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Biosynthesis of the 7-deazaguanosine hypermodified nucleosides of transfer RNA

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Abstract

Transfer RNA (tRNA) is structurally unique among nucleic acids in harboring an astonishing diversity of post-transcriptionally modified nucleoside. Two of the most radically modified nucleosides known to occur in tRNA are queuosine and archaeosine, both of which are characterized by a 7-deazaguanosine core structure. In spite of the phylogenetic segregation observed for these nucleosides (queuosine is present in Eukarya and Bacteria, while archaeosine is present only in Archaea), their structural similarity suggested a common biosynthetic origin, and recent biochemical and genetic studies have provided compelling evidence that a significant portion of their biosynthesis may in fact be identical. This review covers current understanding of the physiology and biosynthesis of these remarkable nucleosides, with particular emphasis on the only two enzymes that have been discovered in the pathways: tRNA-guanine transglycosylase (TGT), which catalyzes the insertion of a modified base into the polynucleotide with the concomitant elimination of the genetically encoded guanine in the biosynthesis of both nucleosides, and *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA), which catalyzes the penultimate step in the biosynthesis of queuosine, the construction of the carbocyclic side chain.

1. Introduction

The post-transcriptional processing of transfer RNA (tRNA) involves a number of functionally distinct events essential for tRNA maturation [1–4]. The phenomenon

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of nucleoside modification is perhaps the most remarkable of these events, and results in a wealth of structural changes to the canonical nucleosides [4]. Although other RNA species also exhibit varying degrees of nucleoside modification, it is only in the tRNA that a rich structural diversity is realized.

Nucleoside modification typically occurs to ~10% of the nucleosides in a particular tRNA, but can involve as many as 25% of the nucleosides [4]. Over 80 modified nucleosides have been characterized [4], many of which are conserved across broad phylogenetic boundaries. The nature of nucleoside modification varies from simple methylation of the base or ribose ring to extensive "hypermodification" of the canonical bases, the later of which can result in radical structural changes and involve multiple enzymatic steps to complete. With the realization that many modifications are conserved in phylogenetically diverse organisms, and that an impressive amount of genetic information codes for tRNA modifying enzymes (an estimated 1% of the total genome in bacteria such as *Salmonella typhimurium*) [5], an appreciation for the importance of modified nucleosides to the basic physiology of the cell has emerged. It is thought that modifications located outside of the anticodon region function in general to maintain the structural integrity of the tRNA and serve as identity determinants for the myriad interactions involving tRNA [4]. In contrast, modifications within and around the anticodon are proposed to play a direct role in increasing translational efficiency and/or fidelity [6,7], and in specific cases, to serve as recognition determinants or anti-determinants for aminoacyl-tRNA synthetases [8,9]. In spite of the importance of modified nucleosides in tRNA, the contributions that specific modifications make to tRNA function are well established in only a few cases [4,5], and our understanding of the biosynthesis of the various modified nucleosides is, in the main, rudimentary.

1.1. 7-Deazaguanosine modified tRNA

Arguably the most remarkable modifications known to occur in tRNA are the 7-deazaguanosine nucleosides queuosine (Q, Fig. 1) and archaeosine (G*, Fig. 1). Both nucleosides share the unusual 7-deazaguanosine core, but differ in the extent of

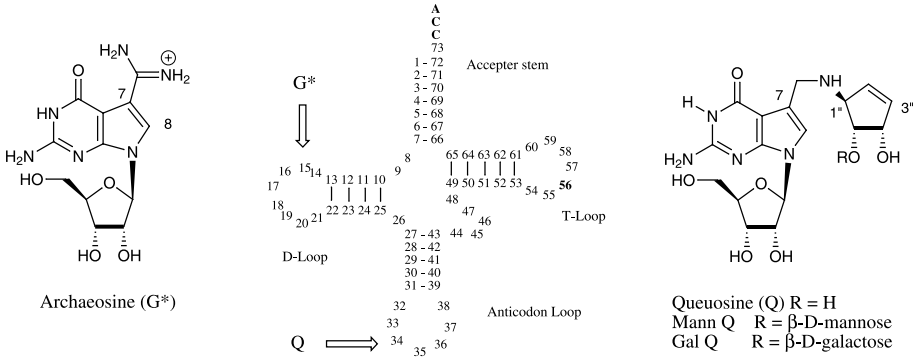

Fig. 1. The chemical structure of queuosine (Q) and archaeosine (G*) and their positions in tRNA.

further elaboration of this core structure; queuosine is characterized by a cyclopentendiol ring appended to (7-aminomethyl)-7-deazaguanosine [10,11], which in some mammalian tRNAs is glycosylated with galactose or mannose at the C5″ hydroxyl [12], while archaeosine possesses an amidine functional group at the 7-position [13]. Queuosine and its derivatives occur exclusively at position 34 (the wobble position) in the anticodons of tRNAs coding for the amino acids asparagine, aspartic acid, histidine, and tyrosine [14]. Each of these tRNAs posses the anticodon sequence GUN (positions 34–36), where N can be any nucleotide. Queuosine is ubiquitous throughout eukaryotic and bacterial phyla (with the exception of the tRNA of yeast and *mycoplasma*), but is absent from the tRNA of the Archaea. In marked contrast, archaeosine is present only in the Archaea, where it is found in the majority of archaeal tRNA specifically at position 15 in the dihydrouridine loop (D-loop) [15], a site not modified in any tRNA outside of the archaeal domain. As with queuosine modification, all of the tRNA in which archaeosine is found originally contain a genetically encoded G at the site of modification.

1.2. Physiological role of queuosine and archaeosine

A definitive picture of queuosines biochemical function or functions has yet to emerge, but research over the last 30 years has established a strong connection between queuosine and a variety of physiological phenomena. In Eukarya, for example, the developmental stages of a cell are closely correlated with the extent of queuosine modification in the tRNA [16]; the relevant tRNAs of mature tissue exhibit full modification with queuosine while undermodification of tRNA is uniformly observed in developmental stages associated with cell proliferation and differentiation [16–19]. Potentially related is the observation that neoplastic transformation and development are correlated with the appearance of (Q^-)-tRNA [17,20–22]. So widespread is this later observation that it has been suggested that the extent of (Q^-)-tRNA be used for histopathological grading of malignancies [21]. Queuosine has also recently been shown to be essential in the biosynthesis of tyrosine in animals [23].

