Investigation in to the Stabilizing Effects of the Modified Base Archaeosine in tRNA and the Identification of the Fluorescent Product of Base Treatment of NAD(P)+ Cofactors

Ben Turner
Portland State University

Let us know how access to this document benefits you.
Follow this and additional works at: https://pdxscholar.library.pdx.edu/open_access_etds
Part of the Chemistry Commons

Recommended Citation

10.15760/etd.5530

This Dissertation is brought to you for free and open access. It has been accepted for inclusion in Dissertations and Theses by an authorized administrator of PDXScholar. For more information, please contact pdxscholar@pdx.edu.
Investigation in to the Stabilizing Effects of the Modified Base Archaeosine in tRNA and
the Identification of the Fluorescent Product of Base Treatment of NAD(P)^+ Cofactors

by

Ben Turner

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
Dirk Iwata-Reuyl, Chair
Niles Lehman
John Perona
Ken Stedman

Portland State University
2017
Abstract

This dissertation covers two projects linked by their involvement in the modification of tRNA bases.

The first project focused on an investigation of a role for the modified base Archaeosine, the ubiquitous modification in tRNA in the archaeal domain. Initial work was performed on a set of in vitro prepared tRNA modified to feature either the canonical guanine base at position 15, preQ₀ (TGT product) or Archaeosine (ArcS product). There was very little difference in the thermal stability of tRNAs containing these modifications in the halophilic *H. volcanii* tRNA$^{\text{Ser}}$ or *E. coli* tRNA$^{\text{Gln}}$. In tRNA$^{\text{Gln}}$ taken from *M. thermautotrophicum* however, there was a 2°C increase in melting point in 50 mM MgCl₂ upon modification to archaeosine.

Benefitting from the development of genetic tools for the generation of specific deletion mutants of the thermophile *Thermococcus kodakarensis*, it was possible to start investigation of tRNAs that have been hypomodified in vivo due to the lack of arcTGT (TK0760) and ArcS (TK2156). In vitro modified equivalents of the Gln$^{CUG}$ isoacceptor were also prepared. Thermal stability of these tRNAs show virtually identical melting transitions, with a biphasic denaturation occurring at all magnesium concentrations tested. Isolation of the CUG isoacceptor from the in vivo matured total tRNA pool allowed melts of specifically hypomodified tRNAs. Those containg Archaeosine (WT) and genetically encoded guanine (Δtgt) showed identical melting profiles with $T_m$ beyond the 98°C limit.
of the experiment. In the preQ₀ containing in vivo RNA the shows a lag in its magnesium response, and a more persistent biphasic melting profile. At 10mM Mg²⁺ concentration the preQ₀ containing tRNA is approaching a Tₘ of 98°C though the turn over point in the melt is not well defined.

The second project was to investigate the product of base treatment of the oxidized cofactor NAD(P)⁺. This cofactor is involved in the biosynthesis of preQ₁ from preQ₀ in bacterial systems and at low concentrations it can be difficult to quantify enzyme activity based on direct quantitation. Under these conditions a fluorescence based method where by the production of NAD(P)⁺ is measured rather than the consumption of NAD(P)H.

Base treatment of the oxidized cofactor generates a fluorescent species with an efficiency of 95%. The assay has been used extensively by our group to track activity of various enzymes including QueF, however the identity of the fluorophore had not been established. Purification of the fluorescent product was achieved by isocratic HPLC in water using a reverse phase column. It was found that the assay conditions previously used (7.5M NaOH for 2 hours) wer actually counterproductive for maximizing fluorescence yield. Incubation at 2M NaOH gave a 35% increase in product yield. The isolated product was determined to have molecular weight of 123.0318 (3.6 ppm by accurate mass ESI MS). ¹H and ¹³C NMR were used to confirm the structure to be that of 2-hydroxynicotinaldehyde. It was also possible to determine the quantum yield for the molecule to 0.11. Work carried out previously on pyridinium based NADP analogs is consistent with the identity of the fluorophore presented here.
Acknowledgements

I would like to thank my thesis committee for the support and encouragement throughout the time working on this project. This extends to the valuable discussion with the many members of the CLEE Group at Portland State University and also Dr David Draper.

I also have to extend my gratitude to Dr Tom Santagelo and Brett Burkhart for providing the TKO knockout cell lines and Dr Ben Rauch, Dr Anna Louise Reysenbach and Mr Liu for advice on how to grow them.

I would like to thank Rob Ross of the Limbach group for processing MS analysis of *in vivo* material.
# Table of Contents

Abstract .......................................................................................................................... i

Acknowledgements ........................................................................................................ iii

List of Figures .................................................................................................................. vi

List of Tables ................................................................................................................... vii

Introduction ..................................................................................................................... 1
  Transfer RNA .................................................................................................................. 1
    Structure and primary function .................................................................................. 1
    Secondary roles ......................................................................................................... 3
  Nucleotide modification in tRNA .................................................................................. 5
    Deazaguanine modified nucleobases ...................................................................... 8
      preQ₀ biosynthesis ................................................................................................. 8
      Queuosine pathway ............................................................................................... 9
      Archaeosine pathways ........................................................................................... 9
      Function of archaeosine ....................................................................................... 11
    NAD⁺ and NADP⁺ biosynthesis .............................................................................. 13
    Roles of NAD⁺ and NADP⁺ .................................................................................... 15
  Determination of NAD⁺ and NADP⁺ concentration .................................................... 18

Materials and Methods ................................................................................................. 20
  Chemicals and Reagents ............................................................................................. 20

Instrumentation .............................................................................................................. 21

In vitro RNA preparation ............................................................................................... 22
  Template preparation ................................................................................................ 22
  Overexpression and purification of T7 RNA polymerase ........................................... 22
  Transcription reactions .............................................................................................. 23

RNA modification enzyme expression and purification and use ................................ 24
  ArcTGT expression .................................................................................................... 24
  ArcS expression .......................................................................................................... 25
  Preparation of preQ₀ tRNAs ...................................................................................... 26
  Preparation of G⁻ tRNAs ............................................................................................ 26

Thermococcus kodakarenis culture .............................................................................. 27
  Media preparation ...................................................................................................... 27
  Cell growth ................................................................................................................ 27
  Confirmation of knockdown by PCR of genomic material ........................................ 28
  In vivo tRNA extraction ............................................................................................. 28
  Isolation of tRNA for mass spectrometric analysis .................................................... 29
  Isolation of isoacceptor tRNA ................................................................................... 29

Biophysical analyses ...................................................................................................... 30
  UV melting studies .................................................................................................... 30
  Magnesium binding .................................................................................................. 31
  HPLC analysis ........................................................................................................... 31

Product synthesis and purification .............................................................................. 32

Spectral properties ........................................................................................................ 33
  UV absorbance and fluorescence spectra and pH dependence .................................. 33
  Quantum yield determination ..................................................................................... 34
Product identification
NMR spectroscopy
Mass spectrometry
IR spectroscopy

Results
Role of archaeosine in tRNA
Thermal stability of in vitro modified tRNA
Preparation of in vitro modified tRNA
UV melting study of in vitro tRNA
Magnesium binding to modified tRNA
In vivo modified tRNA from Thermococcus kodakarensis
Growth of the cell lines
Isolation of tRNA from the knockout cell lines
Purification of the GlnX isoacceptor
Thermal stability of single isoacceptor tRNA

Identification and characterization of NAD(P)+ base product
Stability and assay conditions
Equivalency of substrates
Spectral properties
Absorbance and fluorescence spectra
Quantum yield

Discussion
RNA discussion
In vitro melt study
Growth of TKO mutants
In vivo melts
Identification and characterization of NAD(P)+ base product
Identification of the fluorophore

Tables
Figures
Bibliography
List of Tables

Table 1 Sequences of tRNA used in initial thermal stability experiments.................... 67
Table 2 Sequences of tRNA used in TKO in vitro melting study................................. 68
List of Figures

Figure 1.1 Structure of tRNA ................................................................. 69
Figure 1.2 Naturally occurring modified bases ........................................ 70
Figure 1.3 Biosynthesis of preQ₀ nucleobase ............................................ 71
Figure 1.4 Queuosine biosynthesis .......................................................... 72
Figure 1.5 Archaeosine (G⁺) biosynthesis ................................................ 73
Figure 1.6 Electron density computed for Guanine and modeled G⁺ .......... 74
Figure 1.7 The positioning of Mg²⁺ and Mn²⁺ in the proximity of N7 in guanine 15 .... 75
Figure 1.8 Thermal melting of tRNA structure .......................................... 76
Figure 1.9 The de novo synthesis and salvage pathways of NADH ............ 77
Figure 3.1 SDS PAGE analysis of arcTGT purification .............................. 78
Figure 3.2 SDS PAGE analysis of ArcS purification .................................. 79
Figure 3.3 Modification of the in vitro prepared tRNA .............................. 80
Figure 3.4 Magnesium dependence of thermal stability of tRNA ............... 82
Figure 3.5 Magnesium dependence of thermal stability of tRNA ............... 84
Figure 3.6 Magnesium binding by fluorescence of HQS ........................... 85
Figure 3.7 Scheme for the generation of arcTGT (TK0760) ....................... 87
Figure 3.8 Confirmation of knockout in TKO cell line by PCR ................... 88
Figure 3.9 Analysis of purified total in vivo tRNA ..................................... 89
Figure 3.10 Mass spectrometry of the in vivo modified tRNA .................... 90
Figure 3.11 Affinity tag for isolation of GlnX isoacceptor from TKO .......... 91
Figure 3.12 Purification of the GlnX isoacceptors ..................................... 92
Figure 3.13 Confirmation of in vivo isoacceptor purity .............................. 93
Figure 3.14 Melting profiles of in vitro produced TKO Gln¹⁰°CUG ................ 94
Figure 3.15 Melt profiles of in vivo TKO GlnX isoacceptor tRNA ............... 95
Figure 3.16 The products of acid treatment of reduced NADPH ................. 96
Figure 3.17 Time dependence of fluorophore yield ................................... 97
Figure 3.18 UV spectra of NADPH, NADP⁺ and NMN derivatives ............ 98
Figure 3.19 UV absorbance and fluorescence of isolated fluorophore product 99
Figure 3.20 Determination of quantum yield for the base product ............ 100
Figure 3.21 Accurate mass determination ................................................. 101
Figure 3.22 ¹H NMR of the isolated fluorescent product ........................... 102
Figure 3.23 ¹H NMR of the isolated fluorescent product in basic conditions 103
Figure 3.24 ¹³C NMR of the isolated fluorescent product ......................... 104
Figure 3.26 IR spectrum of the isolated fluorophore .................................. 105
Figure 4.1 Growth phenotype of the T. kodakarensis mutants .................. 106
Figure 4.2 Location of base modifications in the core of tRNA .................. 107
Figure 4.3 Mechanism of base reaction to yield 2-hydroxynicotinaldehyde .... 108
Introduction

The metabolism of nucleic acids is integrated into most regulatory steps of cell proliferation. The process of synthesizing, maintaining and recycling RNA in particular is both highly dynamic and sensitive to the external conditions of the cell. In order to maintain viability under the vast range of condition under which life can persist, strategies capable of adapting fundamental processes such as DNA replication and gene expression have evolved. The archaea are particularly abundant in extreme environments of temperature, pH and osmotic potential. Modification of archaeal RNAs and the enzymes involved therein have been a source of unique biological chemistries. Here we study the effects of one such modification, archaeosine, found exclusively in the archaea. We also examine the pyridine nucleotide cofactors NADH and the phosphorylated NADPH which are utilized in the biosynthetic pathways involved in RNA metabolism, including base modification.

Transfer RNA

Structure and primary function

Transfer RNA, or tRNA, has evolved to serve as the adaptor molecule in converting a nucleic acid based genetic code into an amino acid sequence of a protein gene product. The amino acid to be incorporated into the growing polypeptide is selected for by specific charging of a particular tRNA by an aminoacyl tRNA synthetase. The aminoacyl tRNA (aa-tRNA) product of this reaction is then carried into the translation machinery where it
is held to facilitate the transfer of the amino acid from the 3’ end of the tRNA onto the C-terminus of the growing protein product.

Interactions between macromolecules in the translation process require multiple assembly steps and dynamic contacts. This makes sequence and structure of a given tRNA molecule crucial. Identifiers that ensure the maintenance of fidelity derive from not only nucleotide sequence but also from the correct placement of those nucleotides in space.

Unlike DNA, which is most often found in stable, double stranded form (where every base has a maintained cognate pair), RNA is more likely to be found as a single stranded polymer. This promotes a collapse into a range of more complex secondary and tertiary structures that are grouped into well-defined motifs, often utilizing non-Watson-Crick base pairing. Helical regions dominate the modes of interaction, but are punctuated by single-stranded regions, permitting high degrees of flexibility. Inter-helical and loop-loop interactions allow for further stabilization and longer-range contacts.

tRNA molecules range from 70-95 bases in length. Although there are a large number of codons that must be accounted for by the tRNA molecules, the nature of the interactions within the ribosome means that they must all adopt a very similar general folded structure. The secondary structure of most tRNAs includes four helical regions, allowing tRNA to be represented by a cloverleaf diagram when portraying structural contacts (Figure 1.1a). The stems are referred to as the acceptor stem (comprised of the 5’ and 3’ terminal residues and hence the position at which the amino acid is attached during charging), the
dihydouridine stem (named for the conserved modification of uridine to the dihydro-derivative), the anti-codon stem (the loop formed by this stem contains the 3 base anti-codon which base pairs with the codon found in mRNA during translation) and finally the TΨC stem (featuring the conserved base modification of pseudouridine). As well as these stemloop structures, there is a variable loop which often has no predicted secondary structure contacts, but facilitates the formation of tertiary contacts at the core of the tRNA.

Coaxial stacking of the acceptor stem upon the TΨC stem, and dihydouridine stem upon the anti-codon stem, respectively, initiates the native fold of tRNA. Further rearrangement allows these to form the now extensively studied L-form structure of fully folded tRNA associated with normal function (Figure 1.1b).

Secondary roles

Though the function of tRNA described here is well characterized, alternative roles for these molecules is a significant field in its own right. Regulation of gene expression through control of transcription and translation initiation and processivity have been shown to be dependent on tRNA through numerous mechanisms.

The T box riboswitch is utilized by Gram-positive bacteria in the 5’ UTR to sense levels of aa-tRNA, and so regulating the expression of tRNA synthetase genes based on product levels. This occurs via differential binding to the riboswitch aptamer and effector regions depending on the presence or absence of the amino acylated tRNA. During active translation, occurrences of mis-incorporation will often lead to stalling of the ribosome. It
has been shown that a family of tRNA related molecules (tmRNA) are involved in the rescue of the stalled complex, and tagging the incorrectly formed peptide for degradation\(^9\).

Cleavage of tRNA under oxidative stress and nutritional deprivation induces the production of fragments (cleavage occurs predominantly in the ASL but also in the D-loop and variable loops). These fragments signal various forms of stress response, shutting down proliferation by blocking translation machinery and inducing mRNA degradation by the RISC complex and RNaseZ activity\(^{10}\). tRNA fragments appear to be utilized to halt cell growth, but have also been shown to stimulate proliferation. Indeed, tRNA derived fragments were found to make up a significant population (40\%) of small RNAs in a prostate cancer cell line\(^{11}\).

