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An Embarrassment of Riches: The Enzymology of RNA Modification

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Summary of Recent Advances

The maturation of transfer RNA (tRNA) involves extensive chemical modification of the constituent nucleosides, resulting in the formation of structurally diverse nucleosides. Many of the pathways to these modified nucleosides are characterized by chemically complex transformations, some of which are unprecedented in other areas of biology. To illustrate the scope of the field, recent progress in understanding the enzymology leading to the formation of 2 distinct classes of modified nucleosides are reviewed, the thiouridines and queuosine, a 7-deazaguanosine. In particular, recent data validating the involvement of several proposed intermediates in the formation of thiouridines are discussed, including 2 key enzyme intermediates and the activated tRNA intermediate. The discovery and mechanistic characterization of a new enzyme activity in the queuosine pathway is discussed.

Modification of RNA

The post-transcriptional processing of RNA involves a number of events essential for tRNA maturation [1]. From an enzymological perspective, the phenomenon of nucleoside modification is perhaps the most remarkable of these events, and results in a wealth of structural changes to the canonical nucleosides (Figure 1). While a number of RNA species are subject to nucleoside modification, it is only in transfer RNA (tRNA) that a rich structural diversity is realized. Over 100 modified nucleosides have been structurally characterized [2,3*], many of which exhibit extremely deep evolutionary conservation. The chemistry leading to nucleoside modification varies from simple methylation of the base or ribose ring to extensive "hypermodification" of the canonical bases, the latter of which can result in radical structural changes and involve multiple enzymatic steps to complete. Nucleoside modification is known to play a variety of roles necessary for proper physiological functioning of tRNA [1,4], including tertiary structural stabilization, introduction of recognition determinants, environmental sensing, and modulation of translational efficiency and fidelity.

The Enzymes of RNA Modification

While many RNA modifying enzymes had been discovered and characterized to various extents prior to the mid-1990's, in general modified nucleoside biosynthesis had not undergone comprehensive investigation due to the resistance of these enzymes to traditional biochemical and genetic characterization. For example, the design of assays for simply observing a desired enzymatic transformation was complicated by the difficulty of obtaining the appropriate tRNA substrates, the presence of endogenous RNases in cell-free extracts that degrade the RNA substrates and products, and the typically low abundance of the enzymes involved. Likewise, genetic approaches to enzyme discovery were hindered by the lack of specific phenotypes in

many mutants and the fact that unambiguous identification of a gene involved in RNA modification is ultimately dependent on determining the presence or absence of the specific modified nucleoside in tRNA, a laborious and technically challenging process, especially when working with large libraries of mutants. Consequently, until recently it was estimated that over 50% of the genes encoding tRNA modification enzymes still remained unknown [5]. However, the development of *in vitro* transcription in the late 1980's [6] and more efficient methods for the isolation of *in vivo* synthesized tRNA set the stage for researchers to exploit the flood of information unleashed by the genome sequencing projects for the discovery of new tRNA modification enzymes [7*]. Indeed, since 2000 the application of various genome-mining techniques has resulted in the discovery of at least 50 new genes encoding tRNA modifying enzymes [7*].

Due to the diversity of chemistries inherent in nucleoside modification the enzymology is especially rich, and coupled with the extraordinary progress that has occurred in recent years the choice of topics to cover in a short review is particularly difficult. Two quite different systems are the focus of this review; the sulfur transfer associated with the formation of thiouridines, and the novel nitrile reductase recently discovered in the pathway to the 7-deazaguanosine modified nucleoside queuosine. These systems are chosen to illustrate both the breadth of the field and the disparate problems currently being pursued. Nevertheless, they are also united by the important role that active-site cysteine residues play in catalysis, the central role of redox processes, and the potential involvement of covalent enzyme-substrate intermediates in the catalytic mechanisms.

The incorporation of sulfur to form thiouridines

Sulfur appears in tRNA nucleosides at C2 and C4 of uridine, C2 of cytidine, and C2 of adenosine. These collectively form the basis for 17 unique sulfur containing modified nucleosides present in various tRNA [2,3*]. Sulfur insertion into nucleosides occurs through 2 distinct mechanisms, an oxidative pathway in which the sulfur donor is a protein bound iron-sulfur cluster [8,9], and a non-oxidative pathway in which sulfur is incorporated at the expense of an oxygen atom [10,11]. The former, leading to the thiomethyl derivatives of adenosine, is also observed in other systems in which sulfur is inserted into unactivated C-H bonds, such as biotin and lipoic acid synthases.

