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RNAs molecules fulfill key roles in the expression and regulation of the genetic information stored within the DNA chromosomes. In addition to the four canonical bases, U, C, A and G, RNAs harbor various chemically modified derivatives which are generated post-transcriptionally by specific enzymes acting directly at the polymer level. More than one hundred naturally occurring modified nucleosides have been identified to date, the largest number of which is found in tRNAs and rRNA. This remarkable biochemical process produces widely diversified RNAs further expanding the functional repertoires of these nucleic acids. Interestingly, several RNA-modifying enzymes use a flavin bioorganic molecule as a coenzyme in RNA modification pathways. Some of these reactions are simple while others are extremely complex using challenging chemistry orchestrated by large flavoenzymatic systems. In this review, we summarize recent knowledges on the flavin-dependent RNA-modifying enzymes and discuss the relevance of their activity within a cellular context.

All living cells invest considerable energy to alter the chemical nature of the elementary components of many RNA polymers (*1, 2*). Non-canonical nucleosides are found in various types of RNA, including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and microRNA (miRNA) (*3, 4*). The studies on RNA modifications have been in a state of latency for some time but with recent development of hypersensitive technical methods for transcriptome analysis and the discovery of new modifications, renewed interest for such biotransformations reemerged with an impressive appetite (*5, 6*). This research field, coined with the term of "epitranscriptomic" is nowadays competing with the well-known epigenetic field related to DNA modifications. In particular, the recent discoveries have made possible to better acknowledge the functional importance of these RNA modifications (*7-10*). Here, by remodeling the chemical nature of the canonical nucleosides, cell disposes a more direct and fastest mechanism to manipulate the existing transcriptome, bypassing the conventional gene expression and regulation mediators such as the activation of transcription factors.

The tRNAs, key molecules linking the genetic code to the amino acids in proteins, are by far the RNA species that carry the greatest number of modifications along with the most impressive chemical diversity (*3, 4, 11*). More than a hundred different types of modified nucleosides have been reported in tRNAs from different organisms spanning the three domains of life and others will certainly be discovered in the near future. This overwhelming diversity is enigmatic because none of the biological macromolecules is subjected to such intense chemical reshaping. Up to 25% of all nucleosides in eukaryotic tRNAs are not A, U, G or C. The modifications serve various cellular purposes as for instance: tRNA discrimination, maintenance of translation efficiency and fidelity (*12*), minimization of misfolding and aggregation of nascent protein (*13*), stability (*14*) and protection of tRNA against its degradation by nucleases (*15*). Beyond these rather conventional functions, tRNA modifications have recently been implicated in regulatory processes under stress conditions (*16*).

A large proportion of cell's genome is devoted to encoding RNA-modifying enzymes (~ 1 to 10% in a given organism). In general, these enzymes modify their nucleotidic substrate directly on the RNA polymer (*17*). This enzymatic richness necessarily implies the existence of sophisticated chemistries, which are likely more frequent in complex mechanisms of RNA-modifying enzymes assisted by inorganic or organic redox cofactors such as iron-sulfur clusters, flavins or acetyl-coenzyme A (*18*). Presently, there are seven RNA modifications that depend on the activity of a flavoenzyme allowing the flavin cofactor to enter the new epitranscriptomic world (Figure 1). We herein review the recent exquisite biological, biochemical and structural knowledges on these modifications and on their corresponding flavoenzyme catalysts.

1.The dihydrouridine and the exquisite family of FMN-dependent dihydrouridine synthases

1.1 The simplest RNA modified base: the dihydrouridine

The evolutionary conserved dihydrouridine (D) is a famous modified base which gave its name to the classical tRNA's D-loop. Dihydrouridine results from the simple reduction of the C5 = C6 double bond of uridine (Figure 1A). Abundant mainly in the D-loop region at position 16, 17, 20, 20a, 20b and occasionally in the variable loop (V-loop) at position 47, this base is also present at position 2449 within the domain-V central loop region of *E. coli* 23S rRNA (*19*) (Figure 1B), which is the site of peptidyltransferase activity and also one of the sites of ribosomal interaction with antibiotics such as lincomycin, clindamycin, chloramphenicol, which inhibit this activity and growth of peptides chains (*20*). Dihydrouridine falls into a unique category since it does not participate in base stacking interactions because of its non-planar character. As a result, the ribose moiety enjoys the flexible C2'-endo conformation (*21*), a structural feature opposite in effect to all other modifications known to enhance regional stability through stabilization of the C3'-endo puckered conformations of the pyrimidines. Accordingly, this particular modification promotes greater conformational flexibility and dynamic motion in RNA regions wherein tertiary interactions and loop formation must be accommodated (*21, 22*). In some instances, this unique structural property ensures the proper interaction between a specific tRNA and its cognate aminoacyl-tRNA synthetase (*23*). The physiological benefit of such flexibility is illustrated in the psychrophilic organisms. Indeed these organisms have a high level of dihydrouridine in their tRNAs to counteract the otherwise significant reduction in conformational motions caused by the low growth temperatures (below 15 ° C) (*24*). Conversely, thermophiles contain a lower content of this modification. Beyond the physiological aspect, it has been observed that in certain human malignant tissues, an increase of dihydrouridine level promotes faster cellular growth (*25*) presumably because this base, along with other modifications, prevent rapid tRNA turnover (*15*).

1.2 The dihydrouridine synthases: a large family of flavoenzymes

The dihydrouridine synthesis is catalyzed by a large family of FMN-dependent enzymes named dihydrouridine synthases (Dus) which employ NADPH as a flavin reducing agent (Figure 2A) (*26*). Phylogenetic analysis classified these flavoenzymes into three major groups and eight subfamilies, all of which evolved through independent duplications of an ancestral *dus* gene (*27*). The first group found in prokaryotes regroups three Dus (Dus A, B and C) while the second one is eukaryotic and contains four different enzymes (Dus 1 to 4). The last group is characterized by a single Dus observed only in archaea. Dus B is the oldest enzyme among this large Dus family.

The substrate specificities of Dus enzymes were determined for model organisms such as *E. coli*, *Saccharomyces cerevisiae* and *T. thermophilus* Dus (*26, 28-30*). In the case of the yeast enzymes, the specificities of the four Dus are well known. However, there are still some ambiguities for *E. coli* (Figure 1B). As a matter of fact, the function of Dus B is unknown and the enzymes that synthesize D_{17}, D_{20a} in tRNA and D_{2449} in rRNA remain to be established (Figure 1B). Recently, Hori's group showed that in *T. thermophilus*, D20 and D20a are derived from the activity of one single Dus (Dus_{TT}) belonging to DusA subfamily (*29*). Another interesting feature that remains to be elucidated concerns *Mycoplasma mycoides* whose genome encodes for a single Dus while its tRNAs contain D at many different positions (*30*). Hence, regional multi-site specificity, a rather uncommon functional property among RNA modifying enzymes, seems to be a major peculiarity of this class of flavoenzymes (*31-35*).

From the few available crystal structures of Dus proteins, one can note an exceptional conservation of the general fold characterizing these enzymes. The N-terminal catalytic domain is organized into a TIM barrel (TBD) and is responsible for the FMN binding while the C-terminal domain adopts an helical fold (HD) and participates to the tRNA binding (Figure 2B) (*36-40*). Structure-function analysis of these flavoenzymes led to a perplexing question. How can they function on specific uridines exposed to spatially distinct faces of tRNA if they bear the same general fold with a conserved active site? This question has recently been answered by crystallographic structures of two bacterial Dus carrying different substrate specificities (*37, 41*) (Figure 2C). Dus_{TT} and DusC from

E. coli ($DusC_{EC}$) were solved to a resolution of 3.5 Å and 2.1 Å, respectively. Both Dus structures were obtained in complex with a $tRNA^{phe}$ substrate. Structural comparison revealed that these flavoenzymes bind the tRNA in an almost reverse orientation differing by a 160° rotation. The spatial orientation appears to be guided by (i) a subfamily-specific clusters of amino acids together with a different positive charges distribution at the solvent-exposed surfaces and (ii) a change in the relative angle between the TBD and HD. To our knowledge, such a modulation of substrate specificity is unprecedented in RNA enzymology.