In a process reminiscent of the role of tRNAs is translation, tRNA dependent aminoacylation of phospholipid phosphotidylglycerol occurs at the cell membrane of bacteria. The enzyme aa-PGS catalyses the transfer of the amino acid group from the 3’ terminus of alanyl-, lysyl-, arginyl- and ornithyl-tRNA to the glycerol group of a phospholipid. These modifications alter the overall charge of the membrane surface, reducing the susceptibility of the bacterium to Cationic Anti-Microbial Peptides\(^{12,13}\). It is clear that in all of these roles, the structures adopted by not only intact tRNA but also by tRNA fragments are crucial for correct cellular functions and cell cycle events to be maintained.
Nucleotide modification in tRNA

When considering the structure of tRNA and its function, it is important to appreciate the extent to which tRNA molecules are modified to contain non-genetically encoded nucleotides. Variable degrees of modification occur within different tRNAs but they are on the whole the richest source of modified bases of any class of RNA, with a significant number of bases in each RNA being modified in vivo. These changes extend the types of interaction that may occur and so can modulate both the structure and function of a mature RNA compared to the unmodified counterpart.

Of the 109 naturally occurring nucleotide modifications currently identified, 92 of these are found in the tRNA pool, and any given tRNA has 10-25% of the genetically encoded nucleotides modified. The degree to which a given base is modified varies greatly. Simple modifications such as methylation and the U to Ψ isomerisation are often directed by the action of a single enzyme, the substrate nucleotide being defined by its position within the tRNA structure. This is also often the case for N-methylation of the four genetically encoded bases that are so modified. O-methylation (Figure 1.2) occurring on the ribose moiety will more often occur in a multi-protein/guide RNA complex (also the case in the Cbf5 family of Ψ-synthases). The guide RNA will base pair with the RNA to be modified and the enzyme binds to the complex in a manner that places it in close proximity of the substrate nucleotide.
More complex modifications require multiple enzymatic steps to produce the final biologically relevant modification. The 7-deazaguanosine modified nucleosides archaeosine and queuosine (discussed below) are prime examples of the potential complexity of both the biosynthetic pathways and the biological function of tRNA modification.

Roles for these modifications in tRNA vary greatly and indeed most are still unknown. Of those characterized, there is a significant number occurring in the ASL, with both the wobble position 34 and position 37 being particular hotspots. Since there is such an important role for the wobble position in successful translation and in the regulation thereof, it is not surprising that many modifications have evolved that relate to correct presentation of the anticodon loop to the translation machinery.

One of the only universally conserved modifications occurring in the ACL is N6-threonylcarbamoyladenosine (t6A)\textsuperscript{18} (Figure 1.2) which is located at position 37. It is produced in all domains of life in a multi-enzyme biosynthetic pathway that has recently been elucidated\textsuperscript{19}. Already a complex modification it can be further derivatized to the 2-methylthio form\textsuperscript{20,21}. Both of these can in turn be cyclized to increase the complexity of the modification\textsuperscript{22}.

The biosynthesis begins with the formation of threonyl-carbamoyl AMP, from CO\textsubscript{2}/HCO\textsubscript{3}, ATP and threonine by TsaC/TsaC2 (bacteria) or Tsc1/Tcs2 (archaea and
eukaryotes. TsaB, TsaD, and TsaE (or their homologs) will then transfer the threonyl-carbamoyl moiety specifically on to ANN decoding tRNA in an ATP driven reaction.

The role of the modification itself was elucidated by structural studies of the isolated anticodon loop. The structure of the base in the stem loop adopts a conformation that sets up an effective third heterocycle, stabilized by an internal H-bond between N1 and N11. The threonyl moiety remains relatively unstructured but the added bulk rotates the adenine ring out of range from predicted stacking interactions with U36. Instead of this interaction, it forms cross strand stacking with A1 of the codon it is set to decode.

7-methylguanine (7mG) (Figure 1.2) is well known in the role of capping mRNA transcripts to stabilize and direct the processing of that RNA in eukaryotic translation. However, the occurrence of the modification at position 46 of tRNA has a significant influence on the viability of bacterial cells living at high temperature. m7G is found in both eukaryal and bacterial tRNA and is generated by methyl transferases trmB homodimer (bacteria), Trm8/82 (yeast) and hMETTL1/hWDR4 (human).

The modification itself involves the transfer of a methyl group (SAM dependent) to position 7 of the genetically encoded guanine. A deletion of the trmB gene in T. thermophilus was shown to have a growth phenotype when incubated above 70°C. Analysis of the produced tRNA showed that lack of the trmB gene product not only removed the modification at position 46, but also produced hypomodification in m2G, m5U, m6A and m1G. The melting point of the class I tRNA of this knockout was also found to be lowered.
by 3.5°C when the cells were grown at 70°C and when grown at higher temperatures, the change was reduced to -1.2°C (transcribed yeast tRNA\(^\text{phe}\) had no significant change in T\(_m\) by \textit{in vitro} methylation). This decrease in thermal stability is compounded by the increased rate of \textit{in vivo} degradation of specific tRNA (Phe and Ile). These effects result in loss of translational fidelity and lead to the observed growth phenotype\(^{28}\).

Structural information is available regarding the interaction between 7mG46 and surrounding bases. The crystal structure of tRNA\(^\text{phe}\) was solved with the modification in place and it shows a non-canonical base pairing system with G22\(^\text{31}\). Hydrogen bonds form between N1(46) and N7(22) and between N2(46) and O6(22) generating a trans WC/Hoogsteen interaction. The bonding has been analysed using molecular modeling and it has been calculated that the methylation confers a stabilizing effect of \(-15.12\text{kJmol}^{-1}\) compared to the unmodified basepair\(^{32}\).

**Deazaguanine modified nucleobases**

\textit{preQ}_0 biosynthesis

GTP is the first substrate in a series of reactions that create the queuosine and Archaeosine intermediate, \textit{preQ}_0 (7-cyano-7-deazaguanine). The first enzyme, GTP cyclohydrolase I (GCYH-I) acts upon GTP to form 7,8-dihydronopterin-3'-triphosphate, releasing formate\(^{36}\). This step is shared with the pterin and folate pathways. The product is then converted to 6-carboxy-5,6,7,8-tetrahydopterin (CPH\(_4\)) by the enzyme QueD\(^{37}\). The next step is a rearrangement performed by QueE generating 7-carboxy-7deazaguanine (CDG),
the first deaza-moiety in the pathway\textsuperscript{38}. The final step is an amido transferase (QueC) reaction, using ammonia as a nitrogen source to create the nitrile of preQ\textsubscript{0} (Figure 1.3)\textsuperscript{37}. From this point the biosynthetic pathways diverge for the complete synthesis of Q and G\textsuperscript{+}.

\textit{Queuosine pathway}

In bacteria the free base preQ\textsubscript{0} is transformed into 7-aminomethyl-7deazguanine (preQ\textsubscript{1}) by the only known example of a nitrile reductase, QueF\textsuperscript{35}. PreQ\textsubscript{1} is integrated into substrate tRNA by the enzyme tRNA-guanine transglycosylase (TGT). This enzyme concomitantly removes the genetically encoded guanine base at position 34 (of those tRNAs with GUN anticodon) and replaces it with preQ\textsubscript{1}. PreQ\textsubscript{1} is the substrate for QueA, an S-adenosylmethionine:tRNA ribosyl transferase isomerase. In the penultimate step of this pathway, the ribosyl moiety from SAM is not only transferred to the aminomethyl of preQ\textsubscript{1}, but it is also isomerized to a carboxylate to give epoxyqueuosine (oQ-tRNA)\textsuperscript{39}. The final step in the pathway has been in part solved by the identification of an epoxyqueuosine reductase (QueG) that utilizes cobalamine for the production of queuosine tRNA\textsuperscript{40} (Figure 1.4). Though queuosine is integrated into the tRNA of both bacteria and eukarya, \textit{de novo} synthesis of the modified nucleotide is only possible in bacterial systems\textsuperscript{41}. In eukaryotic systems the free queuine base can be integrated into tRNA by a eukaryotic specific TGT enzyme\textsuperscript{42}.

\textit{Archaeosine pathways}

Unlike the queuosine pathway, the first step to occur in archaea after preQ\textsubscript{0} synthesis is the direct insertion into substrate tRNA. This occurs in tRNA that feature a genetically encoded
guanine at position 15. ArcTGT has been shown to perform a similar reaction to bacTGT in which a transglycosylation reaction replaces the genetically encoded guanine with the preQ₀ base⁴³, ⁴⁴. The conversion of preQ₀ to archaeosine was first shown to be performed by the enzyme Archaeosine Synthase (ArcS). This enzyme (isolated from *M. jannaschii*⁴⁵) has been shown to be an ATP independent amidinotransferase, utilizing glutamine, asparagine or ammonia for direct transfer to the nitrile carbon of preQ₀⁴⁵.

Though this discovery has completed the biosynthetic pathway in species which possess the arcS gene, it is not the case that the enzyme is universally conserved. In those species which do not produce ArcS, two alternative genes have been implicated in this final step⁴⁶. With particular bias toward the Crenarchaeota, the genes queF-like and gat-queC have been found to exist either in isolation or in some cases, in combination with arcS. *E. coli* cells deleted of the queF-like gene, but transformed with a plasmid containing either queF-like or gat-queC were found to produce tRNA modified with G⁺ (Figure 1.5). Furthermore, mass spectrometric analysis of the RNA gave evidence for the modification taking place at position 34, suggesting the activity of bacTGT was still functioning to replace that genetically encoded guanine. The *in vitro* activity of QueF-Like has since been confirmed to be the generation of archaeosine, acting on preQ₀-tRNA as its substrate⁴⁷. The enzyme has also been tested on free preQ₀ base which showed no activity. Finally, the enzyme is more selective for the nitrogen source used compared to ArcS as only ammonia has been shown to be active in this role and no G⁺ was generated when Gln or Asn were used.
Function of archaeosine

The modification of G15 to G+ has been shown to be a ubiquitous modification throughout the Archaeal kingdoms except for the unusual species *Haloquadratum walsbyi*\(^{46}\). Since it is absent from the other domains, it suggests that the role of G+ is either particular to the Archaea, or its function has been supplanted by an alternative modification in the Bacteria and Eukarya. The location of G+ at the very core of the tRNA structure, at the universally conserved Levitt base pair, is also suggestive of a structurally significant role for this modification.

Little information has been gleamed as to how this modification functions *in vivo*. The nature of archaeosine’s role may be in forming enhanced contacts that stabilize a folded RNA against more challenging environmental conditions. *In silico* studies, founded on the structure of tRNA\(^{Phe}\), have shown that the presence of G+ will significantly change the bonding energies between two bases of the Levitt base pair (Figure 1.6)\(^{48}\). The positive charge developed at position 7 on the deazaguanine, is believed to subsume the effects of coordinated metal ions (in the model described, a magnesium is bridged between the N7 and a solvent water oxygen).

This coordinated metal ion also serves as second mode by which the tRNA is stabilized. Metal ions have been found to localize in the pocket between the N7 position and a series of phosphate groups from U7, A14 and G15 itself (Figure 1.7). This dense pocket of negative charge is neutralized by coulombic interactions with the metal ion (has been found
to be Mg$^{2+}$ or Mn$^{2+}$ in crystals structures). The formation of the positively charged center of the formamidino ground of archaeosine, may be making a similar coulombic contribution to the stabilization of the tRNA structure.

In this study we aim to investigate if archaeosine does in fact directly contribute to the stability of tRNA. RNA structural stability is often analyzed by dissecting it into individual stems, stem loops and junctions that represent components of the overall folded RNA\textsuperscript{49-51}. These can then be used to build an ensemble image of both folded structure and dynamic rearrangements that occur during the folding process.

UV absorbance hyperchromicity is one of the most direct methods of tracking the stability of a given RNA. The absorbance of UV light as an RNA unfolds will increase as the molar absorptivity increases. Using combinations of fluorescence, circular dichroism\textsuperscript{52} and various other biophysical techniques\textsuperscript{53-57}, the transitions seen in UV melts has been correlated to specific elements of the tRNA falling apart. The first differential plot of change in absorbance per temperature change reveals subpopulations of the structural transitions that are often unclear in the direct melting profile (Figure 1.8). The generalized folding pathway for tRNA established the importance of the interaction between the D-stem and T-stem in holding the native structure together\textsuperscript{58, 59}. Loss of these structures and intramolecular interactions trigger the complete loss of structure in biologically relevant concentrations of Mg$^{2+}$, leading to an effective two state model for the unfolding process.
We plan to use this technique to exam the effect of G\(^+\) on the stability of \textit{in vitro} transcribed and modified tRNAs. This will provide a foundation to then study the effects of the modification in the context of \textit{in vivo} produced tRNA which has been maturated under native cellular conditions and therefore contains all other modifications. Access to knockout cell lines of the archaeaon \textit{Thermococcus kodakarensis}, allows for the progression of studying the genetically encoded guanine to preQ\(_0\) and G\(^+\) by extracting tRNA from cells lacking arcTGT, ArcS or wildtype cells respectively.

\textbf{NAD}\(^+\) and NADP\(^+\) biosynthesis

The activity of nicotinamide adenine dinucleotide (NAD) was first shown in an extract of yeast in the early 1900\textquotesingle s\textsuperscript{60}. A dried extract which showed no independent fermentive activity was able to enhance the rate at which a second yeast extract was able to ferment glucose. This work would contribute to the awarding of the Nobel prize in 1929 to Harden along with Von Euler for their work on coenzyme discovery and the fermentation of carbohydrates. Indeed, von Euler was the first to identify the components of NAD, those being the two ribose units, two phosphate units and one each of the adenine and nicotinamide nucleobases. The involvement of nicotinic acid and its amide derivative in NAD formation were implicated in a feeding study of dogs suffering from pellagra\textsuperscript{61}. Within the next twenty years, research had elucidated the complete biosynthetic pathway to NAD and in a double publication both the intermediates involved in the synthesis and the enzymes responsible for the chemistry were laid out\textsuperscript{62, 63}. 

13
De novo synthesis of NAD (Figure 1.9) begins with the generation of nicotinic acid mononucleotide (NaMN) which can proceed by two primary routes. Aspartic acid or tryptophan can both be used as the starting reactant, undergoing 2-enzyme or 5-enzyme steps respectively. Both routes yield quinolinate as the immediate precursor which undergoes a phosphoribosyl transfer step generating nicotinic acid mononucleotide (NaMN). Adenylation of NaMN yields nicotinic acid dinucleotide (NaAD) and this is the final intermediate before the action of NAD⁺ synthetase generates NAD⁺ using an exogenous supply of nitrogen.

As well as the de novo pathway there are multiple steps at which salvage pathways can be implemented to replenish NAD⁺ levels. The Preiss-Handler pathway established that nicotinic acid can be ribosylated directly to generate NaMN and then move through to NAD⁺ as per the de novo route. Nicotinamide released by most of the non-redox related processes of NAD⁺ metabolism is also ribosylated to yield nicotinamide mononucleotide (NMN) and then adenylated to form NAD⁺. Finally, nicotinamide riboside (NR) has been shown to be regenerated to NAD⁺ by nicotinamide riboside kinase generating nicotinamide mononucleotide which then undergoes an adenylation step to generate NAD⁺. This process can be performed by a single gene product in some systems by the enzyme nadR.

Although globally the two routes to quinolinate are split between the domains (Asp typical of prokaryotes and Trp largely found in eukaryotes), there is a high degree of interplay within them. There are organisms from all three domains which apparently do not possess
the genes needed for *de novo* synthesis. Likewise, there are bacteria that utilize tryptophan and fungi that metabolize aspartate. Other examples have redundancies that allow for switching between pathways depending on nutrient supply.