The requirements for the synthesis of 2- and 4-thiouridine (s^2U and s^4U) are similar [11], and include ATP to activate the C-2 or C-4 oxygen atom, the cysteine desulfurase IscS as the source of the sulfur atom, and an enzyme to orchestrate the activation of the nucleoside and insertion of sulfur. The relevant enzymes are MnmA [12] and ThiI [13,14], which are responsible for s^2U and s^4U formation, respectively. While ThiI, IscS, tRNA, and ATP are sufficient for robust s^4U synthesis, this isn't the case for s^2U [12], which has recently been shown to require 5 additional proteins, TusA–E [15**]. These proteins participate in a complex sulfur relay in which the activated sulfur of IscS, presumably as the persulfide, is first transferred to TusA, followed by transfer to TusD, which is part of a heterohexameric complex with TusB and TusC ($\alpha_2\beta_2\gamma_2$), then to TusE, which finally transfers the sulfur to MnmA for incorporation into the tRNA. The rationale for such a complex sulfur transfer pathway is unknown, however the enhanced ability to regulate the delivery of sulfur to multiple pathways is one obvious explanation [11,15**], although no evidence is available yet to support that hypothesis.

A molecular mechanism for sulfur transfer from IscS to tRNA was first proposed by Mueller and colleagues in 2000 [14] when they demonstrated that, consistent with the presence of a "rhodanese" domain in ThiI, ThiI was capable of supporting sulfur transfer from thiosulfate to cyanide to form thiocyanide, suggesting that the catalytic mechanism for thiouridine formation might involve a protein persulfide. A persulfide intermediate was also implicated in a

simultaneous report by Lauhon and colleagues [13] in which they demonstrated sulfur transfer from IscS to ThiI. The proposed catalytic mechanism, as originally formulated and elaborated on shortly thereafter [16], is also relevant for the formation of 2-thiouridine, and is shown in figure 2. The first step is sulfur transfer from IscS to give a protein persulfide intermediate (**I**, localized on Cys^{456} of *E. coli* ThiI), followed by binding of the tRNA and activation of the uridine oxygen by ATP to form an adenylated intermediate. Activation as the adenylate was proposed based on the presence in ThiI of a "P-loop" motif [17], a characteristic motif found in enzymes that activate carbonyl oxygens for nucleophilic substitution by adenylation with ATP [18,19]. The critical sulfur transfer to the tRNA was envisaged to occur through either of 2 potential routes, direct attack on the adenylated uridine by the terminal sulfur of the persulfide or by free bisulfide generated after attack by another active-site sulfur (Cys^{344} of *E. coli* ThiI) on the internal sulfur of the persulfide. Subsequent decomposition of either tetrahedral intermediate then liberates AMP and thiouridine, with ThiI in the oxidized disulfide form (**II**). Reduction of the disulfide, either with added reductant *in vitro* or with an endogenous redox system *in vivo*, regenerates the dithiol form of the enzyme, ready to undergo another trans-persulfidation reaction with IscS. While indirect evidence for the overall elements of the reaction has accumulated over the last decade, it wasn't until recently that definitive evidence was reported for the existence of several key intermediates in the reaction, specifically the persulfide and disulfide forms of ThiI, and the adenylated uridine moiety.

Although the nature of the activated sulfur on ThiI and MnmA was proposed to be a persulfide, IscS was shown to readily form trisulfides and higher order polysulfides on another protein partner, the iron-sulfur cluster "scaffold protein" IscU [20], complicating the interpretation of the ThiI and MnmA data. Working with a mutant ThiI in which 4 of the 5 Cys residues had been changed to Ala, leaving Cys^{456}, the putative site of persulfide formation, unchanged, Mueller and colleagues showed [21**] that after the sulfur transfer step from IscS the mutant ThiI could be labeled with *I*-AEDANS, a fluorescent derivative of iodoacetamide, and that treatment of the labeled protein with a reductant such as DTT liberated the fluorescent label. These data demonstrated the nucleophilic nature of the activated sulfur on ThiI, and that the label and protein were linked by an S-S bond. To identify unambiguously the chemical structure of the linkage the protein-label adduct and the reduced protein were separately subjected to trypic digests and MALDI-MS analysis. While the mass of the peptide containing Cys^{456} from the reduced protein corresponded as expected to that with a free thiol, the mass of the peptide bearing the label was consistent only with the presence of a disulfide linkage, clearly demonstrating that a persulfide was the relevant form of activated sulfur on ThiI during catalysis.