Although there is considerably less data available on the role(s) of queuosine in bacterial physiology, what is known is nonetheless striking. *Escherichia coli* mutants defective in queuosine biosynthesis exhibit an apparently normal phenotype during favorable growth conditions, even out-growing a wild-type control strain in mixed population experiments [24,25]. Upon entry into stationary growth phase, however, the viability of the (Q^-)-strain drops dramatically, such that after a short time the number of viable (Q^-)-cells comprise less than 1% of the control. Recent studies with the pathogenic bacterium *Shigella flexneri* have identified tRNA-guanine transglycosylase (TGT), a key enzyme in queuosine biosynthesis (vide infra), as the gene product of *vacC* [26], a virulence-associated chromosomal locus associated with epithelial cell invasion. Among the physiological characteristics of the *vacC* mutant were significantly decreased levels of key invasion-associated proteins, as well as reduced capacity for provoking keratoconjunctivitis. When the cloned *tgt* gene from *E. coli* was introduced into the *vacC* mutant, the virulence phenotype was fully restored.

While the molecular basis for these disparate phenomena is unknown, the presence of queuosine in the anticodon is compatable with a role in modulating translational fidelity. Indeed, translational effects unique to individual tRNAs have been observed both in vitro and in vivo. For example, the -1 frameshifting events essential for correct translation of the retroviral Gag-Pol-Pro polypeptides of HTLV-1 and BLV appear to be dependent on (Q^-)-$tRNA^{Asn}$ [27–29]. A similar dependance on tRNA hypermodification has been observed with the analogous frame shifting events in HIV and Rous sarcoma virus, where frame shifting has been shown to be more efficient with a $tRNA^{Phe}$ that lacks the hypermodified nucleoside wyebutoxine (Wye) normally present in the anticodon loop [28]. In other experiments, (Q^-)-$tRNA^{Tyr}$ has been shown to exhibit amber suppresser activity in both a context-dependent and independent manner [14,30,31], while queuosine in $tRNA^{His}$ eliminates the codon bias exhibited by the (Q^-)-$tRNA^{His}$ for the degenerate codons in vivo [32]. Furthermore, the loss of pathogenicity observed in *S. flexneri tgt* mutants has been traced primarily to mistranslation of the virulence factor *virF* [33], a transcription factor responsible for up-regulation of a suite of virulence associated proteins. Finally, the role of queuosine in mammalian tyrosine biosynthesis appears to be related to the (Q^-)-tRNA dependent mistranslation of mRNA coding for the enzyme phenylalanine hydroxylase.

Interestingly, a growing body of evidence also suggests that queuine, the free base of queuosine, may exert important effects on eukaryotic physiology independent of its role in tRNA. It has been shown, for example, that queuine is essential in HeLe cells for relieving hypoxic stress [34], and queuine has been implicated in lactate dehydrogenase isoenzyme distribution, protein phosphorylation patterns [35,36], and in the modulation of cell proliferation and signaling pathways [37–41].

The molecular physiology of archaeosine has yet to be investigated, but its location at position-15 in tRNA has led to speculation that it may function in stabilizing the tertiary structure of the tRNA [13], an especially critical role given the prevalence of thermophiles within the Archaea. This proposal is based on global interactions between the D-loop and the T-stem and loop [42] that are conserved in the tertiary structure of all tRNA. Specifically, the nucleotide at position-15 (always a purine) base pairs with a pyrimidine at position-48 (the junction of the T-stem and variable loop), and is involved in a stacking interaction with nucleotide-59 (usually a purine). Loss of this structural element triggers complete denaturation of the tRNA to random coil [43]. Thus, stabilization of the D-loop/T-stem interaction is essential to maintenance of tRNA tertiary and secondary structure. Archaeosine may contribute to the stability of this structural element through electrostatic interactions of the positively charged formamidine group, which based on the crystal structure of yeast $tRNA^{Phe}$ [44] should sit in a cleft of high negative electrostatic potential containing the 5′-phosphate groups from nucleotides 7, 14, and 49.

2. Biosynthesis of queuosine and archaeosine

The biosynthesis of the 7-deazaguanosines is only partially understood (Fig. 2), and has been studied primarily in the context of queuosine formation. Whole organ-

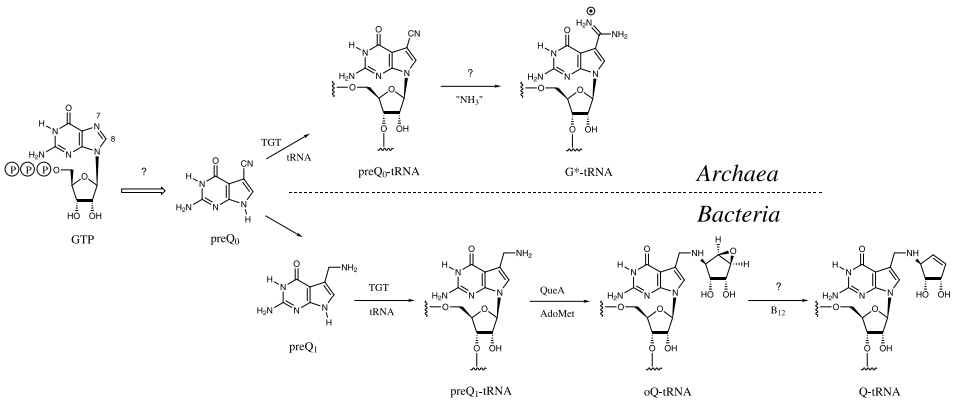

Fig. 2. The proposed biogenesis of queuosine and archaeosine. Although GTP has been shown to be a precursor to queuosine, it has not been investigated in archaeosine biosynthesis. Question marks above arrows denote steps for which the enzyme(s) have yet to be identified.