1.3 The particular case of human dihydrouridine synthase 2

Another interesting characteristic of this family of enzymes relates to the particular case of animal Dus2, which presents a complex modularity. Compared to other Dus enzymes, the animal Dus2 carries an additional C-terminal domain exhibiting the canonical structural motifs of the double stranded RNA binding domain (dsRBD) (*27*) (Figure 2B). This domain is commonly observed in proteins involved in mRNA editing, RNA processing, RNA transport, RNA silencing but so far it has never been detected in other tRNA or rRNA modifying enzymes (*42*). In addition to their primary function in dsRNA binding, some dsRBDs have the ability to interact with proteins. We studied the dsRBD function in human Dus2 enzyme (hDus2) using biochemical and genetic tools (*40*). Obviously, this domain is crucial for hDus2 activity *in vivo* and *in vitro* because of its key role in tRNA recognition and binding. The reason why only Dus2 from animals specifically acquired a dsRBD for a cooperative tRNA binding is still unclear, but perhaps it serves to other roles in the cells. In fact, hDus2 was shown to interact with important proteins in various cancer cells. Immunoprecipitation assays revealed that in non-small cancer cells, hDus2 participates in a direct interaction with the large glutamyl-prolyl tRNA synthetase complex to favor an efficient translational process (*25*). Furthermore, Mittelstadt and coworkers showed that in HeLa cells the dsRBD-containing kinase PKR is also a partner of hDus2 (*43*). PKR is an interferon-induced protein involved in regulation of antiviral innate immunity, stress signaling, cell proliferation and programmed cell death via activation of its kinase activity. Here, the dsRBD of PKR and hDus2 interact together to inhibit the kinase activity and allow the cancer cell to escape apoptosis. Thus, it would be interesting to investigate the mechanisms which lead to the inhibition of hDus2 activity and/or impede its interactions with pathological partners in order to eventually develop new promising therapeutic strategies against specific cancers.

1.4 The enzymatic mechanism of the dihydrouridine synthases

A molecular mechanism for dihydrouridine synthesis, likely shared by all Dus, was postulated on the basis of structural information obtained from Dus_{TT}/tRNA complex and a stopped-flow study on Dus2p (*37, 44*). The reaction begins with the reduction of FMN by NADPH followed by subsequent dissociation of the $NADP^+$ product. After binding the tRNA substrate, the enzyme flips the uridine target and stacks it against the isoalloxazine ring of $FMNH^-$ (Figure 2C, boxes). Under such a configuration, the C6 nucleobase carbon is in the vicinity of the N5-$FMNH^-$ and is ready to receive a hydride anion from the flavin hydroquinone. This relative arrangement of the reactants is very similar to that observed in dihydroorotate dehydrogenase and dihydropyrimidine dehydrogenase, two flavoenzymes sharing structural and functional homologies with the Dus enzymes (*26*). In these three enzyme families, a strictly conserved cysteine located near to the C5 atom of the pyrimidine substrate acts as a key general acid/base catalyst (*30, 37, 44*). The hydride transfer step followed by the protonation of the C5 nucleobase carbon yields the final modified base.

2. **Reductive methylation of tRNA and rRNA by new class of flavin and folate dependent RNA methyltransferases**

2.1 The conserved ribothymidine modified nucleoside

Nucleobase and ribose methylation are the most widespread type of modifications in all RNAs (*45, 46*). Among them, there is the classical uridine C5-methylation that leads to ribothymidine, T or 5-methy-uridine (m^5U) found in tRNA, transfer-mRNA (formerly 10Sa RNA; tmRNA) and rRNA of archaea, eukaryotes, and bacteria (Figure 1A). Ribothymidine is known to be more hydrophobic than

the canonical U. On the one hand, this methylated base is more refractory to hydrogen bonds with adenosine but on the other hand it exhibits a better stacking capacity, which promotes a stable tertiary structure of RNA. For instance, $tRNA^{phe}$ and $tRNA^{fMet}$ lacking m^5U exhibit a lower respective melting temperature by 2 and 6°C (*15*). In tRNA, m^5U has been detected so far at the position 54 (m^5U_{54}) in the characteristic T-loop and at two distinct conserved positions in bacterial rRNA (747 and 1939) (*4*) (Figure 1B).

2.2 Flavin and folate dependent RNA methyltransferases

In most living organisms the m^5U is synthesized by S-adenosylmethionine (SAM)-dependent-methyltransferases, which directly transfer a methyl group from the SAM cofactor to the C5-uracil carbon via a simple SN_2 reaction (*47*). However, in Gram positive bacteria and in several mycoplasmas, this methylation proceeds by a much more complex multistep process involving the N5,N10-methylenetetrahydrofolate (CH_2THF) used as a methylene donor, and the reduced flavin adenine dinucleotide hydroquinone ($FADH^-$) (*48*) (Figure 3A). This alternative type of reaction also called reductive methylation is catalyzed by characteristic flavoenzymes denoted as m^5U_{54} tRNA methyltransferase FAD/folate-dependent (TrmFO) (*48-51*) and m^5U_{1939} rRNA methyltransferase FAD/folate-dependent (RlmFO) (*52*) according to the nature of the substrate used, tRNA and rRNA, respectively. The importance of such TrmFO's catalyzed reaction as being part of organism's adaptive mechanism in response to physical changes of their environment was recently uncovered (*53*).

The crystallographic structures of *T. thermophilus* TrmFO ($TrmFO_{TT}$) show that the enzyme is organized into two domains: an FAD-binding domain (FBD) and an insertion domain (ID) (*49*) (Figure 3B). FAD coenzyme lies within the FDB and is stabilized via extensive and conserved characteristic type of interactions notably by the GxGxAGxEA motif conserved among the glutathione reductase family members. The *si*-face of FAD is engaged in a π–π stacking interaction with a conserved tyrosine (*54*) while the *re*-face serves as a folate binding site (Figure 3B, box). In the case of RlmFO, there is no structure reported yet. Nevertheless, its high sequence identity with TrmFO (*M. capricolum* RlmFO ($RlmFO_{MC}$) vs $TrmFO_{TT}$ ~ 38.6 % and $RlmFO_{MC}$ vs *Bacillus subtilis* TrmFO = 47.5 %) suggests that both flavoenzymes likely share the same tridimensional structure (*50*).

2.3 Flavin as a new RNA methylating agent: insight into the mechanism of TrmFO

Our extensive characterization on *Bacillus subtilis* TrmFO ($TrmFO_{BS}$) has led to the recent discovery that flavin can function as an unprecedented RNA methylating agent (*55-57*). This novel agent is in the form of a unique methylene-iminium derivative of FAD (CH_2=FAD, compound **3**) resulting from the nucleophilic reaction of N5-$FADH^-$ on CH_2THF (Figure 3C). The use of flavin as a covalent catalyst is not uncommon and has recent precedents in the flavoenzymology (*50, 58-60*). Furthermore, the same CH_2=FAD species is used by the bacterial flavin-dependent thymidylate synthase ThyX (*61*), a flavoenzyme found in several human pathogens and which catalyzes the vital conversion of dUMP into dTMP. Methylation of tRNA is assisted by a Michael's addition of a cysteine nucleophile (Cys223 in the case of $TrmFO_{TT}$ and Cys226 in $TrmFO_{BS}$) to C6-U54 (*62*). Upon this nucleophilic attack, the non-covalently bound CH_2=FAD is converted to a covalent protein-tRNA-CH2-FAD complex (compound **4**). Deprotonation of tRNA by a conserved cysteine (Cys51 in the case of $TrmFO_{TT}$ and Cys53 in $TrmFO_{BS}$) allows the transfer of a methylene from the flavin to the uridine. Finally, reduction of this exocyclic methylene into a methyl group by the de-alkylated $FADH^-$ achieves the reaction and releases the nucleophile from the tRNA.

This type of chemistry requires that the activated base be placed close to the iminium group of compound **3** in order to maximize the functional carbon transfer reaction and ultimately minimize uncoupled reactions that lead to the formation of formaldehyde and H_2O_2, which are toxic molecules for the bacterial cells. Paradoxically, in the current structure of monomeric $TrmFO_{TT}$, the catalytic nucleophile Cys223 is located more than 20 Å away from the N5-FAD (Figure 3C, inset) rendering such chemistry unrealistic under this particular state. Rather, we and others have speculated that the active enzyme could possibly be under a homodimeric state that would form during catalysis (*50, 62*). A structural model satisfying the mechanistic criterion was proposed and showed that Cys223 of a monomer could be found in the vicinity of FAD present within the second monomer without invoking major conformational changes. Accordingly, the actual active site is structured by a combinatory interface of the two monomers and can suitably accommodate the target U_{54}, which would have to flip

out from its buried position in the tRNA T-loop. A similar model should also apply to RlmFO. In this regard, a structure of these flavoenzymes in complex with RNA could solve these mechanistic issues.