In the absence of exogenous reductants such as DTT ThiI is only able to carry out a single turnover [16,22], and fewer free thiols are detected after turnover, phenomena consistent with the formation of an enzyme disulfide bond during catalysis as depicted in figure 2. Working with another mutant ThiI in which all the Cys residues except Cys^{456} and Cys^{344} were mutated to Ala [21**], Mueller and colleagues carried out single turnover assays in the absence of DTT, followed as above by tryptic digests and MALDI-MS analysis. In this case an intramolecular disulfide-linked peptide containing Cys^{344} and Cys^{456} was easily detected. Furthermore, this peptide disappeared after treatment with DTT and iodoacetamide, and was replaced by 2 separate peptides, each containing one of the alkylated Cys residues (Cys^{344} or Cys^{456}). These data firmly establish the enzyme disulfide as a key intermediate in the catalytic mechanism.

Finally, in a beautiful display of crystallographic prowess, Nureki and colleagues reported 3 crystal structures of the MnmA/$tRNA^{Glu}$ complex [23**] that they dubbed "snapshots" of the sulfuration reaction, interpreting the structures as an initial binding state (form I), a prereaction state (form II), and the adenylated intermediate state (form III). Notably, in the last structure

they were able to capture the activated uridine in an adenylated form, confirming the presence of yet another putative intermediate on the reaction path.

Although previous *in vitro* sulfur transfer experiments failed to detect ^{35}S-labeled MnmA [15**], leaving open the question of whether sulfur transfer to tRNA occurred from MnmA (as with *E. coli* ThiI) or from TusE, the crystal structures clearly implicate Cys^{199} of MnmA as the site of persulfide formation, and Cys^{102} as the second Cys residue required for transfer and liberation of the sulfur atom via disulfide bond formation with Cys^{199}. Furthermore, the authors also provided supporting biochemical data demonstrating ^{35}S-labeling of MnmA in *in vitro* assays. The authors note that in going from the initial binding state (form I) to the prereaction (form II) and adenylated states (form III) the active-site of MnmA forms a closed conformation in which the activated uridine is protected from solvent. This occurs as a result of the reorganization of a helix and a preceding disordered region in form I to a loop, a β-hairpin, and a short helix in forms II and III. Assuming that the active-site architecture of MnmA and ThiI are similar, the formation of a solvent excluded chamber could explain the exceedingly high concentrations of bisulfide (50 mM) necessary to support efficient ThiI catalysis of s^{4}U formation in the absence of IscS [24].

While the crystallographic data would appear to support the operation of the free bisulfide mechanism since the formation of a closed active-site is considered essential to prevent the easily diffusible bisulfide from escaping the active-site, both mechanisms benefit from a closed active-site as this better protects the reactive adenylated uridine. Thus, none of the recent data reported for MnmA or ThiI allows a definitive conclusion regarding which of the 2 putative mechanisms for sulfur delivery is occurring, leaving this part of the puzzle still unresolved. Nonetheless, our understanding of the complicated process of tRNA sulfuration is now significantly deeper than just a few years ago, and if the recent pace of progress in this area is an indicator for the future, this issue will be resolved soon.

QueF, a biological nitrile reductase

The enzyme QueF (Figure 3) occurs in the biosynthesis of the hypermodified nucleoside queuosine [25*,26**], a pathway rich in new chemistries. Queuosine and the related modified nucleoside archaeosine share a 7-deazaquanine core structure, but differ in the substituent at the 7-position [27]. The biosynthesis of these nucleosides is unique in the realm of RNA modification in that a substantial portion of the pathway occurs at the level of the free base [27], outside the context of the RNA. Thus, the early portion of the pathway leads to a modified precursor base that is subsequently inserted into the tRNA in a transglycosylation reaction [27] with the concomitant elimination of the genetically encoded base (a guanine in both cases).

Although annotated in bacterial genomes as a type I-like GTP cyclohydrolase (GCYH-I), and initially hypothesized to be the first enzyme in the queuosine pathway [26**], the QueF enzyme was shown instead to function as a nitrile reductase [25*], converting the advanced intermediate 7-cyano-7-deazaguanine (preQ$_0$) to 7-aminomethyl-7-deazaguanine (preQ$_1$). Notably, this is the only known example of an enzyme-catalyzed reduction of a nitrile metabolite to the corresponding amine. Despite the misannotation as a cyclohydrolase-like enzyme, sequence and structural analyses were consistent with QueF belonging to the tunneling-fold (T-told) protein superfamily [28], the same structural superfamily to which GCYH-I belongs. This led to the construction of a homology model for QueF [29*] based on the GCYH I structure from *E. coli* [30], setting the stage for subsequent detailed mechanistic studies.