ism incorporation experiments with *Salmonella typhimuruim* established that GTP is the probable primary precursor in the biosynthesis of queuosine. In these experiments positive incorporation into queuosine was observed with [2-^{14}C]guanine, but not with [8-^{14}C]guanine [45]. These results were interpreted to implicate loss of carbon-8 (and possibly N7) in a process analogous to that observed in the biosynthesis of the 7-deazaadenine nucleoside antibiotics toyocamycin [46] and tubercidin [47], as well as the biosynthesis of the pterins and folic acid [48]. In these systems carbon-8 of the purine ring is lost as formic acid, and the carbons of the ribosyl moiety in the nucleotide become incorporated into the deazapurine and pterin rings following an Amidori Rearrangement. In principle GTP cyclohydrolase I or II could serve as the first step in the biosynthetic pathway to queuosine and archaeosine, although it has been reported that in *E. coli* a GTP cyclohydrolase II mutant still produces queuosine modified tRNA [112], rendering a route through cyclohydrolase II unlikely. Alternatively, a unique cyclohydrolase-like activity may be involved in 7-deazaguanosine biosynthesis.

The first established intermediate in the queuosine pathway is 7-cyano-7-deazaguanine ($preQ_0$) [50], which undergoes reduction to 7-aminomethyl-7-deazaguanine ($preQ_1$) by an as yet uncharacterized dehydrogenase. $PreQ_1$ is subsequently inserted into the tRNA by the enzyme tRNA-guanine transglycosylase (TGT), a reaction in which the genetically encoded base (guanine) is eliminated [50,51]. The remainder of queuosine biosynthesis occurs at the level of the tRNA, and involves the unprecedented utilization of *S*-adenosylmethionine (AdoMet) in the construction of an epoxycyclopentandiol ring [49,52,53] to give epoxyqueuosine (oQ), followed by an apparent B_{12}-dependent step in which the epoxide in oQ is reduced to give queuosine [54].

Although queuosine is ubiquitous in both the Eukarya and Bacteria, only Bacteria are capable of de novo queuosine biosynthesis. Eukaryotes acquire queuosine as

a nutrient factor and/or from the intestinal flora [14], and insert queuine, the free base of queuosine, directly into the appropriate tRNAs [55] by a eukaryotic TGT.

The presence of a 7-substituted 7-deazaguanine core structure in both queuosine and archaeosine, along with the structural similarity of $preQ_0$ to archaeosine base, is consistent with identical biosynthetic pathways in Archaea and Bacteria for the formation of $preQ_0$. These pathways would presumably diverge at $preQ_0$, with $preQ_0$ serving as the substrate for an archaeal TGT in the key base substitution reaction. Evidence in support of this scenario came with the isolation of both $preQ_0$ and an archaeal TGT from *Haloferax valcanii* [56], followed by the identification and cloning of a putative *tgt* gene from *M. jannaschii* [57], and the biochemical characterization of the recombinant enzyme as a TGT [57]. The formation of archaeosine can in principle occur through the formal addition of ammonia to the nitrile of $preQ_0$ after incorporation into the polynucleotide, but nothing is presently known about the final steps of the biosynthesis.

To date only two steps in the biosynthesis have been characterized at the enzymatic and genetic level; the substitution of the genetically encoded guanine for a 7-deazaguanine base catalyzed by the TGT enzymes, and the transfer and isomerization of the ribose moiety of AdoMet to $preQ_1$ by the enzyme QueA to give oQ in the biosynthesis of queuosine.

3. The TGT enzymes

The TGTs are a particularly intriguing family of enzymes. They are the only enzymes involved in RNA modification that catalyze the replacement of a genetically encoded base with a modified base [5], and their phylogenetic segregation is correlated with unique modified base substrate specificity and disparate tRNA recognition elements.

TGT activity was first reported in the early 1970s [58,59], and purification of the relevant enzymes from eukaryotic and bacterial sources followed over the next decade. TGTs have now been purified and characterized from organisms representing all three kingdoms [56,60–65], and recombinant forms of the enzyme have been characterized from two Bacteria [66,67] and two Archaea [57,68].

Based on primary sequence data, the TGTs from all three kingdoms share considerable sequence conservation at the amino acid level (22–99% identity based on the *E. coli* enzyme) in a core region of the enzyme [69,70]. In bacterial enzymes this region represents the entire protein, in eukaryotic enzymes it constitutes the catalytic subunit of a putative heterodimer, and in the archaeal enzymes it is present in the N-terminal portion of the protein. A zinc binding domain was identified in the bacterial TGT shortly after the gene from *E. coli* was cloned and over-expressed [71], and based on sequence context it was proposed to serve a structural rather than catalytic role. This hypothesis was confirmed by site-directed mutagenesis and spectroscopic studies [72], which further indicated that the zinc binding domain might play a role in tRNA recognition. This domain is also found in the TGTs from eukaryotes and archaea.

3.1. Base substrate specificity

The base specificity has been investigated in detail for the bacterial enzyme, and more superficially for the eukaryotic and archaeal enzymes. The enzyme from *E. coli* can utilize $preQ_1$, $preQ_0$, and guanine as substrates ($V_{max}/K_M = 1.4 - 6.6\ s^{-1}\ mg^{-1}$), with $preQ_1$ the most efficient substrate [73]. Other 7-deazaguanine analogs are markedly poorer substrates (second-order rate constants 2–3 orders of magnitude lower than $preQ_1$), a result due almost entirely to the higher K_M for these substrates. Some insight into the structural basis of this selectivity was achieved from inspection of the X-ray crystal structure of TGT from *Zymomonas mobilis* [70,74], which shows that the aminomethyl group of $preQ_1$ can form a hydrogen bond with the backbone carbonyl of Leu^{231}, while the cyano group of $preQ_0$ can form a hydrogen bond with the amide nitrogen of Leu^{231}. Compounds that possess smaller substituents at C7 (e.g., CH_3) are unable to take advantage of these interactions, and those with bulkier groups (e.g., CO_2CH_3) are unable to fit into the binding pocket with a productive geometry due to the presence of Val^{223}. While this structural interpretation of the TGT active site is consistent with the kinetic data, the fact that guanine exhibits similar kinetic parameters to $preQ_1$ illustrates that the above analysis is only the first step in understanding the critical structural elements responsible for catalysis by this enzyme.