3. A flavoenzyme complex controls translation via hypermodification of wobble uridine of tRNA

3.1 Hypermodifications of the wobble uridine

Modifications targeting the anticodon stem loop (ASL) of tRNAs are important for the translation since they structure the ASL into the canonical U-turn motif for ribosomal A-site entry, enhance tRNA/mRNA affinity, favor mRNA translocation during translation and ensure the efficiency and fidelity of the translation (*12, 17*). In general, the chemical nature of these modifications is complex and often involves the participation of several enzymes for their biosynthesis. The nucleoside at position 34 (wobble position) located within the ASL has the ability to base pair to two or three different nucleosides (degeneracy of the genetic code) offering to a single tRNA species the capability to decode more than one synonymous codon. In that respect, this tRNA decoding capability becomes restricted or expanded depending on the nature of the modification. For example, xm5U modifications type including the 2-thiouridine (xm^5s^2U) and 2′-O-methyluridine derivatives (xm^5Um), wherein the C5 carbon of the uridine is directly bonded to a methylene group, forces the wobble uridine to pair with purines preventing misreading of the near-cognate codons ending in pyrimidines (*17, 63, 64*). Lack of such modifications causes pleiotropic phenotype in bacteria (*65*), neurological and developmental dysfunctions in worms (*66*) and severe pathologies in humans (*67-69*).

In bacteria, the 5-carboxymethylaminomethyl ($cmnm^5$) and 5-aminomethyl (nm^5) belonging to the xm5U modifications family are synthetized by a conserved enzymatic heterocomplex involving a flavoenzyme component (*70*) (Figure 1A and B). For certain tRNA species, these C5 substituents can serve as intermediates in the metabolic pathways of mnm^5U modification (see below). They can be combined to other modifications such as with the 2-thiolation (s^2) incorporated throughout the activity of multi-enzyme IscS/MnmA pathways or 2'-O-methylation of the ribose (m) incorporated via a single step by the SAM-dependent methyltransferase TrmL. However, all these enzymatic activities are independent from each other's. *E. coli* carries these combined hypermodifications in tRNAs Lys (mnm^5s^2UUU), Glu (mnm^5s^2UUC), Gln ($cmnm^5s^2UUG$), Leu ($cmnm^5UmAA$), Arg (mnm^5UCU), and Gly (mnm^5UCC) (*4*).

3.2 Structure-function of MnmE, MnmG and of their functional complex

The heterotetrameric complex formed by two proteins, MnmE and MnmG, uses an impressively complicated mixture consisting of GTP, K^+, CH_2THF and glycine or $NH4^+$ to catalyze the xm5U modifications (*70, 71*) (Figure 4A). Each of these proteins forms a stable homodimer both inside and outside the complex (*72*) (Figure 4B).

3.2.1 MnmE, a paradigm of bifunctional GTPase protein

As evidenced from the crystal structures, MnmE (formerly known as TrmE) is organized into three domains (*73, 74*). At the N-terminus, an α/β domain (FoBD) involved in the homodimerization of the protein generates at the interface of two protomers a composite binding site for CH_2THF (Figure 4B, left box). In the reaction, this folate derivative provides the methylene moiety, which is directly attached to C5-U34. Interestingly, FoBD shares the same topology as the folate-binding sites of the N,N-dimethylglycine oxidase and sarcosine oxidase, which are both interesting cases of bifunctional flavoenzymes (*75*). FoBD is followed by a central helical domain (HD) generated by residues from the middle and the C-terminal regions. Inserted within the HD, the GTP-binding domain (G-domain) contains the typical motifs characteristic of all G-proteins notably the classical molecular switches found in Ras family (*73*). Ras proteins are characterized by a very low intrinsic GTPase activity combined with high affinities for the substrate GTP and product GDP. Thus, the GTPase cycle proceeds with the intervention of two auxiliary proteins, the GTPase activating proteins and guanine-nucleotide exchange factors to catalyze hydrolysis and nucleotide release, respectively. The G domain of MnmE is at variance with this canonical mechanism since it displays a low affinity for guanine nucleotides and a high GTP hydrolysis rate stimulated by the potassium (*76, 77*). For these specific reasons, MnmE does not need the help of external proteins during its GTP hydrolysis cycle and uses

the G-domain dimerization in a potassium- and GTP-dependent manner as an activation step. Mutational analysis and fast kinetics assays revealed that GTP hydrolysis, G-domain dissociation and Pi release can be uncoupled and that G-domain dissociation is directly responsible for the 'ON' state of MnmE (*78*). The cycle is negatively controlled by the reaction products GDP and Pi. The GTPase activity is essential for the tRNA modifying function of the enzymatic complex (see below) (*79, 80*).

3.2.2 The flavoprotein MnmG

MnmG, also known as GidA, is the flavoenzyme component of the complex (*72*). The crystallographic structures of MnmG's dimer from *E.coli*, C. *tepidum* and *A. aeolicus* show that each protomer is organized into three large domains (*81-83*) (Figure 4B). The first domain exhibits the classical Rossmann fold and carries the FAD-binding site. The second domain (ID for inserted domain) is inserted between the two strands of the Rossmann fold. This domain displays structural similarity to the nicotinamide-adenine-dinucleotide-(phosphate)-binding domains of phenol hydroxylase and 3-hydroxy-3-methylglutaryl-CoA reductase. The protein ends with an α-helical C-terminal domain, which appears to be crucial for the interaction of MnmG with MnmE. It should be noted that MnmG is a paralogue of TrmFO, which means that these two flavoenzyme families evolved from a common ancestor, but acquired divergent cellular functions (*48*). TrmFO is distinguished by its ability to bind the folinic co-substrate and to catalyze the modification by itself, while MnmG requires a stable association with MnmE, which provides the CH_2THF to the reaction. Consequently, the CTD is absent in TrmFO but they both share the same FBD and ID which are differently orientated to satisfy their respective divergent function (*49*). MnmG is the main component involved in the tRNA binding function but MnmE may also participate to this task in the functional complex (*82*). The two functional cysteines of TrmFO are conserved in MnmG and may serve similar purposes during the catalysis (*62, 82*). Indeed we have proposed that for *a. aeolicus* MnmG, the Cys248 placed more than 14 Å from the FAD, could be the nucleophile required for U34 activation whereas Cys48, located within the same monomer, is probably the general base, which abstracts the H5 proton from a nucleobase intermediate.

3.2.3 The flavoenzymatic MnmG/MnmE complex

The lack of a high resolution structure of this complex precludes a clear understanding of how MnmE and MnmG jointly act to orchestrate tRNA modification. Using various spin-labeled MnmE mutants and EPR spectroscopy, Böhme and coworkers showed that MnmG binding induces large conformational and dynamic changes in MnmE (*84*). It stimulates the GTPase reaction by stabilizing the GTP-bound conformation. The recent study combining a SAXS approach along with modeling has shed lights on some mechanistic aspects by which this complicated system works (*85*). Surprisingly, it was observed that the oligomerization state of the complex evolves during the course of the GTPase cycle (Figure 4C). In the free nucleotide state or in the presence of GDP, MnmE and MnmG interact to form an $\alpha_2\beta_2$ complex in an asymmetric "head-to-tail" fashion, wherein the C-terminal domain of one MnmG protomer binds the FoBD and HD from one subunit of dimeric MnmE. Under such configuration, the folate and FAD binding sites are facing each other but separated by 30 Å (*85*). Likewise, upon GTP binding and hydrolysis, the G domains dimerize promoting conformational changes within the complex, which would eventually bring the folate and the flavin in close proximity at certain steps of the reaction (*71, 80, 85*). In contrast, in the presence of GTP and K^+, the $\alpha_2\beta_2$ complex oligomerizes into an $\alpha_2\beta_2\alpha_2$ complex where one MnmG dimer is sandwiched between two MnmE dimer (Figure 4C). It is tempting to speculate that this $\alpha_2\beta_2\alpha_2$ is a functional state in which the reactants are properly poised within the transiently formed active site. Nevertheless, the functional relevance of such interconversion in the catalytic cycle awaits further elucidation.