The activity of the related cofactor, NADP⁺, was discovered in the 1930s by Warburg and Christian, when an erythrocyte extract and later hepatocyte extract were shown to be able to reduce previously oxidized glutathione. The modification to NADP⁺ is a single phosphorylation event at the 2' position of the adenosyl moiety in NAD. The NAD⁺ kinase is conserved through all domains of life and utilizes ATP to transfer the phosphate group on to NAD⁺. This makes the biosynthesis of NADP⁺ explicitly coupled to the biosynthesis of NAD⁺.

**Roles of NAD⁺ and NADP⁺**

The primary role of both NAD⁺ and NADP⁺ have long been known to be in redox reactions where they act as electron carriers. Their structural similarities lead to them having near identical redox potentials under physiological conditions and therefore, it could appear that there is a redundancy in having these two species active in the cell. However, the need to further modify NAD⁺ to NADP⁺ consumes ATP and so the energetic requirement alone would suggest that there is a rationale for this redundancy. Upon inspection of the reactions that the two cofactors are involved in, it becomes clear that they are partitioned between anabolic processes (NADP⁺) and the oxidative processes for generating ATP (NAD⁺). The phosphate group, held significantly away from the redox center, allows for the cofactors to
have different binding modes. Within the cell, in order to help drive these two different directions of reactivity, NAD(H) is held in an excess of the oxidized form and likewise, NADP(H) is largely in the reduced (NAD\(^+\):NADH in humans has been measured to be as high as 1820; NADP\(^+\):NADPH can be as low as 0.017).\(^72\)

Due to the fundamental nature of the reactions that these cofactors are utilized by, they have been studied extensively in their role as redox partners for vast numbers of reactions in all domains of life (2200 of the reactions described on the KEGG database employ NAD\(^+\) or NADP\(^+\)).\(^73\) It has also been determined that both NAD\(^+\) and NADP\(^+\) are involved in more diverse reactions that take them beyond simple cofactors. It expands their role to cell signaling and direct protein modification which brings in to play modulation of activity.

Both NAD\(^+\) and NADP\(^+\) can be processed to form secondary messengers. NAD\(^+\) is acted on by ADP-ribosyl cyclase to generate cyclic-ADP ribose (cADPR), with the elimination of nicotinamide. It has been shown that an increase in the cellular concentration of this molecule (from endogenous synthesis and import) results in activation of the ryanodine receptor channel on the endoplasmic reticulum and so increases cytosolic Ca\(^{2+}\) concentration. There is also activation of cell membrane channels to increase import of Ca\(^{2+}\) ions. This is mirrored in the activity of the NADP\(^+\) metabolite (again with the release of nicotinamide) nicotinic acid ADP, which causes a release of Ca\(^{2+}\) ions from lysozyme related organelles, as well as the ER and activation of the same membrane channels as cADPR. A third calcium mobilizing signal cascade is facilitated by NAD\(^+\) consuming reactions. ADPR is able to upregulate calcium import by the TRMP2 channel. ADPR is
synthesized by multiple routes but can be produced by the direct hydrolysis of NAD$^+$ with release of the nicotinamide moiety.$^{74,75}$

A second route by which ADPR is generated is by the activity of the PARP enzymes that are responsible for the maintenance of genome integrity in the nucleus. PARP or polyADP-ribose polymerase, use ADP-ribose to flag sites of single strand breaks in DNA. Proteins in proximity to the break are modified by poly-ADP ribosylation, using NAD$^+$ as the source of the ADPR. Upon break down of this polymer, ADPR is released and this is translocated in to the cytosol where its effects are described above.$^{76}$

Finally, one of the more recent roles of NAD$^+$ is in the activity of sirtuins. NAD$^+$ is utilized by this universally conserved family of regulatory enzymes to deacetylate proteins that have previously been modified to feature acetyl lysine. NAD$^+$ is consumed, releasing nicotinamide, along with $O$-acetyl-ADP ribose. Along with the deacetylase activity, some members of the sirtuin families have also been shown to exhibit mono-ADP ribosylation activity where the ADPR moiety is connected to the substrate protein via a bond to $C1$.$^{77,78}$ However, the rates of these reactions is found to be several orders of magnitude lower than the deacetylation reaction, and so it may be that the ribosyltransferase is merely a side reaction. This notwithstanding, the activity of sirtuins implicates NAD$^+$ in the regulation of inflammatory response, apoptosis, glucose homeostasis, and the cellular stress responses.
**Determination of NAD$^+$ and NADP$^+$ Concentration**

During the studies of these systems, there is an inherent need to obtain to determine the concentration of the cofactors at a given point in a reaction being studied. Luckily, given the nucleobase component of the molecules, there is significant absorbance within the UV spectrum and the redox state of the cofactors results in a significantly different absorbance spectrum. Both forms of NAD(P) show a strong absorbance peak centered at 260 nm. However, the reduced form alone has a second significant peak centered at 340 nm. This is true for both NADH and NADPH. The extinction coefficient for this 340 nm peak is well characterized as being $6.22 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$. This permits the trajectory of an NAD(P) dependent reactions to be followed by either loss or appearance of this peak.$^{79}$

Use of the absorbance is limited to instrumental sensitivity. This can be overcome thanks to a phenomenon where by treatment of NAD(P)$^+$ with strong base can generate a degradation product which is fluorescent. This was initially observed in the analog molecules diphosphopyridine nucleotide and methyl nicotinamide, where treatment with 5N KOH would yield a fluorogenic product with varied fluorescence yields.$^{80}$ This treatment was found to be applicable to the NAD(P)$^+$ systems and so a work up method was established that allowed for robust quantitation of trace amount of NAD(P)$^+$ in a background of high NAD(P)H.

This section of work initially aims to reappraise the conditions used in this standard assay. The conditions have remained unchanged since those proposed in the 1950s and though it
is robust and consistent, it has not been investigated as to whether these can be improved upon for working up NAD$^+$ or NADP$^+$. We also aim to finally confirm the identity of the fluorescent species produced in the assay using these biological cofactors. The fluorophore is usually measured in the background of other assay components, and so a purification strategy will be employed to identify the fluorophore using standard spectroscopic and spectrometric techniques for small molecules.
Materials and Methods

Chemicals and Reagents

Buffers and salts of the highest grade available were purchased from Sigma unless otherwise noted. DEPC (diethylpyrocarbonate) treated water was used for all solutions used for RNA related assays. All buffers and solutions were otherwise prepared with Millipore MQ grade water. Dithiothreitol (DTT), isopropyl-β-D-thiogalacto-pyranoside (IPTG), kanamycin sulfate, DEPC and ampicillin were purchased from RPI Corporation. The radiochemicals [8-14C]-guanine and [U-14C]-L-glutamine were purchased from Perkin Elmer. Adenosine, guanosine, ATP, GTP, UTP, CTP were all purchased from Sigma. Nickel- nitrile tetraacetic acid (Ni²⁺-NTA) was purchased from Qiagen and Sigma. Whatman GF-B filter disks were purchased from Fisher. Amicon centrifugal concentrators were from Millipore. Dialysis tubing was obtained from Pierce. Plasmid Mini-Kits were from Fermentas and Qiagen. Oligonucleotides were obtained from IDT. All reagents for SDS-PAGE were purchased from BioRad. SDS-PAGE analysis was carried out using 12 % (29:1 Acryl:Bisacrylamide) gels and visualized with Coomassie Brilliant Blue. DNA sequencing was carried out by the OHSU core facility in the Department of Molecular Microbiology and Immunology. The substrate preQ₀ was synthesized as described previously and purified by reverse phase HPLC and stored at room temperature in DMSO. β-Nicotinamide mononucleotide (NMN) was obtained from Sigma-Aldrich. Both β-nicotinamide adenine dinucleotide (NAD⁺) and the phosphorylated form (NADP⁺) were obtained from RPI Corporation. Sodium hydroxide, hydrochloric acid, sulfuric acid,
ammonium acetate and acetonitrile were purchased from Fisher. Quinine sulfate was obtained from AnaSpec Inc. Deuterated solvents (water and sodium hydroxide) were obtained from Sigma-Aldrich.

**Instrumentation**

Analytical HPLC was performed on an Agilent 1100 series HPLC (G1312A binary pump, G1315A diode array detector). Preparative scale separation was achieved using a Hitachi HPLC (L-6200 pump and L-4000 single wavelength detector). Rapid fluorescence screening was performed using a Gemini XPS microplate reader from Molecular Dynamics. Steady state fluorescence was carried out using a Quantamaster 400 from Photon Technologies International. UV-Vis spectroscopy was carried out on a Varian Cary 100 Bio spectrophotometer fitted with a thermostatted multi-cell holder. Mass spectrometry was performed on a ThermoElectron LTQ-Orbitrap Discovery high resolution mass spectrometer with a dedicated Accela HPLC system utilizing ESI in the Department of Chemistry at Portland State University. NMR studies were performed on either 400 Hz or 600 Hz Bruker instruments belonging to the Department of Chemistry at Portland State University. Infrared spectroscopy was carried out on a Nicolet iS10 spectrometer using a diamond ATR attachment.
In vitro RNA preparation

Template preparation

Double stranded template DNA was prepared from two DNA primers which were designed with a ten base pair overlap at the center of the target sequence. The native gene sequence was supplemented with the standard T7 promoter (5’TAATACGACTCACTATA3’) at the 5’ end of the upper oligo. The complete template is generated by primer extension using the Klenow fragment (Fermentas) to create two fully complementary strands. The two oligos are mixed to a final concentration of 4µM each, in the presence of dNTPs (600µM each) and using the manufacturers reaction conditions. The primers are extended by cycling 25 times between 37°C and 10°C in 30 second pulses (Applied Biosystems 2720 thermal cycler). The DNA was then extracted by phenol chloroform extraction (equal volume of 25Phenol:24Chloroform:1Isoamyl alcohol vortexed and then centrifuged at 13000 rpm for 5 minutes) and ethanol precipitation of the aqueous phase. The template was then resuspended in water at 10µM.

Overexpression and purification of T7 RNA polymerase

The del172-73 mutant of T7 RNA polymerase was prepared by the overexpression of a 6His-tagged clone provided by Dr John Perona. Frozen cell stock (E. coli BL21(DE3)) was grown on 200 µg/mL ampicilin agar plate and a fresh colony picked to start a liquid culture. Cells were grown in LB with 200 µg/mL ampicilin and raised to an OD of ~0.9 at 37°C with shaking at 220 rpm. Expression was carried out at RT with induction occurring upon the addition of IPTG to a final concentration of 0.5 mM and growth occurring overnight.
Cells were harvested by centrifugation at 5000 rpm (Sorval SLC-4000) and homogenously mixed in buffer (100 mM Phosphate (pH 7.5), 100 mM NaCl, 1 mM β-ME, 200 µM PMSF). To this was added Lysozyme (to 1mg/mL), and incubated for 20 minutes at 37°C to breakdown cell wall before three rounds of -80°C freeze/thaw to lyse cell membranes. Fully lysed cells are treated with DNaseI (20 µg/mL) and the lysate clarified by centrifugation at 15000 rpm (Sorval SS-34) for 20 minutes at 4°C. Cell free extract was loaded on to a Ni-NTA resin column, pre-equilibrated with 100 mM Phosphate (pH 7.5), 100 mM NaCl, 1 mM β-ME. Non-bound proteins were washed off with 10 column volumes and then fractions were eluted with a stepwise gradient of 10, 20, 50 and 200 mM imidazole. The target protein elutes with some contaminating proteins and so the 200 mM fraction was dialysed (100 mM Phosphate (pH 7.5), 100 mM NaCl, 1 mM β-ME overnight at 4°C using snake skin dialysis membrane) to remove the imidazole present and then run through a Heparin column (GE Healthcare, HiTRAPP, 5 mL), the cleaned protein eluting at 600 mM NaCl on a linear gradient. The protein was dialysed (overnight at 4°C) and stored in 100 mM Phosphate, 100 mM NaCl, 1 mM β-ME, 25% glycerol and stored at -80°C.

Transcription reactions

RNA molecules were transcribed from 1µM DNA template in 30mM Tris-HCl (pH 8.0), 40mM MgCl₂, 10mM DTT, 0.1% Triton X-100, 100µM spermidine, 2.5 mM NTP (individual nucleotides obtained from Sigma, stock made up in DEPC water and stored at -80°C), 50µg/mL of T7 RNA polymerase and 1U/mL of inorganic pyrophosphatase (Sigma). The reactions are run for 4hrs at 37°C and quenched by ethanol precipitation. The
recovered pellet was solubilized in DEPC water and then mixed with an equal volume of formamide/5mM EDTA. The reaction products were denatured at 95°C and then separated by denaturing urea PAGE (7M urea, 10% acrylamide, 1x TBE, gel run at 18W). The full length product band is excised from the gel and the RNA extracted by overnight crush and soak in to 800mM ammonium acetate. The purified RNA was then precipitated and the pellet resuspended in 1mM sodium citrate and stored at -80°C.

**RNA modification enzyme expression and purification and use**

*ArcTGT expression*

The tRNA-Glycosyl Transferase gene from *M. jannaschii* had been previously cloned in to pET30, providing a 6-His fusion protein for expression in BL21 (DE3) *E. coli* cells. The vector confers kanamycin resistance and so cells were grown in LB (pH 7.5) with kanamycin at 50 µg/mL, to an OD of approximately 0.9 at 37°C. Expression of the fusion protein is induced by addition of IPTG to a final concentration of 1 mM. Induction occurred at 37°C for around 6 hours and resultant cells were harvested by centrifugation at 5000 rpm (Sorval SLC-4000) for 15 minutes. These were resuspended in lysis buffer (50 mM Tris-HCl at pH 8.0, 300mM NaCl, 1 mM β-ME, 200µM PMSF, 1 mg/mL lysozyme) and incubated at 37°C for 20 minutes before being put through three cycles of freeze thaw to lyse. DNase I (20 µg/mL) treatment followed by centrifugation (15000 rpm for 15 minutes in a Sorval SS-34 rotor) generates a cleared lysate that can be passed over a Ni-NTA column for affinity chromatography of the target enzyme. The protein elutes at 50 mM
imidazole from a stepwise gradient (0, 10, 50 and 200mM). The appropriate fraction is collected, concentrated and dialysed in to storage buffer.