The reduction of a nitrile to an amine is a 4-electron process, and the electron donor in the QueF reaction is NADPH, which as a 2-electron reductant is required in a 2:1 stoichiometry to the substrate preQ$_0$. Because NADPH is the only cofactor required in the conversion and the protein does not possess any inherent redox capacity, the reaction must occur in a stepwise

fashion and necessarily involves the intermediacy of an imine, the 2-electron reduced product. This presents a chemical challenge in that the imine should be especially reactive towards nucleophilic attack by water, potentially leading to hydrolytic loss of the nitrogen atom. This could conceivably occur in the enzyme active-site itself after departure of the product $NADP^+$ generated in the first reductive cycle, or free in solution if the intermediate escaped the active-site prior to binding of the second NADPH.

So how does the enzyme catalyze the overall chemical transformation, and in particular, what strategy is employed to prevent the unproductive loss of the nitrogen atom through hydrolysis? The answer appears to be through a clever form of "protecting group" chemistry, in which the imine is temporarily masked as a covalent thiohemiaminal adduct. This adduct arises as an obligate intermediate in the catalytic mechanism due to the initial formation of a covalent thioimide adduct, which serves as the actual substrate for the first reduction cycle.

Insight into the catalytic mechanism of QueF began by considering the sequence conservation within the QueF family and the homology model metioned above [29*]. Of particular interest was the presence of a universally conserved cysteine residue (Cys^{55} in the *B. subtilis* enzyme) that aligned with a conserved cysteine residue in the sequences of GCYH-I enzymes. Importantly, in GCYH-I this cysteine is located in the active-site where it serves as a ligand to the essential Zn^{2+} ion, which in turn coordinates the water molecule that is responsible for hydrolytic ring opening at C-8 of GTP in the first step of the cyclohydrolase reaction. The homology model placed Cys^{55} in close proximity to the nitrile group of $preQ_0$, suggesting a direct role in catalysis.

Experiments designed to probe the role of Cys^{55} confirmed an essential role in catalysis [31*]; chemical labeling with iodoacetamide inactivated the enzyme, while labeling in the presence of $preQ_0$ protected the enzyme from inactivation, and conversion of Cys^{55} to serine or alanine via site-directed mutagenesis produced inactive enzyme. However, the key experiment in elucidating the role of Cys^{55} came from spectroscopic analysis of the enzyme in the presence of $preQ_0$ [31*]; titration of the wild-type enzyme, but not the Cys^{55} mutants, with $preQ_0$ was accompanied by the appearance of a new absorption band at 376 nm, consistent with the formation of an α,β-unsaturated thioimide and suggesting the covalent adduct **III** (Figure 3). The absorption reached a plateau when $preQ_0$ became stoichiometric with enzyme, and remained after extended dialysis. When excess NADPH was added to the dialyzed enzyme only 2 equivalents (based on enzyme concentration) were consumed, and reaction with NADPH abolished the absorption at 376 nm. The presence of a covalent adduct also nicely explained the crystallographic behavior of QueF [29*]; in the absence of $preQ_0$ only poorly diffracting crystals formed (>8 Å), but the addition of $preQ_0$ was accompanied by a new crystal form that diffracted to 2.25 Å.

The minimal mechanism consistent with the experimental data is outlined in figure 3, and involves initial binding of $preQ_0$ to the enzyme followed by nucleophilic attack of the thiol of Cys^{55} on the nitrile group of $preQ_0$ to form a thioimide intermediate (**III**). The first NADPH molecule then binds to the enzyme and reduces the thioimide intermediate, giving a new covalent adduct, the thiohemiaminal (**IV**). Release of the oxidized cofactor is followed by binding of the second molecule of NADPH, collapse of the thiohemiaminal to the imine (**V**), and reduction to give $preQ_1$.

With the general features of the QueF mechanism now in place, the elucidation of the molecular details of the reaction, and the potential to reengineer the enzyme for applications in the reduction of other nitrile containing compounds, are now on the horizon.