3.3 Mechanism of tRNA modification by the flavoenzymatic complex MnmE/MnmG

There have been very few studies aimed at determining the exact role of flavin as well as the chemical nature of the reactional steps catalyzed by this complex. Nevertheless, one recent biochemical studies showed that the MnmE/MnmG complex can catalyze $cmnm^5U$ or nm^5U formation in tRNA without the need of NAD(P)H in the reactional mixture (*86*). Although NADP(H) is not essential for the modification, it was suggested that it could prevent accumulation of abortive oxidation of the flavin hydroquinone formed at a specific step during the catalytic cycle. This has led

Armengod and coworkers to propose a hypothetical mechanism in which FAD undergoes, within the functional complex, an oxidation-reduction cycle during the modification (Figure 4D). In this mechanism, several Schiff's base intermediates are generated by the action of flavin. Similarly to TrmFO, Michael's addition chemistry is supposed to activate the U_{34} nucleobase before receiving the C5-modification.

3.4 GTPBP3/MTO1, a human complex homologous to MnmE/MnmG

GTPBP3 and MTO1 are the human homologues of MnmE and MnmG, respectively (*87, 88*). These proteins are synthesized in the nucleus and transported to the mitochondria in order to modify organelle tRNAs. Currently, there is no biochemical and structural characterization of these enzymes, but it is known that the GTPBP3 / MTO1 complex catalyzes the formation of 5-taurinomethyluridine 34 (τm^5U_{34}) using taurine instead of glycine (Figure 1A and 1B). MTO1 and GTPBP3 are important since several genetic mutations of these proteins are associated to severe diseases such as mitochondrial myopathy, hypertrophic cardiomyopathy, encephalopathy, lactic acidosis (*67-69*).

4. MnmC, a bifunctional flavoenzyme relay in wobble hypermodification

In γ-Proteobacteria and some other bacterial groups, the $cmnm^5$ and nm^5 produced by the MnmE/MnmG complex are converted to methylaminomethyl (mnm^5), which is a modification more adapted for certain tRNAs (*4*). Both metabolites are modified by the same monomeric and bifunctional flavoenzyme called MnmC (*89, 90*) (Figure 5A).

MnmC's N-terminal domain contains a SAM binding site (MTD) and catalyzes nm5U methylation reaction (MnmC2 activity) to mnm^5U using most likely a classical SN_2 mechanism whereas the C-terminal domain holding the FAD binding site (FBD) is involved in the deacetylation of $cmnm^5$ to nm^5 (MnmC1 activity) (*91, 92*) (Figure 5B). Interestingly, this FBD shares structural homology with *Bacillus Subtilis* glycine oxidase although both enzymes exhibits relatively low sequence identity. Glycine oxidase catalyzes the FAD-dependent oxidation of glycine to iminoglyoxylate, with the molecular oxygen being the final electron acceptor in the reaction, which is analogous to MnmC1 function. Indeed, superposition between glycine oxidase structure bound to N-acetyl glycine and MnmC placed the glycine alpha-carbon at 3.6 Å from the N5-FAD within MnmC1 (*91*). Consistently, a chemical mechanism similar to that of glycine oxidase and involving a direct hydride transfer from the glycine to the FAD was postulated for MnmC1 (Figure 5C).

The two active sites are separated by ~45 Å (Figure 5C, inset). However, both domains carrying the respective active sites appear to be rigidly fixed by a substantial interdomain interface. A conformational rearrangement, which could eventually bring the two active sites into close proximity, is an unlikely scenario. Instead, tRNA is likely released from the enzyme after oxidation at the MnmC1 active site and then binds to MnmC2 for its subsequent methylation. This scenario is supported by the fact that nm^5U_{34} derived from MnmE/MnmG complex is the substrate of MnmC2 exclusively (*86*). Moreover, the MnmC orthologue in *Aquifex aeolicus* has exclusively the MnmC2 SAM-dependent methyltransferase component indicating that nm^5U coming from either MnmC1 activity or directly from the MnmE/MnmG patway can be proceeded to mnm^5U without the help of MnmC1 (*93*) (Figure 5B). Notably, recent studies demonstrated that the activities of the MnmC1 and MnmC2 domains are independent from each other's (*94*). Hence, the bifunctional MnmC may have evolved to avoid nm^5U34 accumulation and to efficiently drive its conversion to the final desired mnm^5U34 stable product.

5. Flavin in hydroxywybutosine synthesis of tRNA anticodon loop

Wyosine and its derivatives, including wybutosine (yW) and hydroxywybutosine (OHyW), are highly complex modifications observed exclusively at position 37 of $tRNA^{Phe}$ from archaea and eukaryotes (*4, 95, 96*). These critical hypermodifications promote selectivity of tRNA in the ribosomal A-site and prevent frameshifting (*97, 98*). The N1-G37 methylation by Trm5 SAM-dependent methyltransferase constitutes the first step in the course of wyosine biosynthesis. Following this methylation, eukaryotes employ five additional enzymes (Tyw1-5) working in a sequential mode to finally yield hydroxylated OHyW derivative. The unique tricyclic core characterizing the wyosine

nucleosides is formed by the eukaryotic Tyw1 and its archaeal counterpart Taw1 (Figure 6A). Both enzymes add to m1G37 precursor two carbons derived from the pyruvate to create an imidazole ring in a clearly complex reaction. Interestingly, Tyw1 from higher organisms has a unique domain architecture consisting of an N-terminal flavodoxin and a C-terminal catalytic domain. In contrast, the archaeal homologs have only the catalytic domain. This latter is a typical radical SAM domain containing a CxxxCxxC iron-sulfur (4Fe-4S) binding motif (*99, 100*). The role of the flavodoxin like domain and more particularly that of FMN have not been examined yet. However, since all radical SAM enzymes require reductive activation of their radical SAM iron sulfur cluster by a one electron transfer to the $[4Fe\text{-}4S]^{+2}$ to generate the redox active $[4Fe\text{-}4S]^{+}$ state, it is possible that the flavin assume this latter function (*100*). By carrying its own reductive activation machinery in cis, Tyw1 may minimize potential abortive cleavage of SAM, which is known to be generally more frequent when artificial reducing agents are used. Regarding Archaea, it was speculated that an additional protein such as thioredoxin reductase could intervene to reduce the cluster of Taw1 in trans, however, there are no experimental evidences yet. The postulated chemical mechanism of tyw1 is outside the review scope since it involves cluster-dependent radical-based chemistry. We, therefore, refer readers interested in these mechanisms to more specific reviews on the subject (*99, 100*).

Conclusion and perspectives

In this review, we described for the first time all the exquisite flavoenzymes involved in RNA modifications. Their originality lies in the fact that, contrary to the great majority of flavoenzymes which react with small molecules, the flavin-dependent RNA-modifying enzymes modify a specific atom within a large nucleic acid polymer formed by thousands of atoms. These reactions can be simple as those catalyzed by the dihydrouridine synthases, which reduce uridines to dihydrouridines using the flavin hydroquinone as a hydride transfer agent. On the other hand, the chemistry can be diverse and very complex, as illustrated with tRNA and rRNA reductive methylation catalyzed by the respective flavoenzymes, TrmFO and RlmFO, and in which an unprecedented electrophilic $FAD(N5)=CH_2$ species is used as a *genuine* methyl transfer agent. The complexity of these flavoenzymatic systems reaches its paroxysm with MnmE/MnmG complex, which modifies the tRNA wobble uridine. No less than seven different substrates and cofactors are required to yield the cmnm5U and nm5U modified bases by the complex. Besides the challenging chemistry, large conformational changes were shown to take place to assemble a transient and composite active site during catalysis. Unfortunately, there is no structure of such complex, which clearly prevents us from having a clear understanding into how this exciting flavoenzymatic system works. The flavin versatility is also observed with MnmC, a unique case of bifunctional flavoenzyme whose role is first to deacetylate cmnm5U34 previously produced by MnmE/MnmG and subsequently to methylate the resulting nm^5U_{34} using SAM as cofactor to finally form mnm^5U_{34}. Curiously, the two active sites are separated by 45 Å, which therefore raises many questions about the communication between these sites during enzymatic catalysis. Finally, the metabolic pathway leading to the important wyosine and its derivatives in the tRNA anticodon loop also need the help of a bifunctional FMN-dependent protein, Tyw1, which employs an iron sulfur cluster for radical chemistry to create the unique tricyclic nucleobase ring characterizing these hyper-modified bases. Collectively, by its direct participation in the catalysis of several RNA modifications, flavin is a key cofactor in translation and, as such, it has entered into the prestigious world of epitranscriptomic.