Although a large number of compounds have been screened as potential inhibitors of the eukaryotic enzyme [75], quantitative kinetic studies of alternate substrates have in general not been carried out. It has been shown, however, that while queuine is the most efficient substrate, the enzyme does catalyze the insertion of a number of other compounds into the tRNA, including dihydroqueuine, $preQ_1$, 7-deazaguanine, and guanine [75]. In order to gain insight into the basis for substrate selectivity in the eukaryotic and archaeal enzymes, Romier et al. [69] developed a putative model for the active sites by using the *Z. mobilus* structure as a template and modeling in the amino acids from the *C. elegans* and *M. jannaschii* TGTs, respectively. The structural basis for the expanded substrate specificity of the eukaryotic enzyme, and in particular its ability to utilize queuine as a substrate, appears to be due to the substitution of Val^{233} and Cys^{158} with glycine and valine, respectively. These substitutions substantially enlarge the binding pocket for the base substrate, and allow accommodation of the cyclopentendiol side chain of queuine.

The archaeal enzymes have been studied the least with respect to base specificity, and from preliminary screens may possess the most stringent specificity. Enzyme activity was observed only with $preQ_0$ and guanine for both the *M. jannaschii* [57] and *H. volcanii* [56] enzymes; no activity was detectable when $preQ_1$, 7-carboxamide-7-deazaguanine, 7-carboxy-7-deazaguanine, or archaeosine base were investigated as potential substrates for the enzyme. This is consistent with the active site model developed by Romier et al., which predicts that the archaeal enzymes possess a much more crowded active site. In particular, the loop formed by residues Val^{196} to Glu^{201} in the archaeal enzyme (corresponding to Leu^{231} to Glu^{235} in the *Z. mobilis* enzyme) appears to be critical to reducing the volume of the active-site due to the substitution of a Leu for Val at position 199 (position 233 *Z. mobilis* numbering), and the

presence of an extra residue (Pro) immediately proceeding Leu[199]. The former serves to push the loop down into the active-site due to steric interactions with Pro[161] (*Z. mobilis* numbering), while the latter serves to fill up the active-site in the vicinity of the C7 substituent.

3.2. RNA recognition by the TGT enzymes

The observation that in bacterial and eukaryotic tRNA queuosine was limited to a subset of tRNA containing a GUN sequence in the anticodon (Tyr, His, Asp, and Asn tRNAs) led to the inference that this sequence was an essential recognition element for the bacterial and eukaryotic TGTs. Curnow et al. [76] showed that an unmodified tRNA transcript ($tRNA^{Tyr}$) was as effective a substrate as the modified $tRNA^{Tyr}$, demonstrating that other post-transcriptional modified nucleosides were not required for recognition by the enzyme. Furthermore, they also showed that the enzyme efficiently utilized a mini-helix RNA substrate corresponding to the anticodon stem-loop of *E. coli* $tRNA^{Tyr}$. Subsequent work unequivocally demonstrated that for bacterial TGT the minimal sequence requirement was a minihelix with a 7-base loop containing a UGU sequence [77,78] at the positions corresponding to positions 33–35 in a tRNA.

The issue of whether such a restricted recognition motif might allow for recognition at UGU sequences in other structural contexts has been addressed recently by the Garcia laboratory [79–81]. They showed that *E. coli* TGT was able to catalyze the transglycosylation reaction in a mini-helix RNA when the UGU sequence was moved to correspond to the anticodon (positions 34–36) in full-length tRNA, but only when the nucleotide preceding the UGU sequence (corresponding to the invariant U33 in tRNA) was not a uridine [80]. The failure of RNA containing U33UGU to function as a substrate is especially interesting, and appears to implicate loop conformation as a determinant in TGT recognition. This has obvious physiological benefits in that it prevents modification of the UGU sequence present in the anticodon of $tRNA^{Thr}$. It was further shown that TGT was able to catalyze the tansglycosylation reaction at G53 within a UGU sequence in the TΨC arm of in vitro transcribed yeast $tRNA^{Phe}$ [79], as well as mini-helix analogues of this stem-loop structure. However, reaction at this site was not observed in normally modified $tRNA^{Phe}$. These studies were extended to the context of DNA, and while the DNA analogue of a mini-helix RNA was not recognized as a substrate [81], the replacement of thymidine with uracil to give the dU containing DNA resulted in a functional substrate with less than a 10-fold reduction in k_{cat}/K_M [81]. While it is unlikely that recognition of the UGU (and dUGU) sequence in the above structural contexts is biologically relevant (k_{cat}/K_M for these alternate substrates were up to 50-fold lower than for natural tRNA), the results do allow for the potential that TGT might have substrate RNAs other than tRNA.

No in vitro studies have been carried out with the eukaryotic enzyme to elucidate the specific RNA structural elements required for TGT recognition, but in vivo studies with *Xenopus laevis* oocytes and micorinjected tRNA have shown that like the bacterial enzyme, a UGU sequence in a 7-base loop is essential for enzymatic activity

[82]. However, unlike the bacterial enzymes, it also appears that the eukaryotic enzymes require an intact tRNA [83].

Unlike the presence of queuosine in bacterial and eukaryotic tRNA, a survey of archaeal tRNA sequence data revealed no obvious consensus sequence that was unique to archaeosine modified tRNA. While all archaeosine containing tRNA possessed a genetically encoded guanosine at position-15, not all tRNA with a guanosine at position-15 appeared to undergo modification to archaeosine [15,84,85]. Furthermore, there was no apparent higher order structure unique to archaeosine modified tRNA; archaeosine is present in tRNA with small D-loops (seven nucleotides) and large D-loops (12 nucleotides), and in both type I and II tRNA.