ArcS expression

Archaeosine synthase from *M. jannaschii* had previously been cloned in to a range of plasmids, but for all expression in this work the clone is in pET32-Xa. This provides a thioredoxin domain at the N-terminus to assist in the folding process. Expression of the protein requires it be transformed into Rosetta (DE3) *E. coli*. These cells were grown in LB supplemented with kanamycin (50 µg/mL), sorbitol (0.5 M) and betaine (5 mM) at 37°C. At an OD of approximately 0.9 the cells were induced to express with IPTG at a final concentration of 0.25 mM. Induction was carried out at room temperature for 8 hours. The cells were harvested by centrifugation at 5000 rpm (Sorval SLC-4000). Cells are resuspended in lysis buffer (100 mM HEPES pH 7.0, 500 mM NaCl, 1 mM β-ME and 200 µM PMSF). Cells are then incubated with lysozyme (1 mg/mL) and freeze thaw cycles used to lyse cells. Lysate was cleared by centrifugation at 15000rpm (Sorval SS-34 rotor) for 15 minutes. The enzyme was obtained by passing the protein suspension over a Ni-NTA column isolating the 6His-TRX-ArcS fusion. Elution occurs at 50 mM imidazole and the fraction was collected before being concentrated, dialysed into imidazole free buffer. Factor Xa was added so that 1 µg was present for every milligram of fusion protein. The tag is removed by running back over a column of nickel resin to which the cleaved protein no longer binds. The protein is concentrated and dialysed in to storage buffer (100mM HEPES pH 7.0, 500 mM NaCl, 1mM β-ME, 25% glycerol) and kept at -80°C.
**Preparation of preQ₀ tRNAs**

All appropriate tRNAs were modified to feature preQ₀ base by the action of arcTGT. Standard reaction conditions were 50 mM succinate (pH 5.5), 20 mM MgCl₂, 100 mM KCl, 2 mM DTT, 100 µM tRNA and 1 mM preQ₀. RNA was heated at 80°C for 3 minutes before adding in ArcTGT to a final concentration of 10µM and incubating for a period long enough to ensure complete incorporation. Activity of the enzyme was assayed by reacting the RNA with the enzyme in the presence of ¹⁴C-Guanine in place of the preQ₀ native substrate. Incorporation of radionuclide was determined by scintillation counting of ethanol precipitated RNA trapped on glass fiber filter papers. The reaction was terminated by the addition of 1/10th volume of 8M ammonium acetate. Reaction components were removed by phenol/chloroform extraction, and the aqueous phase precipitated by the addition of two volumes of ethanol. The reaction was repeated for two more rounds of incorporation to ensure complete integration of preQ₀ base.

**Preparation of G⁺ tRNAs**

The modification to Archaeosine tRNA was produced by the enzyme ArcS. Previously modified preQ₀-tRNA is suspended (50µM) in 100 mM HEPES (pH 7.0), 0.5 M NaCl, 20 mM MgCl₂, 5 mM Glutamine, 1 mM DTT with 10µM ArcS. The sample was reacted for 1 hour at 40°C. The modified RNA was isolated by ethanol precipitation using 800mM NH₄OAc, quenching reaction and removing the other reaction components.
**Thermococcus kodakarensis culture**

**Media preparation**

Stock solutions (500x) were prepared for both vitamins and trace elements. The components and end concentrations of these are; vitamin mix (8 µM biotin, 5 µM folic acid, 50 µM pyridoxine, 15 µM thiamine, 15 µM riboflavin, 40 µM nicotinic acid, 20 µM Ca-pantothenate, 7 µM p-aminobenzoic acid and 75 nM B12) and trace minerals (50 µM FeCl$_3$, 5 µM MnCl$_2$, 18.5 µM CoCl$_2$, 7 µM CaCl$_2$, 7.5 µM ZnCl$_2$, 1.5 µM CuCl$_2$, 1.6 µM H$_3$BO$_3$, 1 µM (NH$_4$)$_2$MoO$_4$, 5 µM NiCl$_2$, 850 nM NaSeO$_4$, 2 µM AlCl$_3$).

Additional components per liter of growth medium:

Yeast extract (2.5g), tryptone (2.5g), NaCl (10.2g), MgCl$_2$.6H$_2$O (2.4g), MgSO$_4$ (0.8g), CaCl$_2$.2H$_2$O (0.4g), KCl (0.3g), sodium pyruvate (2.5g), agmatine sulfate (0.6g).

Media is prepared under N$_2$ to remove all dissolved O$_2$ (resazurin added to 1 mg/L) and autoclaved to sterilize. Before inoculation the head gas was exchanged for 80:20 N$_2$/CO$_2$ to 10 psi. To ensure fully anaerobic conditions, the growth media was spiked with further Na$_2$S (from a 2.5% w/v stock) until resazurin remained colourless.

**Cell growth**

Starter cultures were grown at 60°C overnight in 10mL cultures in Huntgate tubes with a 1 mL inoculation from stock culture. The cells were then grown in 1 litre culture volumes. The media and starter culture were brought to target growth temperature before the entire
starter culture was transferred to the larger flask and cells allowed to grow for at least 16 hours.

*Confirmation of knockdown by PCR of genomic material*

Harvested cells were resuspended in 50mM Tris.HCl at pH 8.0. The cells are melted by pheno/chloroform extraction. The genomic material is isolated by collecting the extraction aqueous supernatant and adding 1/10th volume of 8M ammonium acetate and 2 volumes of cold ethanol. The genomic DNA is removed by sterilized glass rod and transferred in to 50mM TE. Primers used for amplification of the genes (Table) were provided by the Santangelo group and were designed to anneal upstream and downstream of the gene itself, producing bands larger than the gene if it is intact, and greatly reduced in size if it has been removed. PCR was performed using Phusion DNA polymerase (Thermo) with standard manufacturers reaction conditions and performing 25 rounds of amplification at 55°C annealing temperature (15 seconds) and extension at 72°C (90 seconds). Reactions were separated on a 0.6% agarose gel in TEAE buffer with ethidium bromide for visualization by transillumination.

*In vivo tRNA extraction*

Incubation of whole *T. kodakarensis* cells from culture with phenol:choloroform:IAA (25:24:1) is sufficient to release cell contents. In order to help separate protein, lipid and DNA from the bulk RNA species, the cells are first suspended in 100mM ammonium acetate (pH6.5) with 10mM MgSO₄ and 0.1 mM EDTA. Upon addition of saturated phenol mix RNA remains in the aqueous phase and most other components move to the organic
phase following centrifugation. The bulk RNA is precipitated by adding 1/10\textsuperscript{th} the volume of 8M ammonium acetate and two volumes of ethanol and cooling to -20°C for two hours. The precipitated RNA is pelleted by centrifugation at 15000 rpm (Sorval SS-34 rotor) for 25 minutes at 4 °C. The pellet is resuspended in 100mM ammonium acetate (pH6.5) with 10mM MgSO\textsubscript{4} and 0.1 mM EDTA, an equal volume of 8M LiCl was added and the mix cooled at 4 °C overnight. Precipitated rRNA species were removed by centrifugation, followed by tRNA precipitation with the addition of ammonium acetate/ethanol as above.

**Isolation of tRNA for mass spectrometric analysis**

Total RNA was extracted as above, however to prepare total tRNA for MS analysis solid phase extraction was employed to reduce the counter ion species present. Nucleobond RNA/DNA 400 columns (Macherey-Nagel) were employed to separate high mass RNA molecules and total tRNA. Pelleted total tRNA was suspended in the appropriate buffer according to the manufacturers guidelines. The fractionation is based on a step gradient of salt concentration with tRNA eluting in 0.65M KCl and higher mass molecules eluting in 1.15M KCl. The RNA population in sub-fractions were confirmed by urea PAGE. The isolated tRNA is precipitated in 800mM ammonium acetate/ethanol. This is repeated three times to substitute the K\textsuperscript{+} for ammonium ions. The sample was then dried and sent to the Limbach lab for analysis (University of Cincinnati).

**Isolation of isoacceptor tRNA**

The Gln isoacceptors for *T. kodakarensis* were isolated by affinity purification from the total tRNA pool. Streptavidin agarose (Thermo Scientific) was loaded with a biotinylated
DNA oligo that complements a particular stretch of the target tRNA sequence. The resin was activated by binding the biotinylated oligo to the streptavidin (oligo at 15µM in 10mM Tris-HCl (7.5), 100mM NaCl). For annealing of the tRNA to the immobilized DNA, the total tRNA is dissolved in annealing buffer (10mM Tris-HCl (7.5), 900mM NaCl, 1mM EDTA) and heated to 95°C for 5 minutes. After cooling to 85°C the resin (pre-equilibrated in annealing buffer) is added and the slurry is allowed to fully cool to room temperature with occasional mixing. The unbound RNA is removed by centrifugation and washing with elution buffer (10mM Tris-HCl (7.5), 100mM NaCl) until OD_{260} was below 0.01/mL. Elution was achieved by re-suspending the resin in 0.5 ml of elution buffer and heating to 75°C for 5 minutes before centrifuging to collect the Glx tRNA.

**Biophysical analyses**

**UV melting studies**

All thermal denaturation studies were performed on the Cary 100 Bio UV-Vis spectrophotometer. Single wavelength absorbance was used to record the unfolding of the tRNA species being studied. Temperature was adjusted by a thermostatted cell block holder under peltier control. The thermal melt cycle was controlled by the Thermal program in the Cary Win UV software suite. Samples were prepared in 10mM sodium cacodylate at pH 7.0 with a background of 100mM NaCl. This was supplemented with either EDTA or MgCl₂ where indicated. RNA was slow cooled from 98°C to 55°C, at which point the sample was spiked with the additives mentioned previously and allowed to cool down to room temperature. During analysis, sample volume (120µL) is covered with mineral oil to
prevent evaporation. The sample is monitored at 260nm during the melt and the T_m
determined by performing the first derivative of the absorbance value.

Magnesium binding

In these experiments competition is set up between the binding affinity of the folded RNA
for Mg^{2+} ions and that of the fluorescent molecule HydroxyQuinoline-5-Sulphonic acid
(HQS). The dye was prepared by recrystalisation from boiling conc. hydrochloric acid
(repeated six times). All solutions were prepared from Chelex treated deionized water to
minimize metal ion contamination and stock buffers were kept with aliquots of resin to
maintain this. The dye was quantified and diluted to 25 \( \mu \)M in 10mM Sodium cacodylate
pH 7.0, 100mM NaCl, 10\( \mu \)M EDTA and kept at 37 °C. Magnesium chloride stocks were
prepared from high grade salt and the concentration of an approximate 2M stock was
accurately determined by back titration with EDTA. The dye solution was transferred to a
quartz fluorescence cell and the signal at 550 nm (excitation at 395 nm) recorded upon
addition of magnesium chloride in minimal volume steps. The titration was then repeated
in the presence of 25 \( \mu \)M natively folded tRNA (purification of RNA in TE buffer
effectively removes divalents and refolding was performed with sodium chloride as
described previously)

HPLC analysis

All HPLC steps were performed on Agilent 100 system fitted with a multiwavelength diode
array detector. Analytical work was performed on Gemini columns, either small scale (5
\( \mu \)m C18, 30 x 4.6 mm; 1.0 mLmin\(^{-1}\)), or full analytic resolution (5 \( \mu \)m C18, 250 x 4.6 mm;
0.3 mL min\(^{-1}\)) both from Phenomenex. Large scale purification used a Luna column (5 \(\mu\)m C18, 250 x 10 mm; 3.0 mL min\(^{-1}\)) from Phenomenex. All solutions were prepared in MQ water and using HPLC grade salts and solvents.

Preparation of nucleoside digest of tRNA samples begins by dissolving the sample in water. 100 \(\mu\)g of RNA are adjusted for the first step by addition of 1/10\(^{th}\) volume of 100mM sodium acetate at pH 5.5. Nuclease P1 (\textit{Penicillium citrinum} from Sigma) is added at 0.5 \(\mu\)g per 100 \(\mu\)g of RNA. This is allowed to digest at 50\(^{\circ}\)C for 4 hours. The pH is adjusted by direct addition of 1/10\(^{th}\) volume of 1M ammonium bicarbonate. Snake venom phosphodiesterase is then added to the reaction mix (0.01U) and allowed to react for 1 hr at 37\(^{\circ}\)C before adding 2U of bacterial alkaline phosphatase for a further 1hr at 37\(^{\circ}\)C. This mixture can be directly analysed by RP-HPLC.

**Product synthesis and purification**

Each of the three possible substrates NMN, NAD\(^{+}\) and NADP\(^{+}\) were handled in the same way in order to generate the target fluorophore species. The required mass of material was dissolved in MQ water to a concentration not greater than 300 mM. To this was added an equal volume of 4 M NaOH leaving the reactant at 150 mM and the base concentration at 2 M. This mixture was allowed to react for 2 hrs in the dark. At this time the reaction was quenched by the addition of conc. HCl until pH was neutral. The reaction mixture was then initially analysed by reverse phase HPLC using a Gemini column (5 \(\mu\)m C18, 30 x 4.6 mm; 1.0 mL min\(^{-1}\)) from Phenomenex and a linear gradient from 100% 100mM NH\(_4\)OAc (pH
6.8) to a final concentration of 30% acetonile in the same aqueous solution. This utilized the Agilent instrument and recorded spectra from 220-400 nm throughout the elution. Purification was scaled up and utilized an isocratic elution of either 100mM NH₄OAc (pH 6.8) or just MQ water. Large scale purification employed a Luna column (5 µm C18, 250 x 10 mm; 3.0 mLmin⁻¹) from Phenomenex, and elution tracked by monitoring the absorbance at 360 nm. The initial fraction containing the fluorophore was reinjected through the same column and mobile phase conditions for further purification. This fraction was recovered in 1 mL fractions and the fluorophore content determined by adding 10 µL in to 200 µL of 2 M NaOH and collecting fluorescence spectra (ex 360 nm and em 400-500 nm). The purity of the fraction was also determined by injecting a 10 µL aliquot of the fraction on to the Gemini column described above.

**Spectral properties**

*UV absorbance and fluorescence spectra and pH dependence*

Absorbance spectra for the isolated fluorophore were measured on the Cary 100. HPLC fractions, containing the highest purity of the fluorophore, were diluted in to either Millipore water or 0.5 M NaOH in order to determine solvent effects on the chromophore. The spectra were collected using 2nm slits on the excitation path and scanning between 220 and 400nm. In measuring emission spectra, the samples were analysed on the PTI Quantamaster measuring emission between 390 and 600 nm with excitation centered at 360 nm and 2 nm slits (5 nm slit on the emission path)
Quantum yield determination

The fluorescence quantum yield for the product was measured in 0.5M NaOH and carried out at concentrations that gave an absorbance between 0.01 and 0.1 OD to minimize any internal quenching effect. At each concentration the absorbance was measured at 25 °C on the Cary 100 UV-Vis spectrometer (200-400 nm scans with excitation slit set to 2 nm and 0.5 second averaging time). The samples were held in a 9F-Q-10-GL14 cuvette (Starna Cells) and 1mL of sample was measured to minimize noise. A second aliquot of the sample at each concentration step was then measured in the steady state fluorimeter (PTI Quantamaster 400) using the same cuvette. The fluorescence spectra were recorded at 25 °C (thermostatted with a circulating water bath) from 400 to 600 nm (5 nm slits on emission) with excitation at 360 nm (2 nm slits on excitation). Each absorbance and emission spectrum was collected in triplicate and averaged before being plotted to determine the linear response. This process was repeated using the standard quinine sulfate in 0.5 M H₂SO₄. Concentrations in the same absorbance range were prepared and the absorbance and emission spectra were collected with exactly the same instrumental set ups. The two linear responses were then used to calculate the quantum yield of the base product using the standard value for quinine of 0.54 and the following relationship:

\[
\Phi_x = \Phi_{\text{quinine}} \left( \frac{\text{Gradient}_x}{\text{Gradient}_{\text{quinine}}} \right) \left( \frac{\eta^2 x}{\eta^2_{\text{quinine}}} \right)
\]
**Product identification**

*NMR spectroscopy*

After bulk purification as detailed above (mobile phase was Millipore water), the sample was concentrated in order to perform NMR studies. This was achieved by drying under a low flow of nitrogen gas at room temperature in the dark. To the concentrated aqueous sample was added 10% D$_2$O to allow for locking signal. Water suppression experiments on the neutral solution along with sample supplemented with sodium deuteroxide to 100mM were collected on the 400Hz Bruker instrument. This system was also used to collect C$^{13}$ and H$^{1}$/C$^{13}$ HSQC spectra. Finally, N$^{15}$ and H$^{1}$/N$^{15}$ HSQC experiments were run on the 600Hz instrument. Standard pulse sequences provided by the core facility were used in each case.