The exact role of the flavin in the majority of these enzymatic mechanisms has not yet been validated. Furthermore, we still lack valuable structural information, particularly on how these flavoenzymes recognize and bind their substrates, especially the RNA. Therefore, in the future, a particular effort should be focused on solving these issues in order to unravel the enzymatic mechanisms and possibly discover a new reactivity of flavin. Finally, we would like to mention that although these enzymes work mainly on tRNA and occasionally on rRNA, it is not excluded to envisage that in the future new research will eventually establish their involvement in mRNA modification. For instance, pseudouridine synthases were originally considered as tRNA and rRNA specific enzymes. However, recent works showed that some of them are also active on mRNA discrediting old dogma and offering a new golden area for the dusted pseudouridine modified base

(1,2). All this presages a happy and certainly exciting future for the flavoenzymology of epitranscriptomics.

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Figure 1. **Chemical structure of flavin-dependent modified nucleosides and their location on the corresponding RNAs.** (A) Chemical structure of modified nucleosides. The conventional symbols used to name these modified bases are shown in red. Dihydrouridine (D), 5-methyluridine (m^5U), 5-aminomethyluridine (nm^5U), 5-carboxyaminomethyluridine ($cmnm^5U$), 5-methylaminomethyluridine (mnm^5U), 5-taurinomethyluridine (τm^5U), 4-demethylwyosine (imG-14). (B) Schematic representation of the RNAs and positions where a given flavin-dependent nucleoside has been found. These modifications are boxed and the name of their corresponding RNA-modifying flavoenzymatic systems catalyzing the modification is indicated below each boxes.

Figure 2. **Dihydrouridine and dihydrouridine synthases**. (A) Reaction catalyzed by the dihydrouridine synthases (Dus). The flavin mononucleotide (FMN) is the coenzyme and the nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) is used as a hydride source for FMN reduction into the functional hydroquinone state ($FMNH^-$). (B) Structural organization of the dihydrouridine synthase of *Thermus thermophilus* (Dus_{TT}), *E. coli* DusC ($DusC_{EC}$) and *Homo sapiens* Dus2 (hDus2). The TIM Barrel domain (TBD), helical domain (HD) common to all Dus are in pink and green, respectively. The double stranded RNA binding domain (dsRBD) specific only to Dus2 from animals is in cyan. FMN is represented as yellow ball-sticks. The delimitations of each domain are indicated above the schematic representation of the sequence organization. N represents the total number of protein residues. The PDBs code for the crystallographic structures of Dus_{TT}, $DusC_{EC}$, the catalytic domain and the dsRBD of hDus2 are 3B0P, 4BFA, 4WFS and 4WFT, respectively. (C) Crystallographic structures of Dus_{TT} and $DusC_{EC}$ in complex with the homologous tRNA phenylalanine substrate. Right panel: crystal structure of the $Dus_{TT}/tRNA^{phe}$ complex (PDB: 3B0V). Left panel: crystal structure of $DusC_{EC}/tRNA^{phe}$ C98A mutant (PDB: 4YCO). The D-loop sequence for each tRNAs is also indicated. The active site is illustrated in the boxes. (D) Postulated mechanism for the synthesis of dihydrouridine by Dus enzymes. R= ribityl-phosphate. Cys93 is the general acid and the numbering is based on Dus_{TT}'s sequence.

Figure 3. **Reductive methylation of tRNA and rRNA by flavin and folate methyltransferases, TrmFO and RlmFO.** (A) Reaction catalyzed by m^5U_{54} tRNA methyltransferase FAD/folate-dependent (TrmFO) and m^5U_{1939} rRNA methyltransferase FAD/folate-dependent (RlmFO). CH_2THF is the N5,N10-methylenetetrahydrofolate. (B) Structural organization of TrmFO from *Thermus thermophilus* ($TrmFO_{TT}$). The FAD binding domain (FBD) and the inserted domain (ID) are in blue and brown, respectively. The FAD coenzyme is represented as yellow ball-sticks. The delimitations of each domain are indicated above the schematic representation of $TrmFO_{TT}$. The crystal structure is that of $TrmFO_{TT}$ is complexed with tetrahydrofolate (THF) (PDB: 3G5R). The active site is illustrated in the box showing THF product as green. (D) Proposed mechanism for the reductive methylation of C5-uridine by TrmFO and RlmFO. R1 = adenosine-5'-pyrophosphate-ribityland and R2 = (p-aminobenzoyl) glutamate. The numbering of the cysteine residues is based on $TrmFO_{TT}$'s sequence. The inset structure shows the distance between the two catalytic cysteines.

Figure 4. **Hypermodifications of tRNA by the flavoenzymatic complex, MnmE/MnmG**. (A) Reactions catalyzed by the MnmE/MnmG complex. When the complex uses glycine the product of the reaction is $cmnm^5U_{34}$ while when it uses $NH4^+$ instead of glycine the final product is nm^5U_{34}. (B) Structural organization of the homodimer of MnmE and MnmG outside the complex. Left panel: schematic representation of the MnmE shows the folate binding domain (FoBD) in red, the helical

domain (HD) in blue and inserted G-domain in cyan. The crystal structure is that of homodimeric C. tepidum MnmE (PDB: 3GEE). Below the structure: zoom on the folate binding sites at the interface of two FoBD. Right panel: structural organization of MnmG from *Aquifex aeolicus* ($MnmG_{AA}$). The FAD binding domain (FBD), the inserted domain (ID) and the CTD are in green, pink and brown, respectively. The crystal structure of the homodimer is that of $MnmG_{AA}$ (PDB: 2ZXH). Zoom on the FAD binding site: FAD cofactor is represented as yellow ball sticks and the distance between the two catalytic cysteines within the same subunit is also reported. (C) Model of the MnmE/MnmG complex during the tRNA modification cycle. The transition between $\alpha2\beta2$ to an $\alpha2\beta2\alpha2$ species occurs upon GTP and K^+ binding within the G-domains of MnmE. (D) Postulated mechanism for $cmnm^5U_{34}$ and nm^5U_{34} synthesis by MnmE/MnmG complex. R1 = adenosine-5'-pyrophosphate-ribityland and R2 = (p-aminobenzoyl) glutamate. The numbering of the cysteine residues is based on $MnmG_{AA}$'s sequence.

Figure 5. **Hypermodifications of tRNA wobble uridine by the bi-functional flavoenzyme, MnmC**. (A) Reactions catalyzed by MnmC. S-adenosylmethionine (SAM) is the methyl donor cofactor. (B) Structural organization of MnmC from *E. coli* ($MnmC_{EC}$) (left panel) and from *Aquifex aeolicus* (right panel). The schematic representation of the $MnmC_{EC}$ shows the methyltransferase domain (MTD, known as MnmC1) in blue and the flavin binding domain (FBD, known as MnmC2) in burgundy. In the crystal structure of $MnmC_{EC}$, (PDB: 3PS9) the SAM and FAD cofactors are represented in cyan and yellow ball sticks. $MnmC_{AA}$ carries only one domain, the MTD in blue and has an Nt and Ct extensions in yellow. The crystal structure of $MnmC_{AA}$ (PDB: 3VYW) shows the SAM in cyan. (D) Proposed mechanism for the mnm^5U_{34} synthesis by $MnmC_{EC}$. R = adenosine-5'-pyrophosphate-ribityland. The inserted box shows the distance between the FAD and SAM in $MnmC_{EC}$.

Figure 6. **Hypermodifications of m1G37-tRNA by Tyw1**. (A) Reactions catalyzed by Tyw1. (B) Schematic representation of the flavodoxin domain containing the FMN cofactor (yellow) and the SAM radical domain containing the iron-sulfur cluster (gray).

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Figure 1.