In studies with the *H. volcanii* TGT enzyme, incorporation of $preQ_0$ occurred specifically at position-15, and only when a guanosine occupied that position as anticipated [56]. And in experiments probing the gross structural requirements for tRNA recognition both archaeal and bacterial tRNA functioned as efficient substrates, but not eukaryotic tRNA (unfractionated bovine tRNA) [56]. This appeared to implicate some structural feature essential for recognition by the enzyme that was absent in eukaryal tRNA, or alternatively, some unique structural feature of eukaryal tRNA that served in this case as an anti-determinant. However, subsequent investigation of the recombinant enzyme from *M. jannaschii* revealed that tRNA from all three kingdoms served as effective substrates for TGT, as did unmodified tRNA transcripts generated from in vitro transcription reactions [57]. In more recent experiments with the recombinant enzyme from *Pyrococcus horikoshii* [68] and a variety of synthetic RNA substrates, the enzyme was most active with RNA substrates that lacked any substantive tertiary structure. In fact, maximal activity was observed at 100 °C, a temperature at which the RNA substrates would lack both secondary and tertiary structure. These observations are consistent with a requirement for only a guanine at the 15th position of an RNA oligomer, but are at odds with the data from direct sequencing of archaeal tRNA (vide supra). Such limited RNA structural requirements would imply that archaeosine modification occurred as one of the first modifying events during tRNA maturation in Archaea, consistent with a role in the structural stabilization of tRNA (vide supra).

3.3. Catalytic mechanism of the TGT enzymes

The β-configuration of the glycosidic bond in both queuosine and archaeosine, identical with the canonical nucleosides, suggested that the TGTs utilize either an S_N1 or a double-displacement (two S_N2 reactions) mechanism for the base-exchange reaction. Band-shift experiments with both the *E. coli* [86] and *Z. mobilis* [87] enzymes were consistent with a covalent intermediate in the reaction and thus supported a double-displacement mechanism. Although data from a recombinant *E. coli* TGT mutant (S90A) were interpreted to implicate Ser^{90} as the catalytic nucleophile in such a mechanism [86], the X-ray crystal structure of TGT from *Z. mobilis* revealed that the position of this residue in the active-site was more compatible with a role in substrate binding and orientation [74]. The crystal structure further revealed that Asp^{102} (Asp^{89} in *E. coli* numbering) was in an ideal position to serve as the

catalytic nucleophile, consistent with the observation that the carboxylate from aspartate or glutamate serves as the nucleophile in retaining glycosidases and nucleoside hydrolases, enzymes that perform chemically similar reactions. This aspartate is absolutely conserved in all TGT sequences reported to date (>25), and mutagenesis experiments in which the aspartate was changed to alanine in the *Z. mobilis* [87], *E. coli* [88], and *M. jannaschii* [89] enzymes resulted in mutants exhibiting significantly reduced enzymatic activity ($\sim 10^{-3}$ activity of wild-type) and no ability to form a covalent complex with tRNA. Similar results were also observed with the asparagine and cysteine mutants [88]. In contrast, the *E. coli* [88] and *M. jannaschii* [90] glutamate mutants (D89E) retain substantial catalytic activity, and are capable of forming a covalent intermediate with the tRNA. Thus, all of the structural and biochemical data are consistent with the carboxylate of Asp^{89} (*E. coli* numbering) serving as the nucleophile in a double-displacement mechanism.

The basic elements of the mechanism are outlined in Fig. 3. In this mechanism tRNA binds to the enzyme first to form an enzyme/tRNA complex, followed by nucleophilic attack of the aspartyl carboxylate at the glycosidic carbon to give a glycosylated enzyme intermediate. In keeping with the results observed for the β-retaining glycosidases [91] and the nucleoside hydrolases [92,93], this step would be predicted to involve a transition state with significant oxocarbenium ion character.

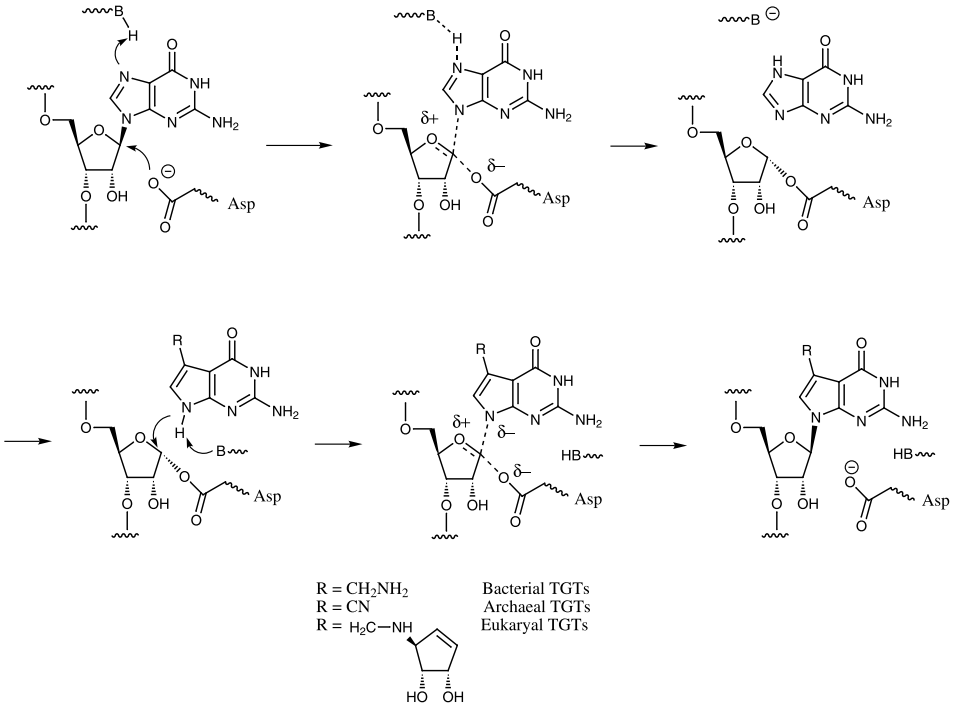

Fig. 3. The proposed chemical mechanism for the reaction catalyzed by the TGT enzymes.