*Mass spectrometry*

In order to obtain an accurate mass for the fluorophore being studied the sample was freshly prepared and passed through the Gemini (30 x 4.6 mm) column. The eluted fluorophore was at this point in water and so to increase volatility for ESI methodology it was mixed 50:50 with methanol. The prepared sample was immediately injected on to the mass spectrometer by direct infusion and the mass profile recorded in positive mode. The ESI interface was operated in the positive mode using the following settings: spray voltage 3.8 kV; capillary temp 300 °C; capillary voltage 13.89; tube lens voltage 120V.
IR spectroscopy

FT-IR spectra were collected on a Nicolet iS10 spectrometer using a diamond ATR attachment. The sample was extracted from aqueous solution using ethyl acetate which was then dried to concentrate. The sample was drop cast on to the crystal and the ethyl acetate dried before analyzing the unknown against the appropriate background zero.
Results

Role of archaeosine in tRNA

Thermal stability of in vitro modified tRNA

The initial work for this section of research began by studying the effects of magnesium on the stability of a set of tRNA molecules that have been modified in vitro to feature either the genetically encoded guanine, preQ$_0$ or G$^+$ nucleobases at position 15. For this reason, Ser$^{\text{CGA}}$ from Haloferax volcanii was initially selected as it had been used to study the activity of the two biosynthetic enzymes in the pathway$^{45}$. A second archaeal tRNA was selected that features a reduced variable loop compared to the type II Serine sequence. Methanothermobacter thermautotrophicus Gln$^{\text{UUG}}$ was chosen here for it having been studied extensively in its folding pathways$^{84,85}$. Finally, the Gln$^{\text{UUG}}$ sequence from Escherichia coli was chosen as a non-archaeal system that could be modified to feature the archaeal modifications.

Preparation of in vitro modified tRNA

The RNA molecules were prepared from duplex DNA template synthesized by primer extension using Klenow fragment$^{83}$. The native sequences for all three RNAs were used, no modification to enhance transcription yield was introduced (Table 1). The Klenow templates were transcribed using in house expressed and purified T7 RNA polymerase. The reaction mixtures were stopped and precipitated before being purified by slab denaturing PAGE. The isolated full length RNA products were then modified in vitro to
have the genetically encoded G converted to preQ₀ by the action of ArcTGT (Figure 3.1). The purified enzyme was assayed for activity by measuring the incorporation of [8-¹⁴C] guanine into a small preparation of the tRNA, establishing the conditions for quantitative incorporation of substrate base. These conditions were then scaled and substituted with preQ₀ base to substitute the guanine (Figure 3.3). A portion of the preQ₀ modified tRNA was set aside for melting studies and the remainder serving as a substrate for the next reaction using ArcS (Figure 3.2) to produce G⁺ modified tRNA. Quantitation of conversion to G⁺ was determined by digesting the tRNA to free nucleoside and confirming the loss of preQ₀ and appearance of the G⁺ in the chromatogram (Figure 3.3).

UV melting study of in vitro tRNA

The nine RNA samples described above, were then carried on to perform melting studies of the tRNA under the effects of increasing magnesium concentration. Each sample was slow cooled in melting buffer (10mM sodium cacodylate pH7.0, 100mM NaCl) from 98 down to 25°C. If magnesium was to be added, the RNA was heated back up to 55°C before the required amount magnesium chloride stock solution was added and re-cooled to 25°C.

The raw melt data was first processed to obtain a melting profile (first differential plot of \( d\text{Abs}_{260nm} \) vs \([\text{Mg}^{2+}]\)) to allow the apparent \( T_m \) to be deduced. The extracted \( T_m \) value was then plotted against magnesium concentration to reveal the magnesium induced stabilization of the tRNA structure. The isotherm was fitted using a two state model giving a calculated lower and upper limit for the magnesium response (Figure 3.4). It also extracts
a half magnesium concentration for each RNA species relating to sensitivity of the response to magnesium concentration.

As summarized in Figure 3.5 it can be seen that by increasing the concentration of magnesium ions (up to a maximum of 50mM) to which a given RNA is exposed produces an increase in melting temperature of around 20 degrees. By comparing the magnesium free $T_m$ values across the samples we see that two Gln$^{\text{UG}}$ isoacceptors have the higher initial melting point compared to the HV Ser$^{\text{CGA}}$. Both HV$^{\text{ser}}$ and EC$^{\text{glu}}$ show very little change in the calculated maxima and minima with the addition of either preQ$_0$ or G$^+$. The only significant change is seen in the MT$^{\text{gln}}$ RNA where the magnesium free $T_m$ increases from 68.3°C to 70.5°C and finally 71.2°C as the modifications are introduced. This is mirrored by the high magnesium concentrations producing nearly a two-degree increase in $T_m$ when the RNA is modified to G$^+$ from preQ$_0$. The lack of any real change in the $[\text{Mg}]_{1/2}$ value indicates that there is not a sudden increase in the sensitivity of the RNA to magnesium upon introduction of the modifications. If the midpoint had shifted to a significantly lower magnesium concentration it might be argued that there was a particular stabilizing effect relating to magnesium binding that was being titrated.

**Magnesium binding to modified tRNA**

Since the formamidino group introduced at N7 in Archaeosine is implicated in changing the electronics of the base and also the coulombic interaction with surround phosphates, normally facilitated by a coordinated divalent ion, we next investigated the effect of modification on magnesium binding. Methodology used extensively by the Draper group
had shown that the fluorescent dye molecule 8-hydroxyquiniline-5-sulfonic acid (HQS) can be used to set up an equilibrium competition experiments that can sense magnesium affinities in RNAs. The binding of magnesium ions is partitioned between the HQS and a target RNA. To examine the change in magnesium binding caused by modification to preQ₀ and G⁺, the modified \textit{in vitro} RNA was used in the assay.

The baseline response to magnesium needs to be established to achieve a differential in the presence of the subject tRNA. The dye was prepared by recrystalisation from boiling conc. hydrochloric acid (repeated six times). This ensures that the dye was completely demetallated before use (spectral properties confirmed before use) (Figure 3.6). The dye solution was transferred to a quartz fluorescence cell and the signal at 550 nm (excitation at 395 nm) recorded upon addition of magnesium chloride in minimal volume steps. The titration was then repeated in the presence of 25 µM natively folded tRNA (purification of RNA in TBE buffer effectively removed divalent metal ions and refolding was performed with sodium chloride as described previously). The increase in the fluorescence in response to magnesium was now delayed, reflecting a preferential binding to the RNA. The position of this equilibrium can be deduced by comparing the two titrations. As the $K_d$ of magnesium binding to the RNA becomes tighter, the apparent $K_d$ for HQS becomes weaker since more magnesium ions need to be added before they become free to associate with the dye. Analysis was performed on normalized data, using a reduced Hill equation to fit the raw data. This provides a minima and maxima for each RNA titration with which the normalization was performed.
Titrating HQS against magnesium gave a $[\text{Mg}^{2+}]_{1/2}$ of 343 µM in the absence of competitor RNA (Figure 3.6). Addition of unmodified tRNA$^{\text{Gln}}$ from M. thermoautotrophicum shifted the observed affinity to approximately 1.65 mM clearly showing that the experiment was reporting the phenomenon appropriately. The preQ$_0$-tRNA shows a very similar response to unmodified tRNA with a $[\text{Mg}^{2+}]_{1/2}$ of 1.62 mM. G$^+$-tRNA however, gives an apparent $[\text{Mg}^{2+}]_{1/2}$ of 961 µM. This means that at a given concentration of magnesium, unmodified and preQ$_0$ have a stronger affinity for magnesium than G$^+$ modified tRNA. By applying the following equation:

$$\Gamma_{2+} = \left( \frac{C_{2+}^{\text{sample}} - C_{2+}^{\text{reference}}}{C_{\text{ref}}} \right)$$

it is possible to deduce a preferential binding constant ($\Gamma_{2+}$) for an RNA at a given magnesium concentration and therefore the change in magnesium ions per nucleotide or RNA molecule. Although there is a change in the amount of magnesium binding to the G$^+$ RNA, it appears to be more in line with diffusely interacting ionic contacts rather than the loss of a singular coordinated ion. The preferential binding coefficient increase with magnesium concentration, and at 1mM we see approximately 20 fewer ions binding per tRNA molecule in the G$^+$ modified tRNA compared to the unmodified transcript.

*In vivo modified tRNA from Thermococcus kodakarensis*

Although it is possible to analyze the effects of singular modifications upon the stability of a given tRNA, *in vivo* samples would allow us to measure the effects of a given modification in an otherwise fully maturated molecule. In order to access tRNA which is
either un-modified, preQ<sub>0</sub> modified or modified with G<sup>+</sup> it is necessary to obtain genetically modified cell lines that have had arcTGT or ArcS deleted from the genome of an archaean species. There are limited numbers of archaean systems that have sufficient genetics tools available to produce selective knockout cell lines. One of these is fortunately the hyperthermophile *Thermococcus kodakarensis*. The work of Tom Santangelo’s lab is systematically cataloguing knockouts of all the genes in that species genome. The group kindly provided cell lines for TS559 (wild type), TK0760 (Δtgt) and TK1256 (ΔArcS). These are stable deletions where the gene of interest is not only removed from the genome but also the recombination vector is lost from the cell. The mechanism for creating the knockout is illustrated in (Fig 3.7)<sup>87</sup>.

*Growth of the cell lines*

The TS559 cell line is auxotrophic for agmatine by removal of the pyruvoyl-dependent arginine decarboxylase (TK0149). This means that although the tgt and arcS genes are stably deleted, in order to grow the cell lines, they must be supplemented with agmatine. Rich media was used to make as much biomass as possible. Under these conditions, cells were either grown overnight (16hrs) at 85°C or 20 hours at 60°C before harvesting by centrifugation. Up to 0.9 grams of cells can be harvested per liter of growth media. Before going forward with tRNA isolation, the deletions were confirmed by PCR amplification of each gene for the three cell lines. The genes of interest are appropriately removed (Figure 3.8).
Isolation of tRNA from the knockout cell lines.

In order to access the pool of tRNA produced by the TKO cells, they are immediately suspended in Trizol solution after harvesting. The non-RNA components are stripped away by multiple round of PCA extraction leaving behind a total RNA pool. The RNA is cleaned of non-tRNA species by LiCl precipitation (Figure 3.9). Analysis of the nucleoside digest of the \textit{in vivo} tRNA shows that the cell lines have the predicted pattern of modified bases. PreQ\textsubscript{0} and G\textsuperscript{+} nucleoside are absent from the \textit{\Delta tgt} strain. PreQ\textsubscript{0} is present in the \textit{\Delta ArcS} strain and only the wildtype show G\textsuperscript{+} detectable by absorbance in the HPLC chromatogram.

For mass spectrometry analysis (performed by the Limbach lab) tRNA was isolated by SPE column. This permits isolation of tRNA enriched samples without introducing any PAGE artifacts to the sample. Analysis of the three cell lines shows that though no G\textsuperscript{+} is seen in either the TGT or ArcS knockouts by RP-HPLC, there is approximately 1.6\% intensity of the wild type observed for the \textit{\Delta ArcS} sample by MS (Figure 3.10).

Purification of the GlnX isoacceptor

Since both isoacceptors for the Gln encoding tRNA are identical except for the anticodon, it was not possible to isolate just that of CUG. However, the environment of G15 in both sequences should be identical, and the sequence of the ACL should be of no consequence to the overall stability of the tRNA. In comparing the sequences of all the TKO tRNA genes, it was found that the area most unique for Gln was from the ACL leading to the 3’ end of the molecule. DNA oligos were designed to walk along the length of the tRNA in 3
nt steps (Figure 3.11). By slow cooling the transcribed tRNA$_{\text{CUG}}$ with the DNA oligos it was possible to identify the best candidate for forming a stable and appropriate complex. The oligo Aff3 was found to sequester most of the RNA into a particle that moved through the gel rather than making a large multiple strand complex as seen in Aff1 and Aff2 (Figure 3.12). The selected sequence was prepared as a 5’ biotinylated strand that was able to be loaded on to streptavidin sepharose to make an affinity resin for the GlnX tRNAs.$^{88}$ Isoacceptor was extracted from all three cell lines by elution of the RNA by heating the resin to 75°C in low salt buffer. The wash fractions and elution are shown in (Figure 3.12). The isolated tRNA was should to be homogenous in both denturing (Urea TBE PAGE) and native (TB, 100mM NaCl PAGE) (figure 3.13)

Thermal stability of single isoacceptor tRNA

To be able to directly compare the melt behavior of the tRNA extracted from the *T. kodakarensis* strains, the thermal stability of *in vitro* produced tRNA$_{\text{CUG}}$ (TKO sequence) was tested under increasing Mg$^{2+}$ concentrations (Figure 3.14). The melts of the wt, preQ$_{0}$ and G$^{+}$ modified tRNA are shown alongside the sequence details. The unmodified *in vitro* tRNA shows a shoulder in the differential plot at around 70°C along with the major melting transition at 85°C under 100mM NaCl. This has shown that the tRNA is inherently more stable than the three other tRNAs studied previously. Under the same salt conditions, the preQ$_{0}$ and G$^{+}$ modified tRNAs also feature a slight shoulder nearer 65°C, but the major melting transition occurs a few degrees above that of the unmodified CUG. As the concentration of magnesium ions is increased up to 10mM, it is seen that the shoulder persists, though the peak does increase to around 71°C for all three RNAs. At the highest
magnesium concentration, the melting transition is not completed by 98°C which is the highest temperature reached in the melt. This indicates that the tRNA is melting fully at least 10-12°C above the HV and MT tRNA with the same modifications in place.

When the in vivo produced and modified tRNA (Figure 3.15) is put through the same melting experiment, we see a significantly different trend. The WT (containing G+) and dTGT (the genetically encoded guanine base) behave almost identically. There is still a slight shoulder at around 71°C and a second melting point at around 83°C. This higher melting temperature is similar for the dArcS (containing preQ0) but there is a more pronounced shoulder at around 63°C with this RNA. The addition of 10µM Mg²⁺ introduced to the system has no measurable effect on the melting transitions. At 100µM Mg²⁺ however, the WT and Δtgt profile has collapsed in to a single and well defined transition at about 82°C. At the same concentration, the tRNA from ΔArcS has the major transition occurring at 73°C with a shoulder at 86°C. At 1mM Mg all three RNAs are melting with a single transition, though ΔArcS is 4°C lower than the WT and Δtgt. This is maintained at 10mM Mg where all three RNAs are melting at a temperature beyond the 98°C limit of the experiment.
Identification and characterization of NAD(P)$^+$ base product

Stability and assay conditions

The assay studied here begins with an initial acid quench step where the total reaction mix is adjusted to be in 150mM HCl. This will not only stop the reaction by denaturing the protein component, but it also decomposes any unreacted reduced NAD(P)H so that no further production of NADP$^+$ can occur after the end point has been defined (Figure 3.16).