Conclusions and future propects

Nucleoside modification engenders a wealth of structurally differentiated nucleosides in tRNA, and the elucidation of the enzymology of these processes is occurring at an unprecedented rate. The systems described above, while providing some measure of the scope of the field, are by no means unusual in exhibiting important and compelling chemistries. MiaB, for example, the enzyme responsible for the thiomethylation [32] of isopentenyl adenosine (Figure 4), belongs to the newly described radical SAM superfamily [33], which utilize a [4Fe-4S] cluster and *S*-adenosylmethionine (SAM) to initiate radical chemistry. MiaB, along with biotin and lipoic acid synthases [8], contains 2 [Fe-S] clusters [34**], one apparently for radical initiation and the second as a source of sulfur for sulfur insertion. Radical SAM enzymes have also been discovered recently in queuosine [26**] and wyosine [35**,36*] biosynthesis, and the enzyme in the latter pathway (Tyw1) is responsible for the cryptic construction of the tricyclic ring system of wyosine. The Tyw1 enzyme has recently been crystallized [37,38], and interestingly, it also contains 2 [4Fe-4S] clusters. An *in vitro* activity has not yet been reported, nor is the identity of the donor molecule responsible for the 2 additional carbons known. While pyruvate and acetyl-CoA emerge as potential candidates for this latter role, regardless of the metabolite ultimately implicated this enzyme seems sure to add a significant new twist to the chemistry of the radical SAM family. These are but a few of the systems that are on the cusp of significant new insights into their mechanism of action. Much of the future work in this area will continue to be informed by bioinformatics analysis, both for the discovery of the remaining enzymes involved in modification pathways as well as for insights into the mechanisms, structure, and evolution of these fascinating enzymes.

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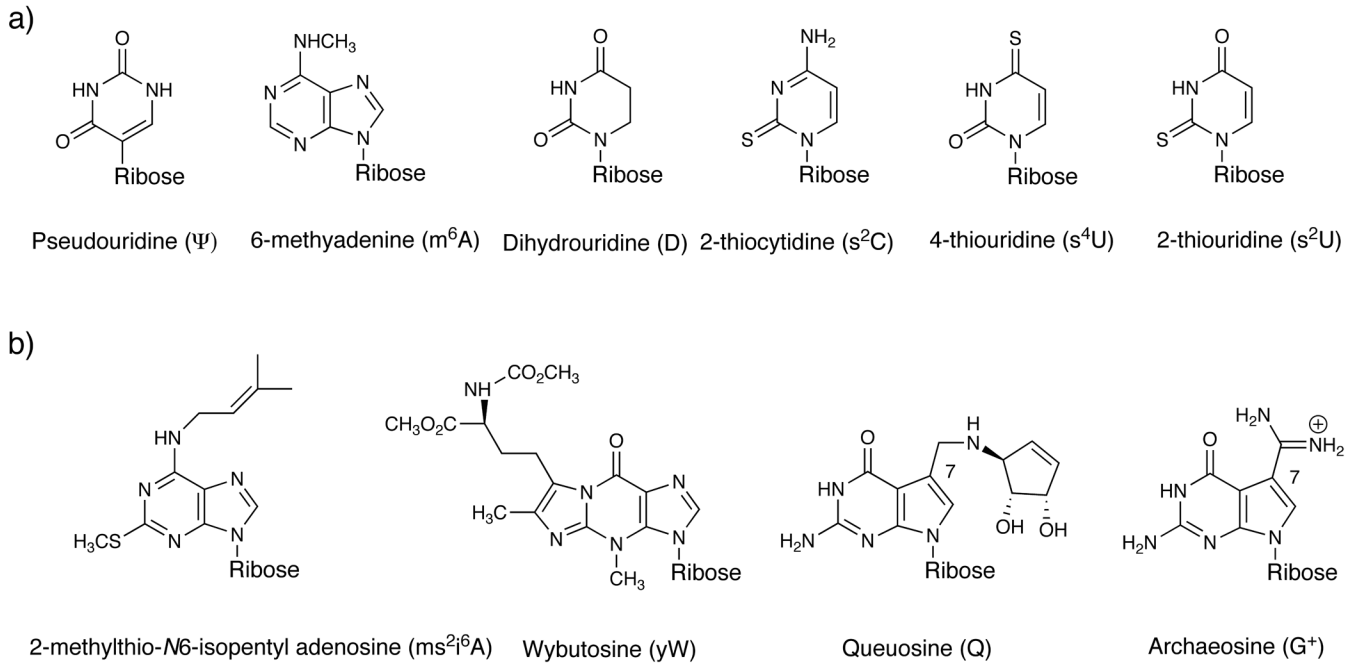

Figure 1.

Examples of modified nucleosides found in tRNA. a) Simple modifications that involve relatively small structural changes to the parent nucleoside. b) Complex or hypermodifications that involve significant structural changes to the parent nucleoside.