Following glycosylation of the enzyme, guanine leaves the active-site and is replaced by $preQ_1$, $preQ_0$, or queuine depending on the phylogenetic origin of the enzyme. Nucleophilic attack of N9 of the modified base at the glycosidic carbon, again through a transition state with oxocarbenium ion-like character, generates the modified tRNA, which dissociates from the enzyme.

While the broad strokes of the mechanism are now generally understood, it is unclear what strategies are employed by the enzymes to activate guanine for elimination and the incoming modified base for nucleophilic attack. A common feature in the mechanism of enzymatic transglycosylation reactions is the presence of an acid/base residue that serves to protonate the leaving group on the substrate so as to facilitate glycosidic bond cleavage, followed by deprotonation of the second substrate to facilitate formation of the new glycosidic bond in the product [94]. In reactions involving cleavage of the glycosidic bond in purine nucleosides N7 is the most efficient site for protonation, but protonation can also occur at either N1 or N3 in adenosine, or the carbonyl group in guanosine. Importantly, the weak nucleophilicity of N9 due to delocalization of the lone electron pair renders it unavailable for protonation, and also requires that the new C–N glycosidic bond formed in the second step of the reaction arise from the nitrogen electron pair orthogonal to the plane of the purine (or 7-deazapurine) ring, and therefore suggests that deprotonation of N9 is required prior to nucleophilic attack (i.e., a transition state in which deprotonation of N9 occurs simultaneously with C–N bond formation via the N9 lone pair is not energetically accessible). Kinetic studies with the *E. coli* TGT showed a correlation of the N9 pK_a in a variety of nucleobase analogs with V_{max} and suggested a mechanism in which deprotonation at N9 of the nucleobase substrate was partially rate limiting [73]. In addition, 7-fluoromethyl-7-deazaguanine has been shown to be an efficient mechanism-based inhibitior (partition ratio of $\sim$7) of the *E. coli* TGT [95], presumably operating through an elimination addition mechanism that would be facilitated by deprotonation at N9. Interestingly however, Romier et al. [69,74] have reported that the crystal structure of the *Z. mobilis* TGT reveals no acid/base residues present in the vicinity of Asp^{102} that could participate in these types of interactions. Whether this is indicative of: (1) a mechanism in which intervening water molecules interact with more distant acid/base residue(s) to accomplish activation; (2) a conformational change that occurs upon tRNA binding that brings acid/base residue(s) into close proximity; or (3) a new mechanism for activation of these substrates, is currently under active investigation.

4. The QueA enzyme

The enzyme *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA) catalyzes the penultimate step in the biosynthesis of queuosine, the formation of oQ via the addition of an epoxycyclopentandiol ring to $preQ_1$ (Fig. 4). The recombinant enzyme was isolated and its activity characterized [53] following the identification of an operon in *E. coli* containing the *tgt* gene along with three unidentified ORFs, one of which (designated *queA*) complemented a mutation in Q biosynthesis

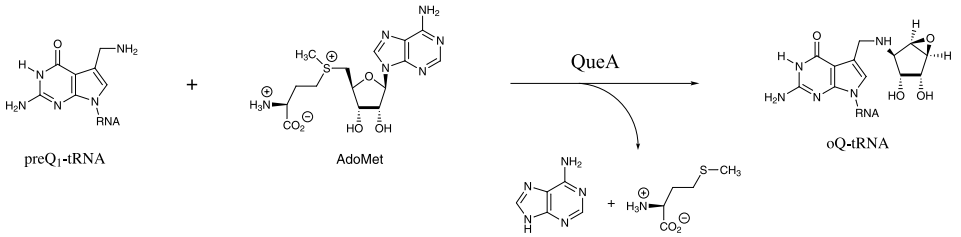

Fig. 4. The reaction catalyzed by the enzyme QueA.

after the TGT step [96]. Remarkably, the epoxycyclopentandiol ring of oQ was found to originate from the ribosyl moiety of *S*-adenosylmethionine (AdoMet) [49,53], the first example of the stoichiometric use of AdoMet as a "ribosyl" donor in an enzymatic reaction. The role of AdoMet in this step clarified earlier studies in which methionine was implicated in an indirect role in queuosine biosynthesis [97,98]. The reaction itself is unprecedented in biological systems, and includes the elimination of methionine and adenine from AdoMet, the transfer of the ribosyl moiety to the tRNA, and its rearrangement to form an epoxy-carbocycle [49,53].

A detailed characterization of the enzyme revealed that it has an unusually high pH optimum (pH 8.7), exhibits no specific metal-ion dependence, and is activated by various organic acids and phosphate [99]. Like the bacterial TGT, the enzyme does not require the full tRNA for substrate recognition [100], and therefore can utilize as substrates mini-helix RNAs corresponding to the anticodon stem-loop that contain $preQ_1$ at the appropriate position [53,100] with only minor changes in the values of the kinetic parameters [99]. Also like the TGTs, the enzyme is not a particularly efficient catalyst, exhibiting a $k_{cat} = 2.5\ min^{-1}$ and $k_{cat}/K_M = 1.1\ \mu M^{-1}\ min^{-1}$ (with $tRNA^{Tyr}$). Unlike TGT, QueA catalysis does not appear to involve a covalent association between the enzyme and the tRNA substrate [100], a reasonable finding given that there is no chemical rationale for a covalent enzyme-tRNA intermediate in the reaction (vide infra). This is also supported by the observation that the enzyme follows an ordered sequential kinetic mechanism [101].