The conversion of NAD(P)$^+$ to the fluorescent product is achieved by adding concentrated base, typically to a final concentration of 7M NaOH. This is incubated at room temperature for 2 hours to develop the fluorescent molecule, however it is unclear from the literature if their conditions are optimal for maximal production of fluorescent product and thus assay sensitivity. To determine the conditions for optimum yield, a 50mM solution of NADP$^+$ was treated with NaOH over a concentration range from 1-10M. Immediately after being added to the sodium hydroxide solutions an increase in fluorescence is observed that correlates to with the concentration of NaOH (Figure 3.17). This trend does not persist upon longer incubation. Left at room temperature overnight and in the dark, the fluorescence yield is maximal at between 2 and 3 M NaOH and produces a 35% increase over the same treatment at 7M NaOH (Figure 3.17).

The 2M base conditions were then used to track the progress of the reaction at lower base concentration. The same 2-hour incubation time prescribed by the original assay was analysed by RP-HPLC. It is shown that intact NADP$^+$ is entirely consumed by 20 minutes
of exposure to 2M NaOH. The remaining time causes two unidentified products to exchange, both eluting long before NADP\(^+\) or the fluorescent product.

**Equivalency of substrates**

In order to ascertain if the adenosine group is involved in the formation of the fluorophore, NADPH and nicotinamide mononucleotide (NMN), the latter of which lacks the adenosine moiety, were treated with the 2M NaOH work up conditions. NADPH, NADP\(^+\) and NMN were all prepared as 50 mM aqueous solutions. The absorbance spectra of both NADP\(^+\) and NMN are dominated by a peak at ~260nm (Figure 3.18). The spectrum of the reduced form, NADPH is further characterized by a peak at 340nm. Upon treatment with sodium hydroxide (equal volume of 4M NaOH added to the 50mM stock for 2 hours at room temperature, neutralized by addition of HCl) the absorbance spectrum of NADPH remain relatively unchanged either when diluted in to MQ water or back in to basic conditions (1M NaOH). The peak at 340nm persists in water but there is a slight red shift when measured in base. In contrast, NADP\(^+\) and NMN show a drastic change to their spectra. In the case of NADP\(^+\) a new band appears at approximately 340nm when measured at natural pH, and shifts to ~360nm under alkaline conditions. This corresponds to the absorption band utilized in the assay. The spectrum still features a large peak at 260nm which corresponds to the adenine moiety. In the reaction involving NMN which is the peaks observed in both water and 1M NaOH match those observed for NADP\(^+\), but the spectrum at lower wavelengths is no longer swamped by the 260nm peak, revealing base-sensitive fine structure below 260nm. This confirms that the fluorophore produced by base treatment of
NAD$^+$ or NADP$^+$ is generated independently of any involvement of the bridging phosphates or adenosyl moiety.

Spectral properties

Absorbance and fluorescence spectra

To characterize the fluorescent product a purification strategy needed to be developed in order to isolate it from the rest of the reaction side products. Analytical scale purification by reversed phase HPLC using 100mM ammonium acetate (pH 7.0) showed good separation with just an isocratic run on a C18 column. In order to obtain the fluorescent product free of other species the preparative scale separation was performed from the neutralized reaction mix, using a mobile phase of just MQ water. The isolated product was then kept in solution and adjusted to be at a useful concentration for further analysis.

Under neutral conditions the absorbance spectrum exhibits a single symmetric peak centered at 330nm for the isolated fluorophore (Figure 3.19). Exciting at that maxima, the fluorescence is minimal when scanned between 400 and 600nm. When this is repeated in 0.5M NaOH fine structure in the absorbance spectra forms, with a shoulder peak at 330nm and a main peak that is shifted to 360nm. Again excitation at absorbance maximum results in the characteristic fluorescence emission at 450nm. Shifting the pH of the solution creates a fluorescence enhancement of ~68 fold compared to neutral solution (Figure 3.20).
Quantum yield

While this assay has been used extensively to measure NAD(P)$^+$ concentrations however it has not been reported how efficient the fluorophore is. In order to do this the quantum yield ($\Phi$) was determined for the base product using the standard quinine sulfate. The absorbance values were then plotted against the fluorescence integral for each of six concentration points. It was found that the quantum yield for the base product is 0.11 (c.f. 0.54 for quinine sulfate) (Figure 3.20).

Identification of the fluorescent species

Mass spectrometry

The first step in characterizing the unknown fluorophore was to obtain an accurate mass value for the product. Using freshly prepared and purified material, ESI MS was performed and provided a molecular ion (MH$^+$) 124.03948 m/z (figure 3.21). A stable sodiated adduct (MNa$^+$) at 146.02176 m/z was also found. To ensure that these masses represented the parent ion and not a daughter fragment, the tube lens voltage was reduced from 120 to 80 V with no appearance of higher mass species. This provided a molecular weight of 123.0315 a.m.u. and a predicted formula of C$_6$H$_5$NO$_2$ with an accuracy of 3.94 ppm.
NMR

$^1$H

The sample was analysed under both neutral and basic conditions by hydrogen NMR. The sample in water was spiked with D$_2$O to a final concentration of 10% to allow signal lock. Under these conditions, a singlet is seen at 9.88 ppm consistent with an aldehyde hydrogen. There are three other resonances seen in the spectrum at 8.18 (dd), 7.81 (dd) and 6.63 ppm (t) (Figure 3.22), consistent with aromatic hydrogens. Upon addition of 1/10$^{th}$ volume of 10M sodium deuteroxide, there was only a slight shift in the resonances. The aldehyde hydrogen resonance is shifted upfield by 0.01ppm and maintains a singlet structure. The aromatic hydrogens shift by a small amount, now appearing at 8.08 (dd), 7.83 (dd) and finally 6.5 ppm (dd) (Figure 3.23). The latter resonance suggests that the triplet seen in the spectrum of the sample in neutral conditions is likely to be a doublet of doublets with a symmetrical J coupling value giving the appearance of a triplet.

$^{13}$C

**Prepared in ammonium acetate and dried under N$_2$.** The carbon spectrum of the isolated fluorophore shows seven peaks. One at 22.95ppm is attributed to the acetate from preparing the sample. The other six are consistent with one being part of a carbonyl (192.09ppm) and the five aromatic carbons (180.98, 148.07, 142.77, 124.25 ppm) (Figure 3.24).
The sample for IR analysis was prepared by extracting the purified material from water using ethyl acetate. The organic phase was concentrated under N\textsubscript{2} gas. The IR spectrum was collected after drop casting onto the diamond surface and the solvent allowed to evaporate before collecting (Figure 3.25). No broad peak was seen for the sample in the region above 3000 cm\textsuperscript{-1} suggesting the absence of any hydroxyl groups in the compound. There are sharp peaks that dominate the spectrum in the range of 2800-3000 cm\textsuperscript{-1} which are likely to correspond to C-H stretches in carbonyl groups. This is supported by strong peaks at 1690 and 1670 cm\textsuperscript{-1} consistent with C=O stretching frequencies).
Discussion

RNA discussion

The first experiments carried out here reported the effects of the G+ modification and the precursor, preQ0 on the structural stability of tRNA from *H. volcanii* (HV), *M. thermautotrophicus* (MT) and for non-archaeal comparison, *E. coli* (EC). These species grow optimally at 45, 85 and 37°C respectively, so the sequences cover moderate and hyperthermophilic species. It is also notable that *H. volcanii* is a moderate halophile and requires around 2.5M NaCl and 1.5M MgCl2 for optimal growth. This correlates to only 37% of the tRNA sequences from *H. volcanii* featuring a guanine at position 15 compared to 60% for *M. thermautotrophicus* and 100% for *T. kodakarensis*.

**In vitro melt study**

The *in vitro* prepared tRNAs were melted at NaCl concentrations of 100mM. It was decided to perform the melts in a background of monovalent cations in order to observe the magnesium specific stabilization of the modified RNA. The background sodium concentration ensures that the secondary structure and most tertiary contacts should be in place before the melting begins.

At this concentration the *in vitro* transcribed HV Ser tRNA in all three modification states are identical, with the RNA melting at around 64.4°C. Upon addition of Mg2+ ions the melting point rises steadily, with the fitted maximal melting temperature being 1°C lower.
than unmodified when preQ$_0$ is present and 0.6°C higher for G$^+$. For the \textit{in vitro} transcribed MT$^{Gln}$ RNA, modifying the transcript to feature preQ$_0$ and G$^+$ increases the T$_m$ in sodium by 1.2 and 1.0°C respectively. The high magnesium melting point for unmodified and preQ$_0$ RNA are identical at 89.7°C. The high magnesium concentration T$_m$ in G$^+$ tRNA is however raised by 2.1°C. In the \textit{in vitro} transcribed EC$^{Gln}$ RNA all three modification states have a maximum T$_m$ at high magnesium of 87.3°C. The sodium T$_m$ for both transcript and G$^+$ are also identical, with the preQ$_0$ being 1°C higher.

The largest change observed in this set of data is in the \textit{M. thermautotrophicus} sample. However, even that is only a 2°C increase in the T$_m$ when the G$^+$ modification is in place. This does not seem a massive shift, especially for a modification that is purported to be involved in a structural role. However, in previous studies that have examined \textit{in vivo} prepared tRNA where all modifications are in place, compared to a unmodified transcript, a change of 5-6°C is all that is observed$^{92}$. Individual modifications have been shown to stabilize active structures (the modification s$^2$T54 in \textit{T. thermophilus} was found to increase the T$_m$ by 3°C) active structures and lock in conformations normally under equilibrium that are conducive for function$^{93, 94}$. The change in Tm observed with G$^+$ formation may be small, but it is not unusually low compared to other similar experiments with core base modifications.
Magnesium binding

The fluorescent chelator HQS has been used in many studies to analyze the observed affinity of a given RNA for magnesium ions. The experimental technique used here was devised by the Draper group, to examine the binding of a tightly coordinated magnesium ion in the adenine riboswitch. The phenomenon of magnesium being partitioned between HQS and RNA has been applied to various RNA structures, including tRNA. In 1975 the yeast tRNAPhe that was the basis for most of the structural information gathered on tRNA was used in a similar experiment.

The results of that study was that the tRNA had two subclasses of magnesium interactions, a strong interaction with a Kd of approximately $9 \times 10^4 \text{M}^{-1}$ and a weaker binding mode of $6 \times 10^3 \text{M}^{-1}$. The occupancy of the two were 19 and 5 ions respectively.

The manual titrations performed using the HQS and the in vitro transcribed M. thermautotrophicus tRNA showed that there was very little difference between the magnesium binding displayed by the transcribed tRNA$^{\text{Gln}}$, and that of the preQ$_0$ modified molecule. The binding displayed for the G$^+$ RNA is significantly different to the first two, but the trend does not correlate to a difference of a singular magnesium ion being lost. At 1mM Mg$^{2+}$ we observe a decrease in the preferential binding factor 10 ions per RNA molecule when we compare the G$^+$ modified tRNA to the unmodified transcript. It appears that the technique used was to insensitive to capture a single loss of a tight binding event. The number of weakly associated ions swamps out the effects that are seen for other systems that have more defined binding pockets.
**Growth of TKO mutants**

The knockouts of the TGT and ArcS genes from *T. kodakarensis* gave us the first opportunity to study the effects of losing the G\(^+\) biosynthetic pathway in a hyperthermophilic archaeon. The cells were confirmed to have maintained the deletion with PCR confirming the loss of the two genes producing truncated extension products. Upon harvesting larger quantities of cells it was also possible to confirm loss of preQ\(_0\) from the delTGT and G\(^+\) from the delArcS cells by total nucleoside digest of the tRNA. The RNA was also prepared for accurate mass analysis by ESI LC-MS (performed by the Limbach group). This further confirmed loss of preQ\(_0\) and G\(^+\) from the respective cell lines. This gave us the confidence to go on with the melting studies using these RNAs.

The Santangelo group who originally generated the cell lines had performed growth experiments (Figure 4.1). This showed that the cells were all able to grow well up to 85°C (the species normal optimal growth temperature). Although cell density in the deletion cell lines does not reach that of the wild type, the rate of growth appears comparable in all three. Raising the temperature to 95°C was found to significantly reduce the growth of both TK0760 (Δtgt) and TK2156 (ΔarcS). In large culture the cells grown in house were found to show significantly reduced growth at 90°C as well. The growth phenotype was seen in cultures grown on both sulphur or pyruvate (cultures grown in house were on pyruvate media to allows for easier harvesting of biomass). The phenotype shows that modification to G\(^+\) or apparently even preQ\(_0\) assists in the growth of TKO cells at high temperature. This is in line with the hypothesis we are trying to investigate, in that these modifications may
be stabilizing tRNA at high temperature, and that their loss, and resultant instability hinders cell proliferation.

In vivo melts

The first step in approaching the thermal melting experiments was to ensure that we could obtain the isoacceptor from the total tRNA pool to make a clearer melting profile. The methodology has been used extensively\textsuperscript{88} to extract isoacceptors from \textit{in vivo} prepared RNA but rarely on a species that grows at such a high temperature. To make sure this was not going to impede the purification process, test oligos were used along with transcribed RNA equivalents of the target tRNA. This allowed for the appropriate sequence to be used that avoided oligomeric high mass particles being formed. This was the case for the oligos that touched down directly on the most unique stretch of the tRNA sequence. Moving further to the 3’ of the tRNA sequence permitted the sequestering of the tRNA in to a discrete complex with the affinity DNA.

The RNA recovered from the affinity tagging (eluted by heating the washed resin at 75°C) was slow cooled and analyzed by denaturing and native PAGE to confirm both integrity of the RNA (no laddering in the denaturing gel) and single species being formed by slow cooling (single band on the native gel). This meant that the RNA was we purified and appeared to be structurally intact before melting analysis was performed. From 1 litre of cell culture the yield of isoacceptor was 30µg (WT), 38.4 µg (dTGT) and 35.6 µg (dArcS). This gave sufficient material for both melting and the above analysis of the same prep.
The transcribed equivalent of the TKO CUG isoacceptor RNA was modified as in the experiment with HV, MT and EC tRNA. The melting transitions show a biphasic melting process, the lower melting occurring at around 65°C which rises to above 70°C at high magnesium, but persists even at 10mM MgCl₂. The second and more substantial melting point occurs at 85°C at the lowest magnesium concentrations, implying that the TK has a significantly more stable tRNA compared to the MT RNA. At high magnesium the Tₘ cannot be determined as the half point occurs beyond the limit of the experimental conditions. Very little difference is seen between the three modification states, except that the unmodified RNA shows a tighter transition for the low Tₘ than the other two samples.

The in vivo tRNA show a significantly different trend. The WT (G⁺) and dTGT (G) both behave almost identically. They both feature two melting transitions, the first, lower Tₘ at 70°C at low magnesium, but at 100µM the two transitions have merged into a single event at 82°C. Increasing the magnesium further raises the Tₘ to 95°C (1mM) and beyond 98°C by 10mM. In the dArcS sample which feature preQ₀, we observe a lower Tₘ occurring at 60°C and remaining two phases until 1mM where the collapse into a singular transition at 90. This third RNA is also melting beyond 98°C however a greater proportion of the RNA is melted at this point seen in the greater dAbs value.

With all the other modifications in place, no significant increase in the Tₘ of the tRNA is produced. However, given the inherently more stable sequence, we are already melting at higher temperatures than in the other RNAs in this study. The pattern of response to magnesium is similar that seen in the E. coli tRNA^{Phe} in comparing in vivo matured vs in
vitro material\textsuperscript{95}. The change in $T_m$ at biologically relevant ionic concentrations are not massively different, but there are clear differences in how components of the folding process respond at different modification states.

Given that we do not see a significant loss of thermal stability at conditions equivalent to growth conditions, and yet we see a growth phenotype at higher temperature, the results may seem to be at odds. However, that assumes that the loss of viability of the cell lines at high temp is simply due to reduction of absolute melting point.