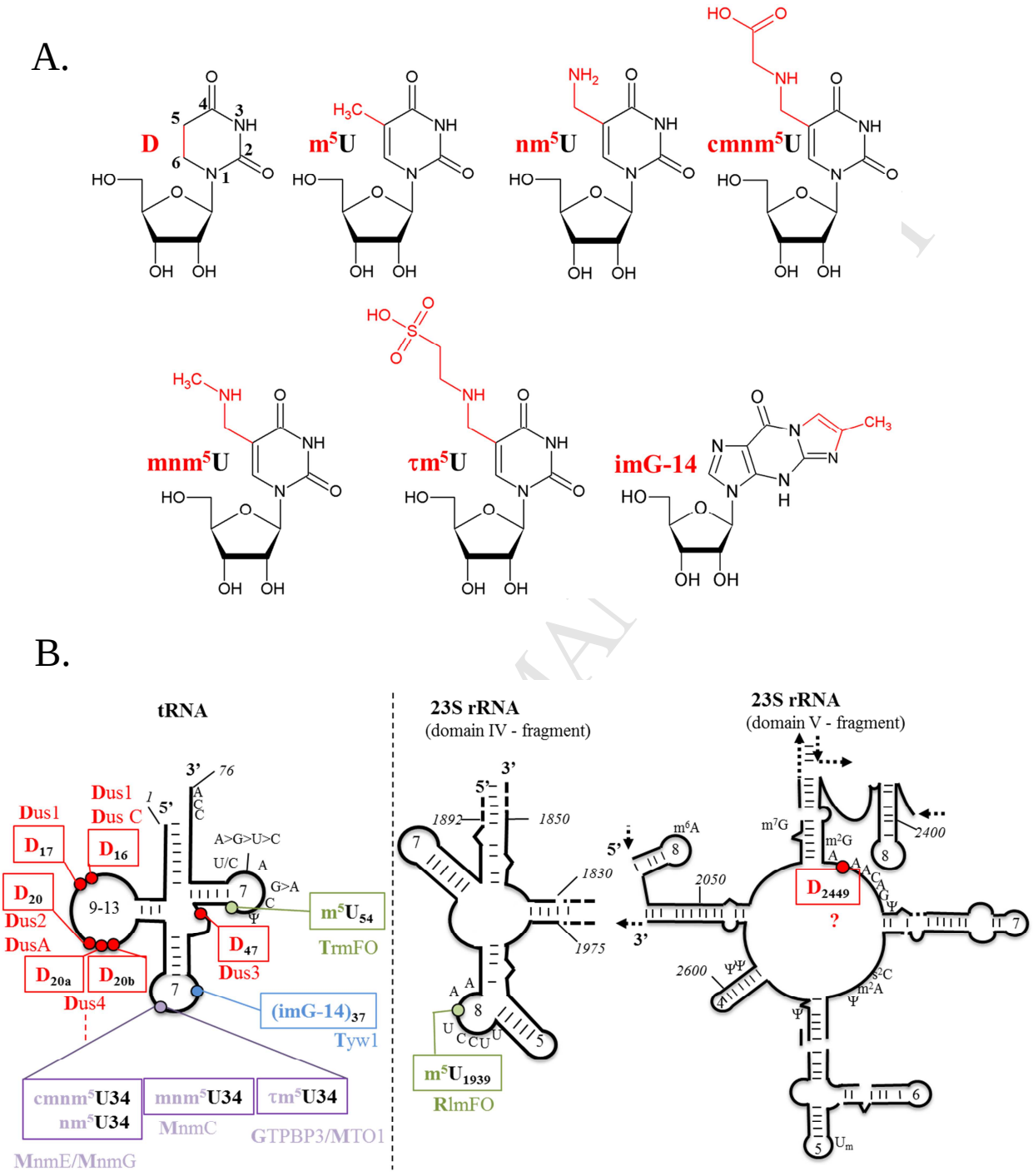

A.
D
m5U
nm5U
cmnm5U
mnm5U
τm5U
imG-14
B.
tRNA
23S rRNA
(domain IV - fragment)
23S rRNA
(domain V - fragment)
Dus1
Dus C
D17
D16
D20
Dus2
DusA
D20a
D20b
Dus4
9-13
A>G>U>C
U/C
G>A
D47
Dus3
m5U54
TrmFO
(imG-14)37
Tyw1
cmnm5U34
nm5U34
mnm5U34
τm5U34
MnmC
GTPBP3/MTO1
MnmE/MnmG
m5U1939
RlmFO
D2449
?

Figure 2.

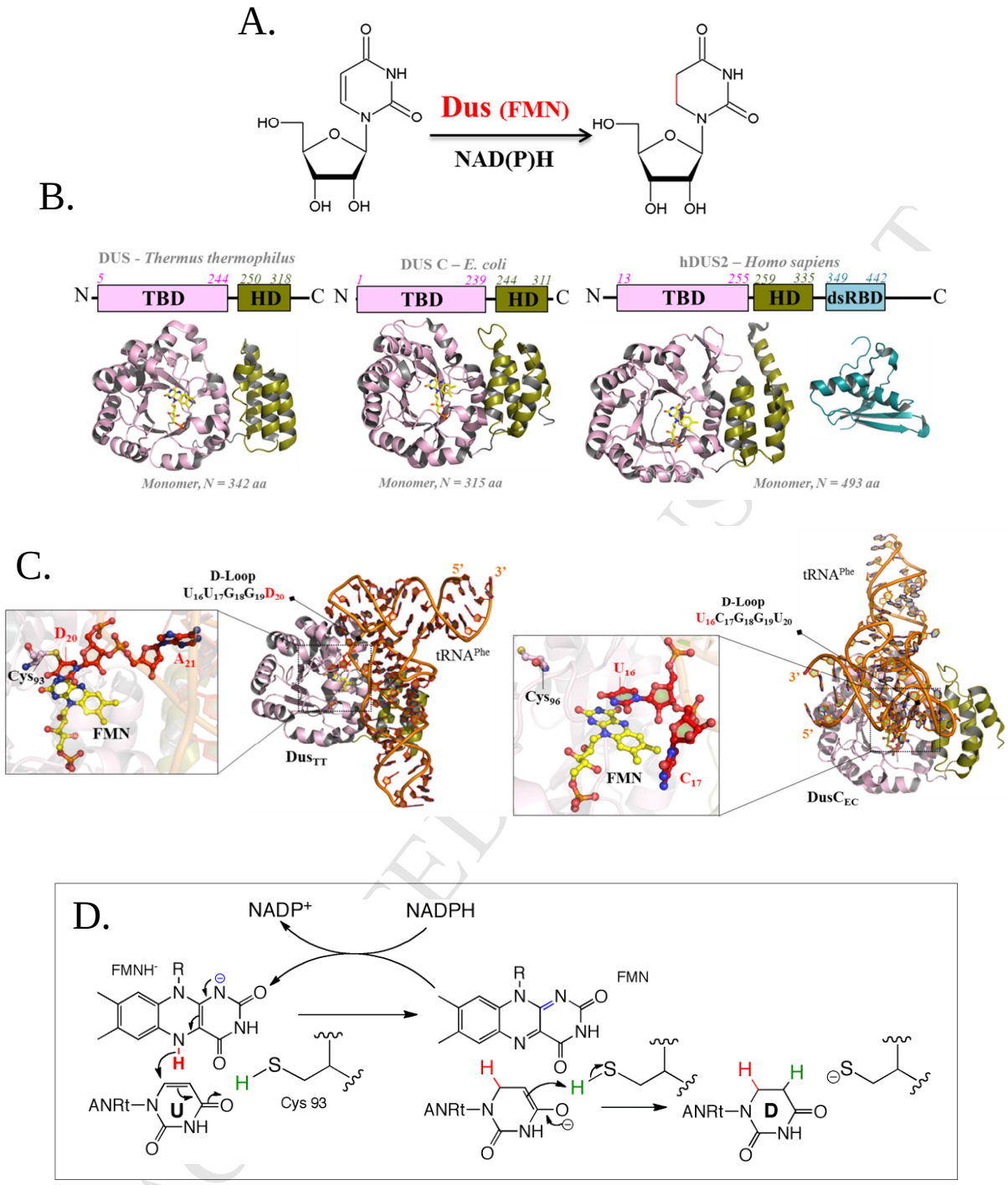

A.
Dus (FMN)
NAD(P)H
B.
DUS - Thermus thermophilus
DUS C – E. coli
hDUS2 – Homo sapiens
TBD
HD
dsRBD
Monomer, N = 342 aa
Monomer, N = 315 aa
Monomer, N = 493 aa
C.
D-Loop
U16U17G18G19D20
D20
A21
Cys93
FMN
DusTT
tRNAPhe
U16
Cys96
C17
DusCEC
D.
NADP+
NADPH
FMNH-
FMN
ANRt
Cys 93

Figure 3.