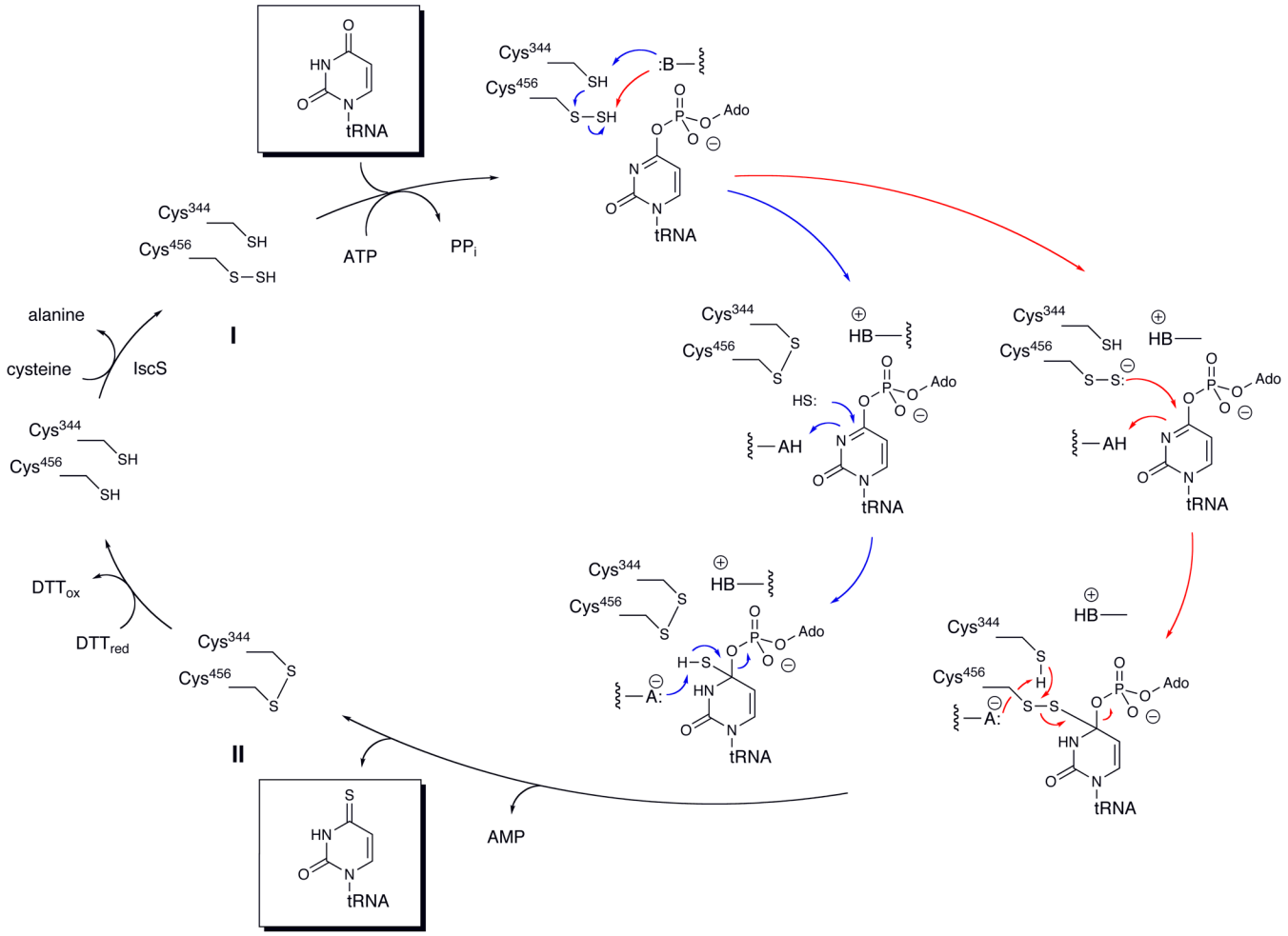

Figure 2.

Proposed mechanism for the formation of thiouridine. The mechanism is explicit for *E. coli* ThiI catalyzed formation of s^4U, but the overall chemistry is relevant for MnmA catalyzed formation of s^2U. Two potential routes to sulfur insertion have been proposed and are compatible with all the experimental data. In a direct sulfur transfer the nucleophilic, terminal sulfur of the enzyme persulfide (shown on Cys^{456}) attacks the activated adenylated uridine (red path) to form a covalent, disulfide linked protein-tRNA adduct. Subsequent nucleophilic attack of a second active-site Cys residue (Cys^{344}) on the Cys-sulfur of the disulfide completes sulfur transfer to the tRNA. In a free bisulfide mechanism to sulfur transfer (blue path) the second active-site Cys residue attacks the enzyme persulfide at the internal sulfur, releasing free bisulfide, which subsequently attacks the activated adenylated uridine. In both routes the breakdown of the tetrahedral intermediates releases AMP and leaves the enzyme in the oxidized disulfide form. Reduction of the disulfide prepares the enzyme for another round of trans-persulfidation from IscS. Although persulfide formation is shown prior to tRNA and ATP binding, formation of the adenylated uridine has been shown to occur with MnmA without sulfur transfer from IscS.