The original hypothesis for the function of archaeosine was that it took on a structural role to stabilize tRNA in thermophilic archaean species. This is born out in the \textit{in vitro} prepared tRNA\textsubscript{Gln} from \textit{M. thermautotrophicus}, where a 2.1°C increase in melting point was measured when archaeosine was present. This was not reproduced in the tRNA of fellow extreme thermophile \textit{T. kodakarensis}, moderate thermophile \textit{H. volcanii} or the bacterium \textit{E. coli}, this would suggest that the effects of $G^+$ modification are dependent on the RNA in which it occurs and possibly the species in which it is being formed. Indeed, in the context of \textit{in vivo} maturated tRNA with all modifications are steady state levels except for preQ\textsubscript{0} or $G^+$, we see that $G^+$ modification confers no additional stabilization beyond the genetically encoded guanine base. Somewhat surprisingly, \textit{in vivo} material with preQ\textsubscript{0} present, is actually less stable that the genetically encoded guanine. This creates the possibility of a more complicated structural consequence of the $G^+$ pathway, one that is dependent on the species, it’s tRNA primary sequence and environmental conditions for the role that $G^+$ ultimately serves.
Previously, similar studies have found that loss of a given modification doesn’t always produce a significant change in overall thermal stability, but increased dynamics of the RNA makes it less functional at higher temperatures. Loss of m7G at position 46 by a deletion of the methyltransferase trm8 in yeast caused reduced modification at other sites\textsuperscript{96}. There was a temperature dependent growth response, where cell taken from 28°C to 37°C showed reduced growth. Analysis of the tRNA stability showed little difference in the stability of the tRNA, but increased dynamics of the RNA meant that subsequent modifications were not being produced. This led to the specific degradation of tRNA valine (other tRNAs normally featured the modification but were not degraded). This first instance of Rapid tRNA Decay (RTD) pathway for the targeted removal of misfolded tRNA showed that the observed temperature dependent growth phenotype might not be directly related to RNA stability but to the modification status of the tRNA.

Similarly, loss of a single pseudouridine at position 55 in \textit{T. thermophilus} was found to have a significantly higher $T_m$ (8.4°C) if it was grown at low temperatures. It was proposed that loss of $\Psi$55 actually increased the amount of other stabilizing modifications which made for a more rigid structure. This then in turn, decreased its ability to function in the cell giving a growth phenotype at low temperatures caused by an overly stable, misfolded RNA\textsuperscript{97}. The degradation of tRNA by a bacterial RTD was also first identified in \textit{T. thermophilus}\textsuperscript{28}.

There is still a dearth of information regarding the function of base modifications that occur in the core region of tRNA (Figure 4.2). One of the most abundant of these modifications
are methylations. Though some of these are specific to the archaea, there is often a parallel modification that occurs in the other domains, serving a similar role. Methylation events are often involved in directing the formation of hydrogen bonding at the core. In *P. abysii* for example, m$^2$G10 creates steric hindrance that directs the formation of the D-stem, in the case of tRNA$^{Pro}$, by enhancing a wobble base pairing with U25, and in tRNA$^{Asp}$, by preventing a 2 base slip that causes the D-loop to be expanded\textsuperscript{98}. In *H. volcanii* the same modification regulates the formation of the AC stem in prolyl tRNA by a similar mechanism\textsuperscript{99}. In the region between the accepter stem and the variable loop, position 48 and 49 will often be methylated (m$^5$C) but in this case, it is believed that the modifications are acting to enhance base pairing by shifting the electronics of the modified bases.

In archaea specifically, further modification of these methylated bases can occur where O-methylation at the 2’OH of the ribose takes place. The gradual addition of methyl groups has been shown to be an adaptive response to increasing growth temperatures\textsuperscript{100}. The population of m$^2$G$_m$ goes up as the precursor m$^2$G decreases (60-70% of the precursor is converted to the O-methyl derivative at the highest growth temperatures used).

Uridine is also commonly modified in the core, and the modifications are also sensitive to sequence and growth conditions. Pseudouridinylation will occur at position 13, stabilizing base pairing between itself and position 22 which forms in the D stem. It is only modified however if the base at position 22 is a U or G, canonical base pairing to A22 does not induce the modification to be carried out. The occurrence of pseudouridine in the T-loop is well known, acting as one of the bases (Ψ55) that creates loop-loop interactions with the
D-loop. In archaea, the position 54 is also found to be modified to $\Psi$, $m^1\Psi$ and also the related high temperature induced $m^5s^2U$. These modifications help to ensure the correct structure is adopted in the T-loop to maintain needed tertiary contacts.

Crystallographic studies have shown that arcTGT binds to tRNA in an alternative $\lambda$-form structure $^{101}$ where the D-stemloop is fully disrupted, allowing the enzyme to “count” the first 15 bases of the substrate. Indeed, biochemical characterization of the $P.\ horikoshii$ enzyme showed that the enzyme became increasingly active the higher the temperature of the exchange reaction was carried out at (up to $100^\circ C$)$^{44}$. It is possible that the addition of preQ$_0$ to a new transcript will occur fairly early in the maturation process. If the disordered RNA needed for TGT activity is to be funneled in to an active form for further modification and function, it is possible that the G-preQ$_0$-G$^+$ status at position 15 serves as a check point for viability of the tRNA. Since the more extreme halophiles are able to thrive with a lower number of their tRNA featuring the modification, it is possible that preQ$_0$ and G$^+$ modifications help direct the maturation process or the folding of extended transcript in to functional tRNA.

Synthetase charging assays would give an immediate answer as to the functionality of the in vivo produced tRNAs. Looking at the folding rates of tRNA in the same, stepwise modification status as has been carried out in this study, would be the likely next step to see if the role of G$^+$ lies in the direction of the folding pathway, rather than the stabilization of an already folded tRNA.
Identification and characterization of NAD(P)^+ base product

The continued use of the methodology established in the 1950s\textsuperscript{102} shows it to be rigorous and flexible enough to still be of use to current research of systems consuming NAD(P). The exact nature and properties of the fluorophore however have been largely ignored since the protocol is so reliable. Here we have characterized the fluorophore to be 2-hydroxy-nicotinaldehyde.

Identification of the fluorophore

Isolated immediately from the assay work up conditions, the first step in determining the structure was recording an accurate mass. Since no mass higher than the mono-sodium adduct of the product species was found, it put an immediate bracket on the structures possible. The strong signal and accurate mass obtain in positive mode ESI meant that we could be confident in the molecular mass of 123.0315 g/mol. This allowed the formula to be calculated at \( \text{C}_6\text{H}_5\text{NO}_2 \).

In order to confirm this, the fluorescent product needed to be characterized spectroscopically to identify structural features to finalize the structure. In order to collect the NMR spectra the product was collected by HPLC in MQ water. From here it was initially attempted to dry down under vacuum to remove the water, however it was found that the product was volatile. Under the vacuum supplied by a speedvac (30 in Hg below ambient) all material volatilized and so could not be recovered. To provide more gentle
drying conditions, the sample was concentrated under nitrogen. After significant concentration, the sample, still in aqueous solution was spiked with deuterium oxide for signal locking. Since there was still such a strong residual water signal, the H\textsuperscript{1} spectra were recorded using a water suppression pulse sequence. Under these conditions, four protons were recorded. This would suggest that there is one exchangeable proton given the predicted formula. The resonances and splitting pattern suggested an aldehyde proton at 9.88 ppm that showed no coupling. The remaining three protons are 8.18, 7.81 and 6.63 ppm. These are characteristic for aromatic protons, so it is highly likely that we have an intact ring structure. This is supported by the C\textsuperscript{13} spectrum which suggests 5 aromatic carbon centers and an aldehyde resonance.

The three ring protons are in three distinct environments, and so it is likely that the ring structure is asymmetrically substituted. The observed resonances for the $\alpha$ (8.18), $\beta$ (6.63) and $\gamma$ (7.81) match the deshielding pattern of pyridine. The splitting patterns for the three protons in water are also in support of this with the constants being $\alpha$ ($J_\beta = 7.27$ Hz, $J_\gamma = 2.19$ Hz), $\beta$ ($J_{\alpha,\gamma} = 6.83$ Hz) and $\gamma$ ($J_\alpha = 2.20$ Hz, $J_\beta = 6.32$).

Maintenance of the pyridine ring, along with the formation of a formyl group leaves an oxygen and a fifth proton left to be accounted for based on the MS derived formula. The proton for a hydroxyl group would be exchangeable and so not seen in the proton NMR. Performing a N\textsuperscript{15}-H\textsuperscript{1} coupled HSQC experiment (data not presented) on the sample also hinted that the nitrogen was not stably protonated as no signal was detected for protons excited by resonance to the only nitrogen atom. Using spectrum prediction software, the
theoretical spectra for the 2-hydroxy derivative rather than the 3-hydroxy most closely matches the pattern seen empirically \(^{103,104}\). Although the simulations are calculated based on DMSO as the solvent, the observed resonances and splitting pattern can again give us confidence in our structural assignments.

Once the identity of the product was confirmed, literature searches for 2-hydroxynicotinaldehyde (2HNA) brought up hits from the early 1970s where analogs of \(\text{NAD}^+\) were studied to investigate the fluorophore first identified by Kaplan in 1951. Comparison of pyridinium and nicotinamide derivatives showed that the carboxamide substitution at position 3 is able to significantly enhance the susceptibility of such molecules to nucleophilic attack at position 2. Indeed, the formation of the 2-hydroxy derivative in nictotimamides are found to have a rate ~1000 fold higher than that of the pyridinium equivalent. This in turn translates in to a \(K_{eq}\) enhancement of 6000 fold for the formation of the open ring form of the two families \(^{105-107}\).

Guilbert and Johnson \(^{108}\) carried out the base work up on 1-(N,N-dimethylcarbamoyl) nicotinamide chloride and diphosphopyridine nucleotide (DPN). They were able to characterize the product of the reaction between these two analogs as being identical to 2HNA and synthesized a control of the substance for full comparison.

Using the dimethylcarbamoyl analog the mechanism of formation was deduced for 2HNA. Initial base treatment of a species featuring an oxidized nicotinamide moiety produced what is referred to as the open ring form of NAD (ONAD). The conversion from ONAD
to 2HNA goes through a second major intermediate that is created upon hydrolysis of the Schiff base form of ONAD. This would release everything connected to what was previously the ring nitrogen of the nicotinamide, leaving 2-carboxamide-glutacondialdehyde (CGDA).

Recyclization occurs upon deprotonation of CGDA which facilitates nucleophilic attack of the amide nitrogen upon carbon 5. This regenerates the pyridine structure and upon further decomposition generates 2-HNA\(^{109}\). The overall mechanism deduced for the generation of 2-HNA from oxidized nicotinamide species is laid out in (Figure 4.3) with key intermediates in the process highlighted. There are secondary reactions at most steps, however the concentration of base used really drives the process to the overall yield of around 95%.

The work up condition specified in the original methodology paper by Lowry et al. exposed the biological sample to 8N NaOH in order to generate the fluorogenic species detected in the assay. With the knowledge gleamed from previous studies and now this one, it would appear that this is far more aggressive than it need be. The early work was carried out on pyridine derivatives and then transferred to the nicotinamide nucleotides as an application to biological systems. The studies by Johnson throughout the 70s showed that the nicotinamide moieties were faster to react, and also more sensitive to base concentration to produce the ring opening derivatization\(^{105}\).
Unlike the pyrimidine system which achieves maximum yields of fluorescence at pH > 13, the equivalent effects can be generated at up to four pH units below this with nicotinamides. In the time courses completed in this study it was shown that fluorescence can be recorded maximally when incubated at 2M NaOH. The reaction time of 2 hours is also more than enough for all NAD(P)$^+$ to be converted to the 2-HNA. As long as the reaction has been successfully quenched in the 150mM HCl step, incubation in 2M base should still provide a significant excess of hydroxide ion concentration to fully convert to 2-HNA. Indeed, the lower base concentration may be of benefit by making the assay conditions more product stable over a prolonged time. Since the chemistry to generate 2-HNA permits going to completion at lower concentrations and is not under equilibrium, the reduced fluorescence observed at higher concentrations may be resulting from decomposition of the target molecule. This considered it may be advisable to reduce the concentration of NaOH used to 2-3 M to permit an increased dynamic range and stability of sample.
## Tables

**Table 1** Sequences of tRNA used in initial thermal stability experiments. *H. volcanii* Ser\textsuperscript{CGA}, *M. thermautotrophicus* Gln\textsuperscript{UUG} and *E. coli* Gln\textsuperscript{UUG} tRNA sequences. The RNAs are transcribed from templates produced by Klenow extension of the corresponding DNA oligo pairs. The DNA primers add the T7 promoter sequence (bold) and the annealing overlap is shown underlined.

### RNA sequences

**Ser CGA (H. volcanii)**

5’ GCCGAGGUAGCCCUAGCCGCAAGGCGUAGAUUCGAUUCUACUGUCCAUCACCAUUCGACACGUG AGUUCAAAUUCUACCCUCGGCG3’

**Gln UUG (Methanothermobacter thermautotrophicus)**

5’ AGUCCCGUGGGGUAGUGUAAUCCUCUGGCGCUUUGGACCCCGGCACAGCGGUUCGAUCCGC UCACCACAAUCA3’

**Gln UUG (E. coli)**

5’ UGGGGUAUCCAGCGGAAGGCACCAGGUUUUUUUGAUACCGGCAUUCUCCUGGUUUCGAUCCAG GUACCACAGGCA3’

### Klenow template sequences

**Ser CGA (H. volcanii)**

5’ GCAGTAATACGACTCATAAGCCGAGGTAGCCTAGCCCGGCAAGGCGGTAGATTCGAA3’

5’ TGCCGCCGAGGGTGAGAATCCTGCTGGGCTT3’

**Gln UUG (Methanothermobacter thermautotrophicus)**

5’ GCAGTAATACGACTCATAAGCCGAGGTAGCCTAGCCCGGCAAGGCGGTAGATTCGAA3’

5’ TGCCGCCGAGGGTGAGAATCCTGCTGGGCTT3’

**Gln UUG (E. coli)**

5’ GCAGTAATACGACTCATAAGCCGAGGTAGCCTAGCCCGGCAAGGCGGTAGATTCGAA3’

5’ TGCCGCCGAGGGTGAGAATCCTGCTGGGCTT3’
Table 2 Sequences of tRNA used in TKO in vitro melting study. The RNA sequence that was used is shown first, below are the DNA oligos used to generate double stranded template for in vitro transcription.

**RNA sequence**

**Gln CUG (Thermococcus kodakarenisis)**

5’AGCCCUGGGUGUAGCGGCCAAGCAUGCGGACUGGAUCCCGCGACCGGGGUUCGAAUCCC CGCGGGGCUACCA3’

**Klenow template sequences**

5’TAACTCACGACTATAGGCCCGTGTGTAAGCCGCAAAGGATGCGGGG3’

5’mUmGTTAGCCCGGGGATTGCAAACCGCGGATCCAGATCCGCATGC3’
Figures

**Figure 1.1** Structure of tRNA(a) Secondary structure of a typical tRNA shown in the cloverleaf representation. (b) The folded structure of the active tRNAPhe from *S. cerevisiae* (PDB accession 1EHZ).
**Figure 1.2** Naturally occurring modified bases. Naturally occurring modified nucleotides which are all found in tRNAs. Both queuosine and N6-threonylcarbamoyl adenine are located at the anticodon loop and maintain translation fidelity. 2’-O-methylcytidine and N7-methylguanine are involved in structural stability. Modified functional groups are shown in blue.
Figure 1.3 Biosynthesis of preQ₀ nucleobase. The first steps that are common to the archaeosine and queuosine pathway is the formation of preQ₀ from GTP. Figure modified from *Biochemistry* 2009, 48, 3847-3852.
Figure 1.4 Queuosine biosynthesis. The fate of preQ₀ in bacteria is to be converted to preQ₁ before being inserted into tRNA by bacTGT. Figure modified from NAR. 2016, 44(20): 9965–9976.
Figure 1.5 Archaeosine \((G')\) biosynthesis. After insertion into substrate tRNA at position 15, preQ\(_0\)-tRNA is further modified by either ArcS (A), QueF-like (Q) or Gat-QueC (G).