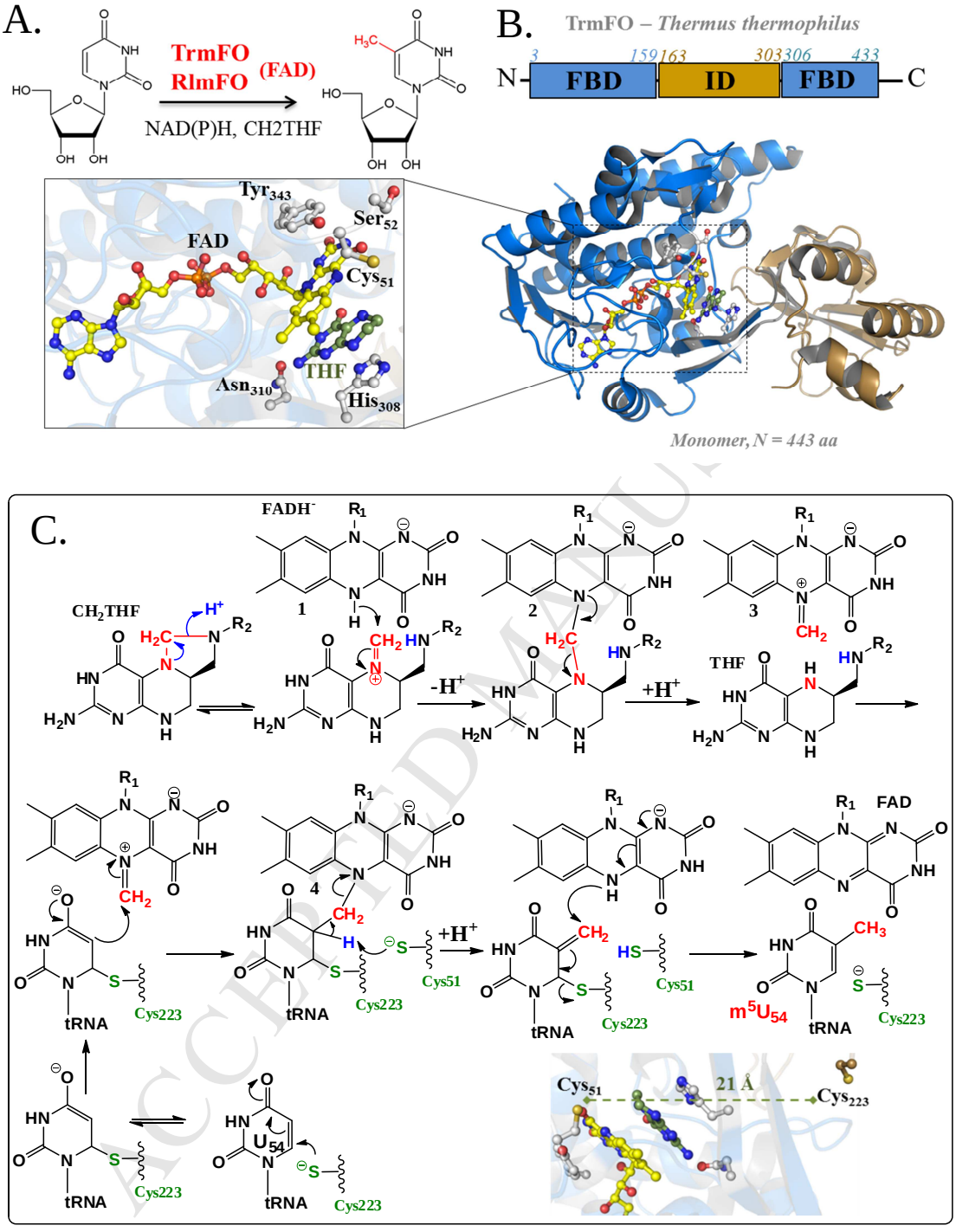

A.
TrmFO
RlmFO (FAD)
NAD(P)H, CH2THF
B.
TrmFO – Thermus thermophilus
3
159163
303306
433
N
FBD
ID
FBD
C
Tyr343
Ser52
FAD
Cys51
THF
Asn310
His308
Monomer, N = 443 aa
C.
FADH-
CH2THF
1
2
3
4
-H+
+H+
tRNA
Cys223
Cys51
m5U54
U54
21 Å

Figure 4.

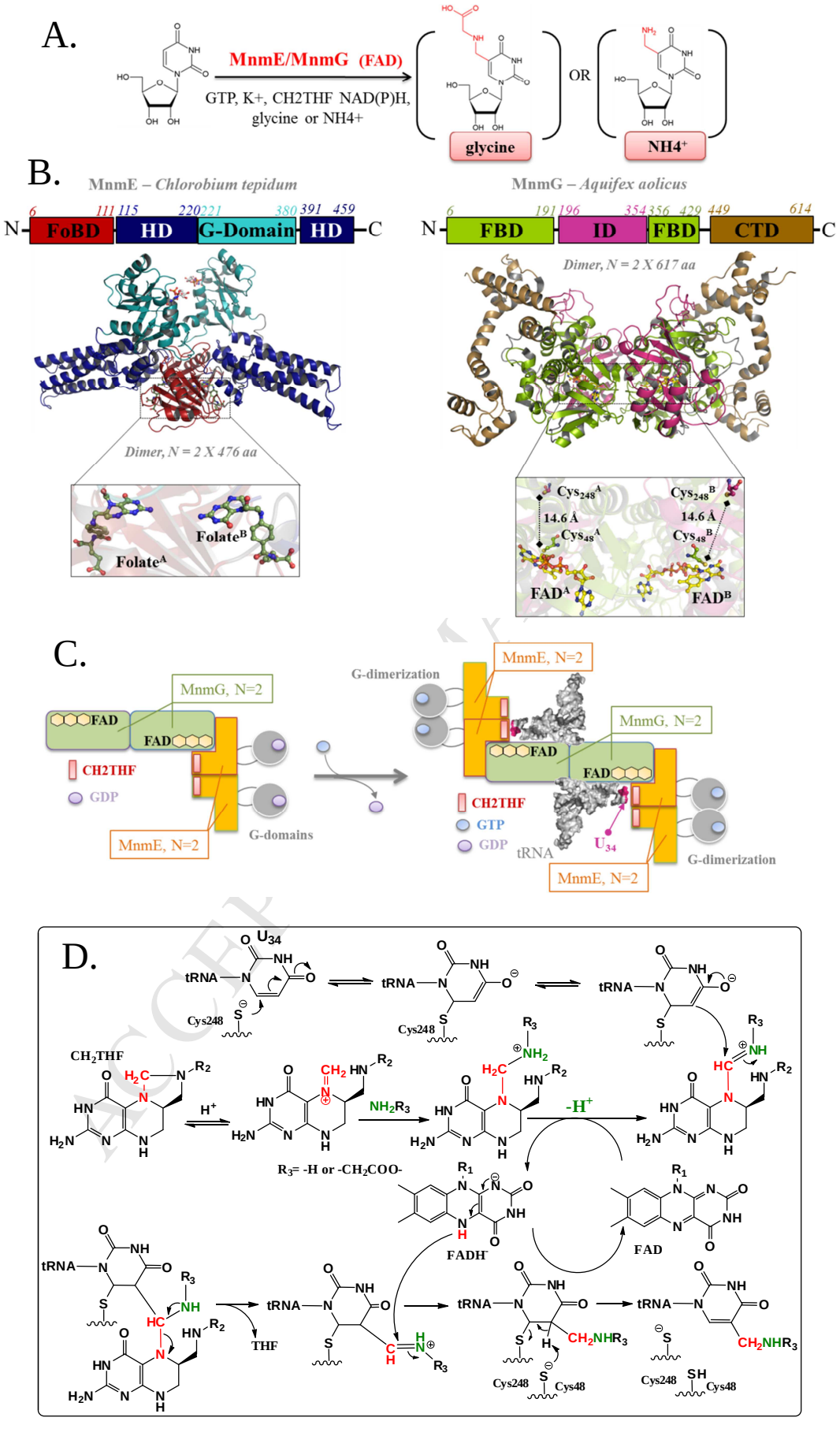

A.
MnmE/MnmG (FAD)
GTP, K+, CH2THF NAD(P)H,
glycine or NH4+
OR
glycine
NH4+
B.
MnmE – Chlorobium tepidum
MnmG – Aquifex aolicus
FoBD
HD
G-Domain
HD
FBD
ID
FBD
CTD
Dimer, N = 2 X 476 aa
Dimer, N = 2 X 617 aa
Folate^A
Folate^B
Cys248^A
Cys248^B
14.6 Å
Cys48^A
Cys48^B
FAD^A
FAD^B
C.
MnmG, N=2
FAD
CH2THF
GDP
MnmE, N=2
G-domains
G-dimerization
GTP
tRNA
U34
D.
tRNA
Cys248
CH2THF
R3= -H or -CH2COO-
FADH-
FAD
THF
Cys48

Figure 5.