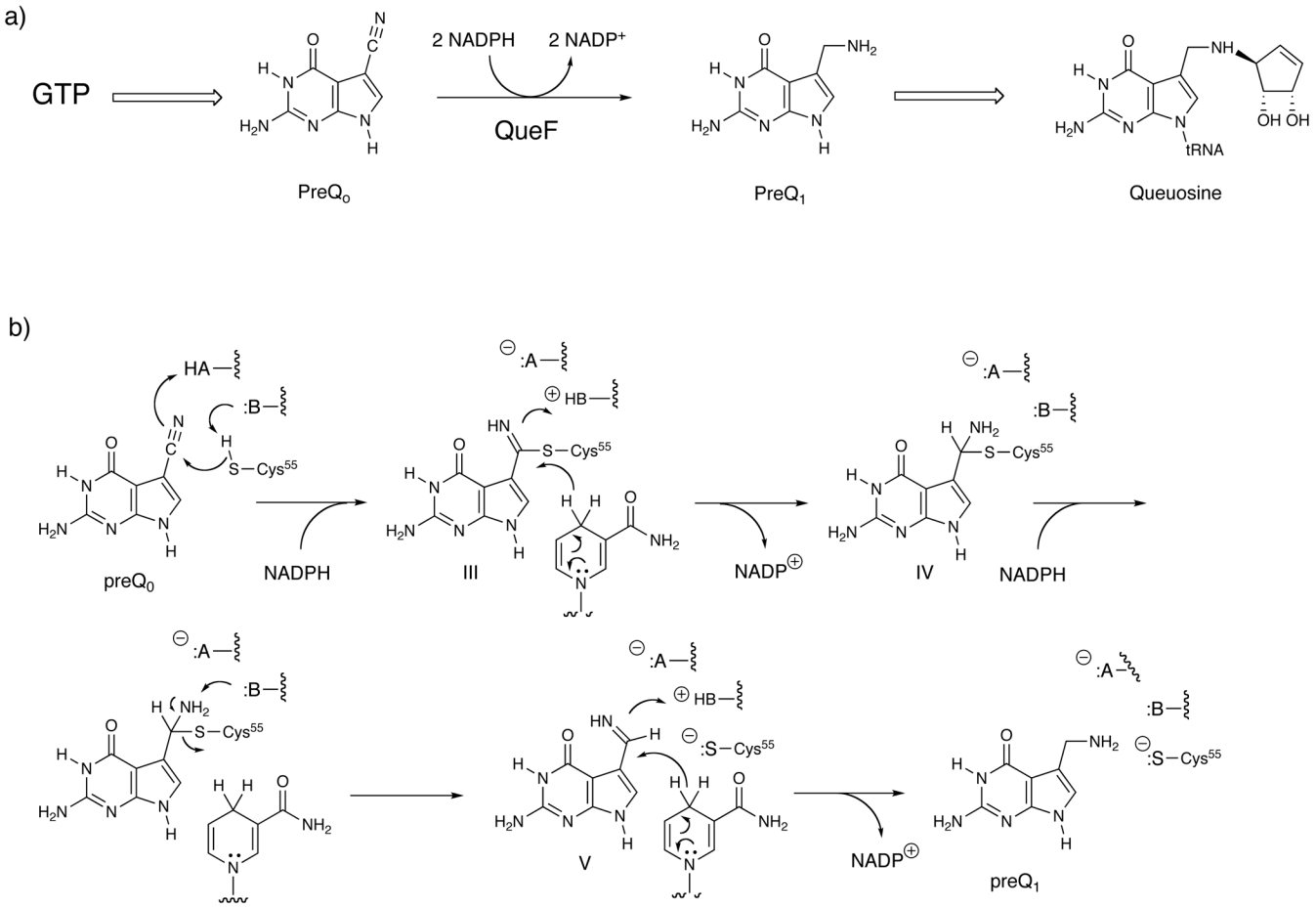

Figure 3.