4.1. Catalytic mechanism of QueA

It is not obvious from the structural changes that accompany ribosyl transfer and isomerization from AdoMet to oQ how the carbons in oQ correlate with the carbons in AdoMet. Mechanistic considerations of the QueA reaction first lead to a proposal in which nucleophilic attack by the aminomethyl group of $preQ_1$ at C1′ of AdoMet initiated the reaction [49], a proposal clearly based on the precedence of enzyme-catalyzed transglycosylation reactions [102]. Importantly, such a process establishes direct correspondence between C1′ of AdoMet and C1″ of the epoxycyclopentandiol ring of oQ. However, if one makes the assumption that the *cis*-diol present in AdoMet remains unchanged in the conversion to oQ, then the absolute stereochemistry of oQ and AdoMet renders C–N bond formation at C1′ of AdoMet untenable

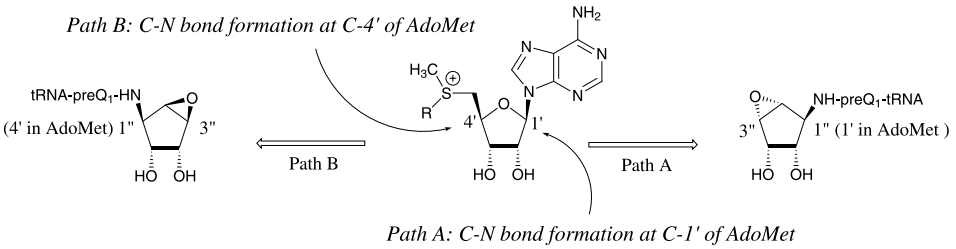

Fig. 5. Regio- and stereochemical constraints on carbon–nitrogen bond formation in the QueA catalyzed conversion of AdoMet to oQ when it is assumed that the diol remains unchanged in the conversion of AdoMet to oQ. (A) The relative stereochemistry of the newly formed C–N bond is shown *anti* to the diol since reaction occurs at C-1′ of AdoMet, and a unique stereochemistry for this carbon is not dictated by the structure of AdoMet. The relative stereochemistry of the epoxide is *syn* to the diol due to the *S* absolute stereochemistry at C-4′ of AdoMet, a carbon which would not be expected to undergo any changes in bonding during the conversion of AdoMet to oQ. Note however that if the stereochemistry at C-4′ did invert, the epoxycarbocycle formed would be *enantiometric* to the epoxycarbocycle found in oQ. (B). Both the newly formed C–N bond and the epoxide are shown *anti* to the diol since bonding changes occur at the relevant carbons, and a unique stereochemistry is not imposed at these carbons. The correct chemical structure of the epoxycarbocyclic ring of oQ is as shown on the left (see also Fig. 1).

(Fig. 5, path A). Instead, C4′ of AdoMet is implicated as the site of C–N bond formation (Fig. 5, path B). On the other hand, if the constraint of leaving the diol intact is removed, then in principle C–N bond formation could occur at any of the five carbons in the ribosyl moiety of AdoMet. Clearly then, establishing the regiochemistry of C–N bond formation was fundamental to any mechanistic exploration of the reaction.

One approach to addressing this problem is through the utilization of [^{13}C]AdoMet specifically labeled in the ribose ring in the QueA catalyzed reaction, followed by identification of the isotopically enriched carbon in the oQ produced in the reaction. We made use of the commercial availability of [1-^{13}C]ribose to synthesize [1′-^{13}C]AdoMet for this purpose. Using a mini-helix RNA substrate [53] prepared by in vitro run-off transcription [103] and modified with preQ$_1$ by reaction with recombinant *E. coli* Tgt in situ, [^{13}C]oQ modified RNA was produced in a reaction with [1′-^{13}C]AdoMet. After digestion of the RNA and dephosphorylation of the mononucleotides, the [^{13}C]oQ was isolated and subjected to NMR analysis [52]. Because oQ had never been isolated in quantities sufficient for the acquisition of a high-quality $\{^{13}C\}$ NMR spectrum, the location of the enriched carbon was instead identified through the effect of ^{1}H–^{13}C coupling on the $\{^{1}H\}$ NMR spectrum by calculating a difference spectrum from the ^{1}H–^{13}C coupled and ^{13}C-decoupled $\{^{1}H\}$ NMR spectra [52]. The results from these experiments clearly eliminated C1″ as the site of isotopic enrichment, and instead were consistent with enrichment at C3″ of oQ, implicating C4′ of AdoMet as the probable site of C–N bond formation.

Based on these results and the structural analysis in Fig. 5, we proposed the chemical mechanism outlined in Fig. 6 for the reaction catalyzed by QueA. In the first step enzyme catalyzed deprotonation of AdoMet at C5′ generates the sulfonium ylide **I**,

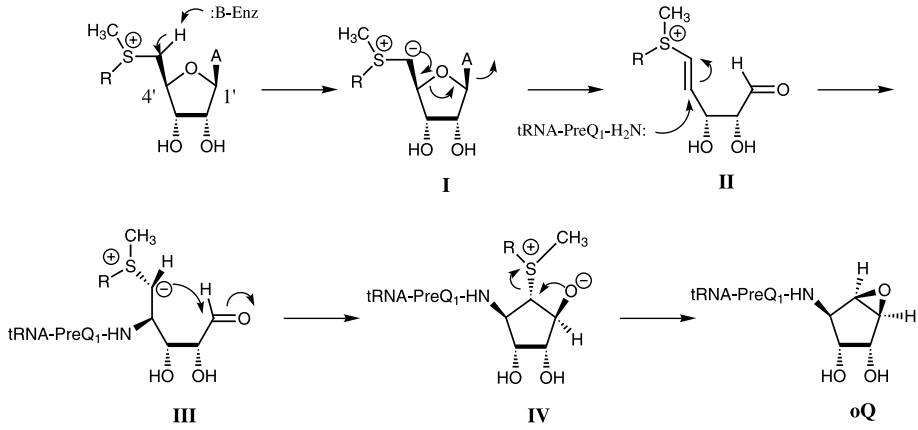

Fig. 6. The proposed chemical mechanism for the QueA catalyzed reaction.

which can collapse to the vinyl sulfonium **II** by opening of the ribose ring with concomitant elimination of adenine. Nucleophilic attack of the $preQ_1$ amine at the *re*-face of C4′ then generates the new sulfonium ylide **III**, which can subsequently attack the *re*-face of the C1′ (C3″ in oQ numbering) aldehyde to give the alkoxy-carbocycle **IV**. Intramolecular S_N2 attack of the alkoxy oxygen on the adjacent carbon and elimination of methionine then gives oQ. The proposed chemical mechanism is consistent with the kinetic data [101], which showed that the order of product release from the enzyme was first adenine, then methionine, followed by oQ-tRNA, and provides a logical series of chemical steps to account for the observed reaction. Good chemical precedence exists for the basic features of the proposal [104,105]; in fact it has been proposed that deprotonation at C5′ of AdoMet followed by ring opening to the vinyl sulfonium **II** and loss of adenine is the mechanism for the conversion of AdoMet to pentosylmethionines in solution [106]. Importantly, the mechanistic proposal reveals a chemical imperative for the unique utilization of AdoMet as a ribosyl donor; the increased acidity of the 5′-methylene hydrogen's of AdoMet ($pK_a = \sim 23$) due to the adjacent sulfonium ion [104] makes the key carbon–carbon bond construction between C5′ and C1′ of AdoMet possible.

