easily treated with common antibiotics begin to develop resistance to such treatments, new methods must be created in order for our society to continue to live with the same standard of health that we have grown accustomed to. One potential target for such a medication is the bacterial ribosome biogenesis factor universally conserved throughout evolution, KsgA. It is one enzyme of many involved in a highly regulated pathway that leads to a fully functional ribosome. The task initially attributed to the enzyme involves the dimethylation of two adjacent adenine nucleotides. However, it was recently discovered that KsgA may be involved in an essential checkpoint that can disrupt ribosome assembly and greatly affect the survival of bacteria¹.

KsgA is responsible for the dimethylation of the two adjacent A1518 and A1519 adenosines in 16S rRNA³. While there are many methylations within the ribosomal RNA (rRNA) of all organisms, the specific nucleotides that are methylated vary between kingdoms and species. The two aforementioned adenosines and the entire structure of the region in which they are found however, is highly conserved across prokaryotes and eukaryotes⁵. This remarkable evolutionary conservation hints at a more important role of KsgA within ribosome biogenesis. Though the specific enzyme varies across the different domains of life, the function is universally conserved and in both bacterial KsgA and the yeast orthologue Dim1p, the methyl groups are donated from S-adenosyl-L-methionine (SAM)^{3,8}.

In a novel experiment utilizing *E. coli*, the bacteria were transformed with a mutant KsgA enzyme that bound to its normal binding site on the pre 30S substrate but did not methylate the two adenosines as wild-type KsgA would^{1,3}. A polysome analysis was employed in an attempt to identify how the accumulation of the different ribosomal subunits varied with differing activity of KsgA. In a normal *E. coli* cell, a polysome analysis generally yields a result that displays relatively low levels of the large ribosomal subunit (LSU) and the small ribosomal subunit (SSU) and a high level of fully assembled ribosomes¹. When using the mutant KsgA, the methylations of adenosine A1518 and A1519 were not performed, and there was an accumulation of LSUs and SSUs and a much lower level of fully assembled ribosomes. However, when KsgA was completely removed from the cells, the levels of LSUs and SSUs dropped and the level of assembled ribosomes increased until all three were nearly equal, indicating KsgA's role as an inhibitory checkpoint within ribosome biogenesis.

An open question that remains is whether or not the pre-30S particles that accumulated in the *E. coli* strain containing the inactive KsgA background were stalled assembly intermediates or dead end products. A method to resolve this question is to 'turn KsgA on' after

the inactive KsgA/pre-30S particle has formed. To do this we will exploit an essential active site tyrosine by replacing it in vivo with a modified, photocaged tyrosine residue that can be restored to a wild-type tyrosine by shining 365 nm light on growing cells. It is anticipated that the photocaged KsgA will be catalytically inactive, but can be fully activated. Depending on the degree of recovery that is seen in the activity of KsgA, it will explain whether or not the pre-30s subunits that accumulate can be recovered in vivo, or whether they are dead end products.

II. Progress Report

During the ten week summer program, the goal of the project was accomplished. The desired mutation of the 116th amino acid within KsgA was achieved, and the plasmid which produces the modified tRNA that would recognize this mutation and encode a photocaged tyrosine was successfully incorporated into the BL-21 strain of *E. coli* bacteria. Upon expression of the protein, it was determined via SDS-page electrophoresis that the mutant KsgA produced from the bacteria was a full length product and contained the o-nitrobenzyl tyrosine. After confirmation of the full length protein product, a large scale growth and expression was performed in order to obtain a sufficient quantity of KsgA to allow for an activity assay.

An activity assay was run using unmethylated 30s small ribosomal subunits, SAM methyl donor groups containing tritiated hydrogen and two groups of mutant KsgA, one subjected to irradiation by 365 nm UV light and the other not. The results of the assay revealed activity in KsgA regardless of UV exposure, indicating no change in catalytic activity as a result of the addition of the o-nitrobenzyl group.

III. Goals for Academic Year

The experiments that will be performed during the academic year hinge on the results of several more tests involving the mutated KsgA. First, a mass spectroscopy analysis must be performed in order to determine whether the o-nitrobenzyl is attached to the tyrosine or whether it is cleaved before an activity assay is run. In order to produce full length, functional KsgA, the bacteria must be grown in media that contains photocaged tyrosine; the modified tRNA will only add that unnatural amino acid to protein when it reaches the mutated codon. Electrophoresis gels that were run confirm the presence of full length KsgA produced by the mutated sequence. It can be inferred that at the time KsgA was expressed, the o-nitrobenzyl group was attached to the tyrosine. However, it is possible that some source of UV radiation could have come in contact with the protein between expression and the activity assay resulting in the unexpected catalytic phenotype seen in the non-UV treated KsgA.

Depending on the results from the mass spectroscopy analysis and further activity assays, the goal for the academic year differs. If the analysis reveals that the o-nitrobenzyl group is in fact attached to the tyrosine in the experimental group not exposed to UV light, then the assumption can be made that the hypothesis was wrong and the interaction between the tyrosine and adenosine is not perturbed by the presence of this modifying group. If this is the case, the procedure for plan one will be followed. However, if further tests determine that the

group was cleaved before the activity assay by another source of UV light, then further analysis can be performed with KsgA containing o-nitrobenzyl tyrosine and plan two will be explored.

IV. Plan for Academic Year

During the summer, two strains of *E. coli* cells from Keio University in Japan were made competent for use in growth assays during the academic year. The first strain is wild type *E. coli* and the second strain has the gene for KsgA knocked out. Both will be used in assays for growth defects associated with mutant KsgA

IV. A. Plan One:

If it is determined that o-nitrobenzyl tyrosine has no effect on the activity, there is another tyrosine analog that can be employed to provide a novel way of cross-linking KsgA to 16S rRNA. Using this plan, I will incorporate two plasmids into the Keio Cells using the same protocol I used over the summer. The first will be the same pET-15b plasmid containing mutant KsgA but instead of incorporating a photocaged protein, I will incorporate a gene that allows for the incorporation of a tyrosine analog that can cross-link with the rRNA. The tyrosine at position 116 will be mutated, which should create a mutation that allows for the creation of a covalent bond between tyrosine and target adenosines.

This experiment will not allow the on/off control of KsgA catalytic activity that the previous experiment would have, but a successful incorporation will give future researchers the ability to study other methyltransferases within ribosome biogenesis. It will also give researchers the ability to stall the biogenesis pathway at different points and by doing this, previously unknown intermediates will accumulate and the biogenesis pathway can be understood in greater depth.

IV. B. Plan Two:

If further analysis reveals a flaw in the protocol used over the summer to incorporate the unnatural amino acid into KsgA, and the o-nitrobenzyl does result in an uncatalytic phenotype, the following plan will be followed. The pET-15b vector containing the mutant KsgA along with the pSUP vector that contains the modified tRNA will be transformed into the competent Keio cells. In vivo assays will be performed involving the dilution plating of bacteria exposed to, and not exposed to UV radiation, in order to explore growth defects that result from the catalytic activity of KsgA.

V. References

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