a) The reaction catalyzed by the QueF enzyme and it's role in the biosynthesis of queuosine. b) The proposed mechanism for reduction of the nitrile group of $preQ_0$ to the aminomethyl group of $preQ_1$. After binding of $preQ_0$ the active-site Cys residue (Cys^{55} in *Bacilis subtilis* QueF) attacks the nitrile group to form a thioimide (**III**). Binding of the first equivalent of NADPH and hydride transfer gives the thiohemiaminal **IV** followed by the dissociation of $NADP^+$. Binding of the second equivalent of NADPH is followed by breakdown of **IV** to give the imine **V**. The second hydride is then transferred to generate $preQ_1$, followed by dissociation of $NADP^+$ and $preQ_1$ from the enzyme. By keeping the intermediate thiohemiaminal form until *after* the binding of the second NADPH hydrolysis of the intermediate imine is avoided.

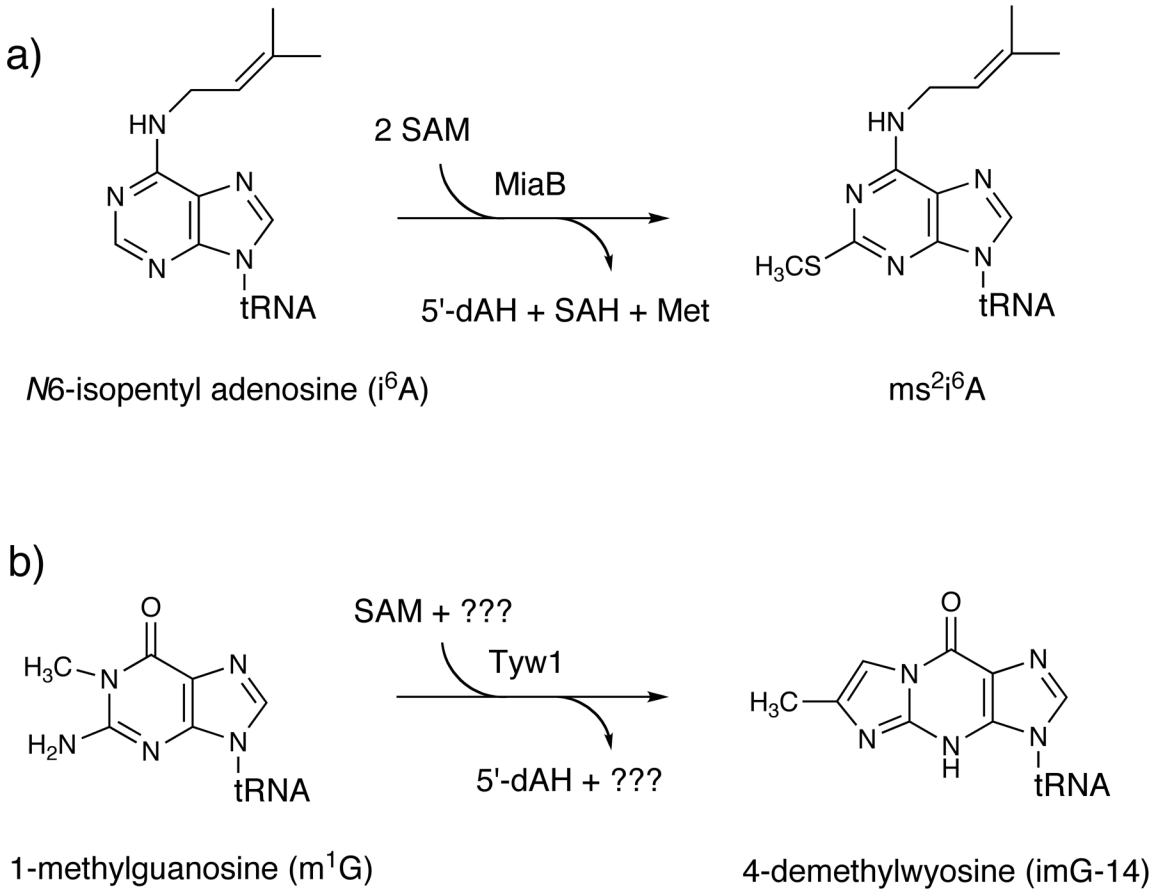

Figure 4.

a) The MiaB catalyzed thiomethylation of i^6A to give ms^2i^6A. b) The reaction catalyzed by the Tyw1 enzyme in the wyosine pathway. The co-substrate(s) in the Tyw1 reaction has not yet been identified.