Figure 1.6 Electron density computed for Guanine and modeled G⁺. The model proposes a substitution of a coordinated magnesium ion in unmodified tRNA for the positively charged formamidino group of G⁺ (RNA. 2007 Sep; 13(9): 1427–1436).
Figure 1.7 The positioning of Mg$^{2+}$ and Mn$^{2+}$ in the proximity of N7 in guanine 15. The crystal structure of tRNAPhe from *S. cerevisiae* showing the unmodified Levit base pair (G15-C48) in stick mode. The neighboring nucleotides are shown in surface render indicating negatively charged phosphates (orange and red) surrounding the manganese ion (purple sphere).
Figure 1.8 Thermal melting of tRNA structure. Using the hyperchromic effect of UV absorbance during thermal denaturation of tRNA it is possible to detect the unfolding events as subpopulations in the melting profile (first differential plot of dAbs vs T). (a) the melting of in vitro and in vivo tRNAPhe in EDTA (dotted), 100µM MgCl₂ (solid) and 4mM MgCl₂ (dashed) (NAR 1998 Jun 1; 26(11): 2723–2728). Subpopulations shown as peaks I or II have been previously deconvoluted by NMR studies to represent individual structures falling apart. (b) the subpopulations are sensitive to the concentration of magnesium ions and at biologically relevant concentrations the melting of tRNA collapses into a single melting event dictated by the breaking of the D-stem/T-stem contacts. (Trends Biochem Sci. 1996 Apr;21(4):145-9).
**Figure 1.9** The *de novo* synthesis and salvage pathways of NADH. Schematic for the biosynthetic pathway of both NAD and ultimately NADP cofactors. (*FEBS Journal*, 2012, 279, 3355-3363)
**Figure 3.1** SDS PAGE analysis of arcTGT purification. CFE is the total soluble protein fraction and RT is the run through fraction that does not interact with the resin. Unbound and non-specifically bound proteins are eluted at 0 and 10mM imidazole respectively. The target 6His fusion protein is eluted at 50mM imidazole.
Figure 3.2 SDS PAGE analysis of ArcS purification. SN is the total soluble protein fraction obtained following lysis and RT is the run through fraction that does not interact with the resin. A stepwise gradient of imidazole concentration was applied and the target ArcS was eluted at 50mM imidazole.
Figure 3.3 Modification of the in vitro prepared tRNA. (a) shows the $[8^{-14}C\text{-guanine}]$ incorporation into substrate tRNA to assay arcTGT activity. (b) HPLC separation of total nucleoside digest of preQ$_0$ modified tRNA, with the modified nucleoside eluting at approximately 21.5 minutes. (c) transformation of preQ$_0$ (black) in to $G^+$ (gray) by the activity of ArcS, again showing total nucleoside digest analysis by RP-HPLC.
Figure 3.4 Magnesium dependence of thermal stability of tRNA. The *in vitro* transcribed tRNA for (a) *H. volcanii*, (b) *M. thermautotrophicus* and (c) *E. coli* have been modified to feature preQ₀ or G⁺. The RNA is melted under increasing concentrations of Mg²⁺ ions in a background of 100mM NaCl. The isotherms are fitted to a two-state model to obtain minimal and maximal melting temperatures.
**Figure 3.5** Magnesium dependence of thermal stability of tRNA. The extracted values for the isotherm fits of magnesium dependence on $T_m$. For each RNA species and modification state there is a minimum and maximum $T_m$ and the transition midpoint for the response.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HV CGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>64.3</td>
<td>64.5</td>
<td>64.4</td>
</tr>
<tr>
<td>max</td>
<td>85.5</td>
<td>84.8</td>
<td>86.2</td>
</tr>
<tr>
<td>$[\text{Mg}^{2+}]_{1/2}$</td>
<td>1.2mM</td>
<td>1.7mM</td>
<td>1.8mM</td>
</tr>
<tr>
<td>MT UUG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>68.3</td>
<td>70.5</td>
<td>71.2</td>
</tr>
<tr>
<td>max</td>
<td>89.7</td>
<td>89.9</td>
<td>91.8</td>
</tr>
<tr>
<td>$[\text{Mg}^{2+}]_{1/2}$</td>
<td>1.2mM</td>
<td>1.5mM</td>
<td>1.9mM</td>
</tr>
<tr>
<td>EC Gln</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>69.2</td>
<td>70.5</td>
<td>69.5</td>
</tr>
<tr>
<td>max</td>
<td>87.3</td>
<td>87.4</td>
<td>87.3</td>
</tr>
<tr>
<td>$[\text{Mg}^{2+}]_{1/2}$</td>
<td>1.6mM</td>
<td>1.7mM</td>
<td>1.5mM</td>
</tr>
</tbody>
</table>
Figure 3.6 Magnesium binding by fluorescence of HQS. (a) Preparation of the demetallated HQS reagent by recrystalization gives a magnesium responsive fluorophore whose absorbance spectra have well defined isosbestic points. (b) Binding of magnesium in the absence (red) and presence of trx (dark blue) and G\(^+\) (light blue) tRNA gives an observed shift in the affinity of magnesium for the dye. (c) The difference between the control titration (HQS alone) and the RNA titration is used to deduce the preferential binding coefficient (colored as previous figure).
Figure 3.7 Scheme for the generation of arcTGT (TK0760). The addition of the TK0149 to the knockout cassette permits temporary prototrophism to be established in the normally agmatine auxotrophic TKO strain. The gene for 6-methylpurine (6MP) acts as a counter selective agent to drive the looping out of the cassette to produce the final stable TK0760 cell line.
**Figure 3.8** Confirmation of knockout in TKO cell line by PCR. Genomic material was recovered from each cell line and amplified by PCR, targeting either the TGT gene cassette (lanes 1, 2 and 3) or the ArcS gene cassette (lanes 4, 5 and 6). Lanes 1 and 4 confirm WT maintains both genes. Lanes 2 and 6 confirm the TGT and ArcS, respectively, have been successfully knockdown with truncation of the cassette. ArcS is still present in the Δtgt cells and TGT is still present in ΔarcS.
Figure 3.9 Analysis of purified total in vivo tRNA. (a) Denaturing PAGE separation of the tRNA pool extracted from the three cell lines. 1 & 2 are WT LiCl precipitation products, 3 & 4 Δtgt and 5 & 6 are ΔarcS. (b) Total nucleoside digest of the samples analyzed by RP-HPLC. PreQ₀ appears at 26 minutes and is absent from the WT and ΔarcS samples. G⁺ elutes at 30 minutes and is present in the wild type sample only.
Figure 3.10 Mass spectrometry of the in vivo modified tRNA. The extracted chromatogram for G$^+$ (324+H$^+$) are shown for each cell line tRNA sample. (a) The WT shows a strong peak at 29 minutes corresponding to the G$^+$ nucleoside. (b) In the TGT knockout, no peak is seen for this mass confirming the expected effect. (c) In the Arcs knockout, there is a peak at approximately 1.6% of the WT intensity seen.
Figure 3.11 Affinity tag for isolation of GlnX isoacceptor from TKO. (a) The target sequences of the GlnX gene that were used to derive affinity oligos (the reverse compliment of sequence shown). (b) Native PAGE analysis of the complexes formed between the four candidate oligos and the transcribed tRNA$_{\text{CUG}}$.

(a)

5’ AGC---CATGCGGGACCTCTGGATCCCGGACCGGGGTTC--CAA 3’

<table>
<thead>
<tr>
<th>Oligo</th>
<th>$T_m$</th>
<th>$T_m$ (predicted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GCGGGACTCTGGATCCCGC</td>
<td>60</td>
<td>66</td>
</tr>
<tr>
<td>2 GGACTCTGGATCCCGGACCCGACC</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>3 CTCTGGATCCCGGACCGG</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>4 TGGATCCCGGACCGGGGT</td>
<td>60</td>
<td>67</td>
</tr>
</tbody>
</table>

(b)
Figure 3.12 Purification of the GlnX isoacceptors. Total tRNA from the three cell lines was incubated with streptavidin sepharose which had been coupled with the biotinylated Aff3 oligo. The RNA (in 10mM Tris.HCl pH 7.0, 900mM NaCl and 1mM EDTA) was heated to 95°C and mixed with pre-equilibrated resin. The suspension was slow cooled to allow the Aff3 to anneal to the tRNA. The wash fractions (W) of annealing buffer were continued until the OD_{260} was below 0.001 units. The RNA was eluted at both 45 and 75°C in 10 mM Tris.HCl pH 7 and 100mM NaCl.
Figure 3.13 Confirmation of \textit{in vivo} isoacceptor purity. The isoacceptors were slow cooled according the melt experiment prep and then run through either a 10\% denaturing PAGE (a) or an 8\% native gel (b). Lane 1: mixture of tRNA$^{CUG}$ (76mer) and a DNA oligo at 49 bases (d$_{49}$) and 19 bases (d$_{19}$) in length. Lane 2: \textit{WT}, lane 3: \textit{Δtgt} and lane 4: \textit{ΔarcS}. Xylene cyanol (XC) and bromophenol blue (BB) are shown for electrophoretic progress.
Figure 3.14 Melting profiles of *in vitro* produced TKO Gln$^{\text{CUG}}$. The Gln$^{\text{CUG}}$ sequence was used for comparison to the *in vivo* material extracted from *T. kodakarensis*. The RNA was modified in vitro to produce G (black), preQ$_0$ (dashed) or G$^+$ (gray) at position 15. The melting profiles were recorded at (a) 100mM NaCl (b) 1µM MgCl$_2$ (c) 100µM MgCl$_2$ and (d) 10mM MgCl$_2$. 
Figure 3.15 Melt profiles of *in vivo* TKO GlnX isoacceptor tRNA. The purified isoacceptor tRNA from *WT* (black), Δtgt (dashed) and ΔarcS (gray) are melted in the presence of increasing Mg$^{2+}$ concentrations; (a) 100mM NaCl, (b) 10µM MgCl$_2$ (c) 100µM MgCl$_2$ (d) 1mM MgCl$_2$ (e) 10mM MgCl$_2$. 
Figure 3.16 The products of acid treatment of reduced NADPH. (a) HPLC separation of NAPDH in water (red) and following treatment with 150mM HCl (blue). HPLC was carried out using an isocratic mobile phase of 100mM NH$_4$OAc (pH 6.8). (b) The extracted absorbance spectrum (red) for the peak at 3.3 minutes of the untreated NADPH separation. The product peaks for the adenosyl moiety (dashed blue) and nicotinamide moiety (solid blue) eluting at around 0.5 and 6.5 minutes respectively.
Figure 3.17 Time dependence of fluorophore yield. (a) Fluorescence intensity of a solution of NADP$^+$ and increasing concentration of NaOH. Immediate quantitation of fluorescence is in red, the blue data points reflect an 18hr incubation at each concentration. (b) HPLC separation of the base treatment of NADP$^+$ at 2M NaOH. The NADP$^+$ is shown in red. Immediate quenching with HCl produced the products for 0 mins (blue dash), 60 minutes (blue dotted) and 120 minutes (solid blue). The loss of NADP$^+$ 260nm absorbance is shown inset.
Figure 3.18 UV spectra of NADPH, NADP$^+$ and NMN derivatives. (a) NADPH in water (dark blue), treated with base in 0.5M NaOH (medium blue) and in water (light blue). (b) NADP$^+$ in water (purple), treated with base in 0.5M NaOH (lilac) and in water (magenta). (c) NMN in water (red), treated with base in 0.5M NaOH (yellow) and in water (orange). Base treatment in each case was 2hr in 2M NaOH before being neutralized with HCl.
Figure 3.19 UV absorbance and fluorescence of isolated fluorophore product. The absorbance spectrum of the product was measured between 250 and 500nm, in both neutral conditions in water (red) and 0.5M NaOH (blue). The fluorescence is measured from 400 to 600nm with excitation at 360nm (dashed lines, colors represent the same conditions as absorbance).
Figure 3.20 Determination of quantum yield for the base product. The fluorescence spectra of a quinine sulfate standard (red) in 0.5M H2SO4 and the base product (blue) in 0.5M NaOH. The area between 400 and 600 nm was integrated and plotted against the corresponding absorbance value at 350nm.
Figure 3.21 Accurate mass determination. Freshly prepared and purified fluorophore in DI water is mixed with an equal volume of methanol before being analyzed by electrospray MS. In positive mode the two main peaks have m/z of 124.03948 and 146.02178. This corresponds to a molecular weight of 123.0315 gmol$^{-1}$. 
Figure 3.22 $^1$H NMR of the isolated fluorescent product. Water suppression $^1$H spectrum of the HPLC purified product shows four resonances: 9.88 (s), 8.18 (d,d), 7.81 (d) and 6.63 (t) (expanded in lower panels). Spectrum collected in 10% D$_2$O.
Figure 3.23 $^1$H NMR of the isolated fluorescent product in basic conditions. Water suppression $^1$H spectraum of the HPLC purified product shows four resonances: 9.87 (s), 8.08 (d,d), 7.83 (d,d) and 6.50 (d,d) (expanded in lower panels). Spectrum collected in 1M NaOD.
Figure 3.24 $^{13}$C NMR of the isolated fluorescent product. Carbon spectrum of the HPLC purified product shows seven resonances: 192.09 (s), 180.98 (s), 148.07 (s), 142.77 (s), 124.25 (s) and 108.47 (s) relate to the compound being studied. The resonance at 22.95 ppm is from the acetate carried over from the purification.
Figure 3.26 IR spectrum of the isolated fluorophore. The sample was extracted from the MQ water with ethyl acetate and concentrated under nitrogen.
**Figure 4.1** Growth phenotype of the *T. kodakarensis* mutants. Both the Δtg (TK0760) and ΔarcS (TK2156) mutants show greatly reduced growth at 95° where the wild type is still showing rigorous growth.
Figure 4.2 Location of base modifications in the core of tRNA. Shown is a schematic of the tRNA form *H. volcanii*, showing type and location of modifications that are specific to Archaea and facilitate the formation of the folded core (bases in the blue box). (figure modified from *BMC Genomics, 2008, 9:470*).
Figure 4.3 Mechanism of base reaction to yield 2-hydroxynicotinaldehyde. The first step in producing 2HNA is the nucleophilic attack of hydroxide at position 2 of a reduced nicotinamide species (i). This yields (ii) which upon further supply of hydroxide ions irreversibly is converted to the ring open form of NAD\(^+\) or ONAD (iii). This decomposes to generate 2-carboxamideglutacondialdehyde (CGDA) (iv) with loss of what was the ring nitrogen of the starting material. Intramolecular re-cyclization results in the formation of the fluorescent product (v) which is in equilibrium with the inactive protonated form (vi).