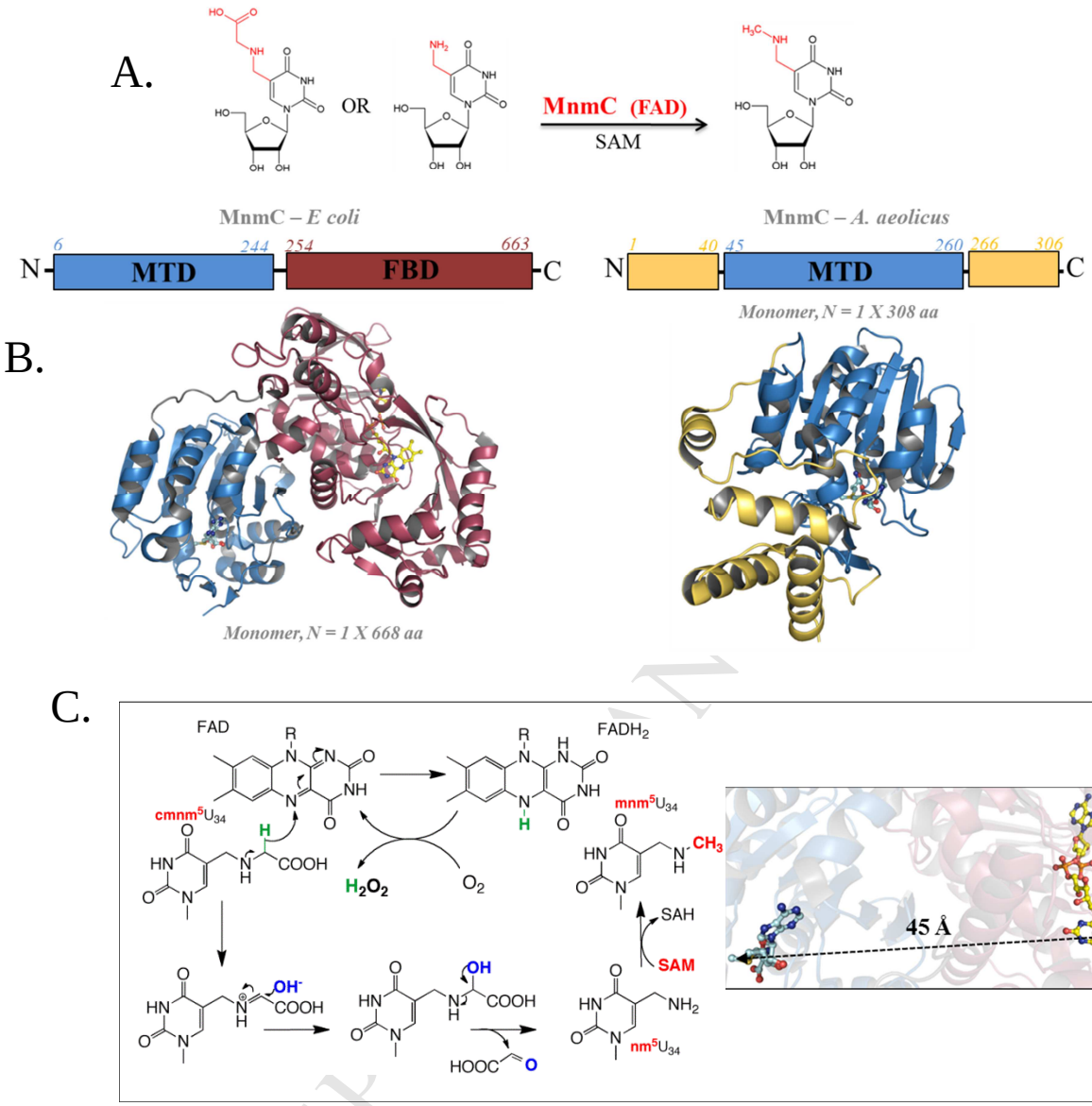
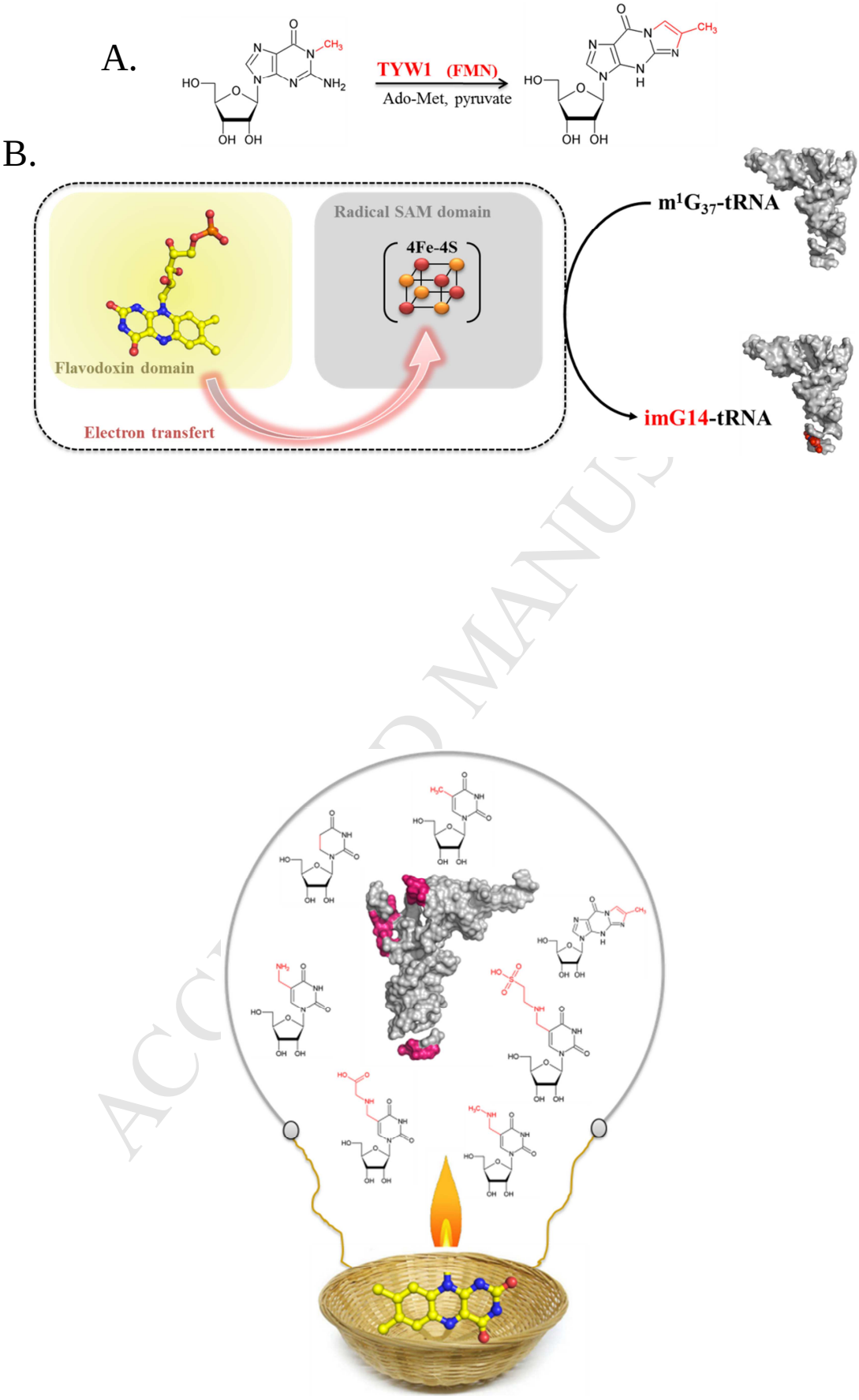

A.
OR
MnmC (FAD)
SAM
MnmC – E coli
6
244
254
663
N
MTD
FBD
C
MnmC – A. aeolicus
1
40
45
260
266
306
N
MTD
C
Monomer, N = 1 X 308 aa
B.
Monomer, N = 1 X 668 aa
C.
FAD
FADH2
cmnm5U34
mnm5U34
H2O2
O2
SAH
SAM
nm5U34
45 Å

Figure 6.


A.
TYW1 (FMN)
Ado-Met, pyruvate
B.
Radical SAM domain
4Fe-4S
Flavodoxin domain
Electron transfert
m1G37-tRNA
imG14-tRNA