5. Future directions

While significant progress has been made in recent years in elucidating details of the biosynthesis of queuosine and archaeosine, the majority of the biosynthetic pathways still remain unknown. It is clear from a cursory examination of the structural changes that occur in the biosynthesis of these nucleosides that the steps yet to be discovered are likely to be as remarkable as those already known. For example, while the cyclohydrolases offer good biochemical precedent for the incorporation of the ribosyl moiety of GTP into the 7-deazapurine ring, no biochemical precedent exists for

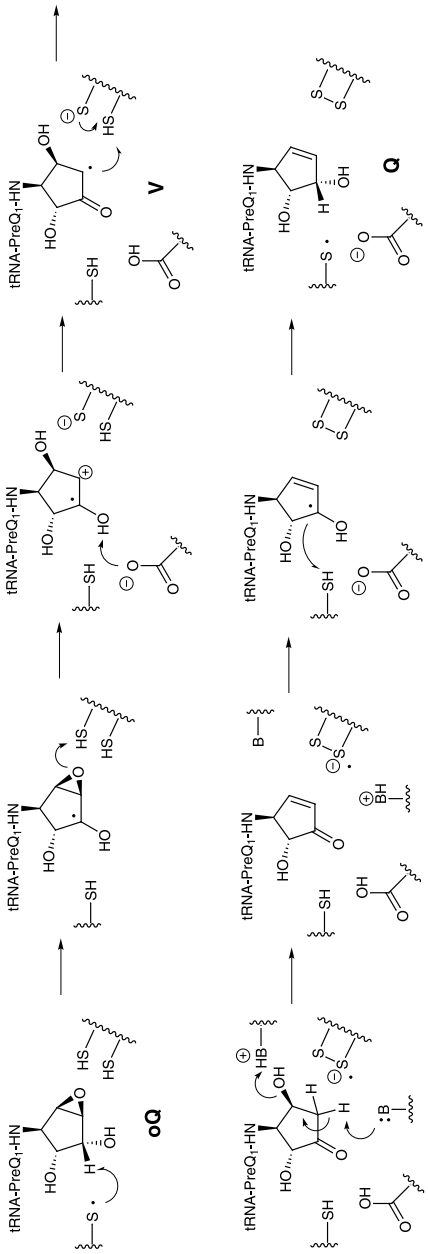

Fig. 7. A mechanistic proposal for the conversion of epoxyqueuosine to queuosine.

the elimination of N7, or alternatively, it's transfer to the exocyclic carbon in $preQ_0$. And the final step in queuosine biosynthesis, the apparent B_{12}-dependent reduction of an epoxide to an alkene (epoxyqueuosine to queuosine), is particularly intriguing; were this reaction not dependent on B_{12} one could envision a process analogous to that proposed for vitamin K epoxide reductase [107,108], in which opening of the epoxide from nucleophilic attack of an active site thiol followed by reductive elimination of water would directly produce oQ. However, the requirement for B_{12} implicates the involvement of radical chemistry, and close inspection of the reaction reveals that it is strikingly reminiscent of the reaction catalyzed by the ribonucleotide reductases (RNR) [109].

The first step in ribonucleotide reduction involves hydrogen atom transfer from the 3′-carbon of the ribonucleotide to a protein radical (a thiyl radical [110] for the B_{12}-dependent RNR) to generate a substrate radical. This step is crucial as it allows the subsequent formation of a 2′-carbocation after protonation and departure of the 2′-hydroxyl [109]. These events can be exactly replicated in the reduction of oQ where protonation and opening of the epoxide (Fig. 7) replaces protonation and departure of the hydroxyl. As in RNR these steps could be mediated by one of two thiols present in the active site, which react further to transfer a hydrogen atom to an α-radical carbonyl intermediate (**V**) and form the disulfide radical anion, again exactly as proposed in the RNR catalyzed reactions. At this point the chemistry of oQ reduction would briefly diverge from ribonucleotide reduction with elimination of the hydroxyl formed from epoxide opening to give the unsaturated carbonyl. Electron transfer from the disulfide radical anion to the carbonyl provides a substrate radical analogous to RNR, which is then quenched by hydrogen atom transfer from the thiol to give queuosine and the starting thiyl radical. Reduction of the enzyme could then be linked via disulfide exchange reactions to NAD(P)H.

An interesting variant to the mechanism in Fig. 7 involves initial radical formation at the amino carbon (C1″) instead of C4″. This has the advantage that the relative positions of the thiyl radical and the two thiols (on opposite sides of the ring) are identical to that observed in RNR [111], and the formation of an intermediate imine renders the subsequent elimination of water more favorable. Mechanisms other than that outlined in Fig. 7 can also be envisioned for this transformation, but the requirement for B_{12} render any proposals that do not involve the initial formation of a substrate radical suspect. The potential similarity of the chemistry involved in ribonucleotide and oQ reduction raises intriguing questions about the evolutionary relationship between the ribonucleotide reductases and the putative epoxyqueuosine reductase, and the identification of this reductase and elucidation of its structure may provide insight into the problem of unraveling the evolutionary history of the RNR family of enzymes [109].

Clearly there is much work yet to be done to elucidate the biosynthetic pathways leading to queuosine and archaeosine, as well as to better understand the chemical steps in the TGT and QueA reactions. These problems are currently being addressed in a number of labs around the world, and the next decade should reveal a wealth of interesting chemistry as the results of this work come to fruition.

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