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## Invited Review

# MnmE, a GTPase That Drives a Complex tRNA Modification Reaction

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### ABSTRACT:

*MnmE is a multi-domain GTPase that is conserved from bacteria to man. Together with its partner protein MnmG it is involved in the synthesis of a tRNA wobble uridine modification. The orthologues of these proteins in eukaryotes are targeted to mitochondria and mutations in the encoding genes are associated with severe mitochondrial diseases. While classical small GTP-binding proteins are regulated via auxiliary GEFs and GAPs, the GTPase activity of MnmE is activated via potassium-dependent homodimerization of its G domains. In this review we focus on the catalytic mechanism of GTP hydrolysis by MnmE and the large scale conformational changes that are triggered throughout the GTPase cycle. We also discuss how these conformational changes might be used to drive and tune the complex tRNA modification reaction. © 2016 Wiley Periodicals, Inc. *Biopolymers* 105: 568–579, 2016.*

*Keywords: MnmE; MnmG; GTPase; G proteins activated by dimerization; tRNA modification*

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### INTRODUCTION

Guanine nucleotide binding proteins (GNBPs or G proteins) regulate a vast amount of cellular processes ranging from sensory perception to protein synthesis, cell growth, and differentiation.<sup>1</sup> One important member of these G proteins, which is conserved in bacteria and eukaryotes, is called MnmE (formerly known as TrmE). MnmE forms a transient complex with MnmG (also known as GidA), and together these proteins play a vital role in tRNA modification.<sup>2–4</sup> tRNA modification is a crucial step in the processing of precursor tRNA toward fully matured tRNA molecules. All four canonical nucleotides can undergo posttranscriptional modifications resulting in a bewildering diversity of modifications acting in a combinatorial manner (reviewed in Ref. 5). These modifications range from simple methylations to complex multi-carbon modifications and occur throughout the whole tRNA molecule. Today, more than a hundred types of RNA modifications are known of which at least 93 are present in tRNAs<sup>6,7</sup> and on average 11.9% of all nucleotides in a given

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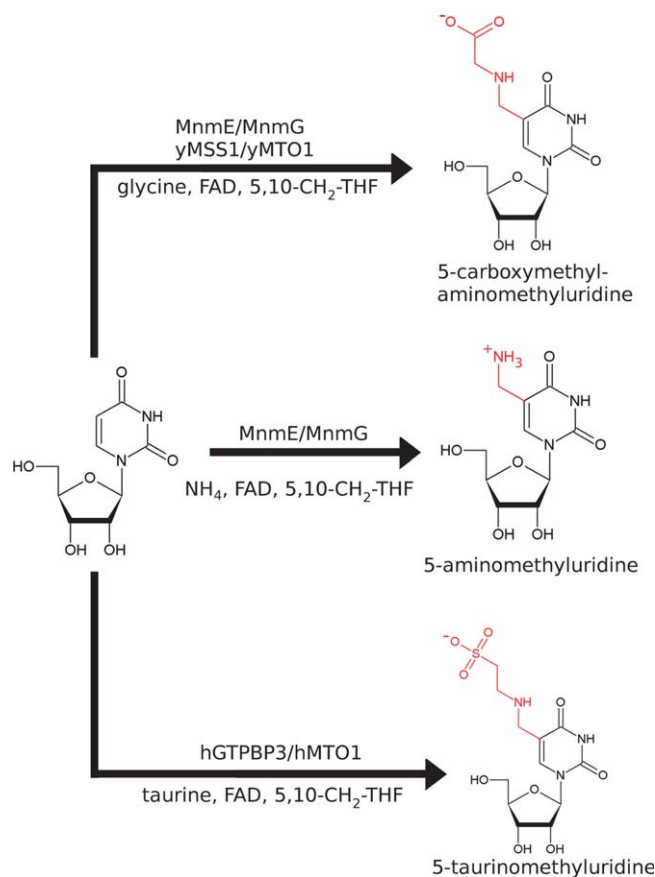
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tRNA molecule are modified.<sup>8</sup> These tRNA modifications can influence the binding of aminoacyl tRNA synthetases,<sup>9,10</sup> the local and global structure of tRNA<sup>11–13</sup> or the codon-anticodon pairing within the ribosome.<sup>14</sup>

Modifications in or adjacent to the anticodon are particularly important, because during the translation process ribosomes have to select the correct tRNA and reject many near cognate tRNAs while sampling them in their aminoacyl site (A-site).<sup>15,16</sup> Of special interest in the process of tRNA recognition within the ribosome is the tRNA modification at position 34, known as the wobble position. Although at the wobble position all four canonical nucleotides can undergo modification, this is especially prevalent and diverse in the case of uridine (U34), which is nearly always modified.<sup>17</sup> One class of modifications at U34 is the (c)mnm<sup>5</sup>(s<sup>2</sup>) [5-(carboxy)methylaminomethyl-(2-thio)]-type modification which ensures the recognition of codons ending in A or G.<sup>17</sup> The selective recognition and interaction of these two different nucleotides by the modified uracil is likely achieved via keto-enol tautomerization of the uracil, which is influenced by the substituent on the C5 position of uridine.<sup>18</sup> The (c)mnm<sup>5</sup>(s<sup>2</sup>) modification moreover makes the anticodon more rigid and therefore prevents the recognition of U and C at the third codon position.<sup>17,19</sup> In contrast, an unmodified U34 can recognize and interact with all four nucleotides<sup>20</sup> and would thereby lead to misincorporation of amino acids in the growing polypeptide chain.

In *E. coli*, six tRNAs are modified by a (c)mnm<sup>5</sup>(s<sup>2</sup>)-type modification.<sup>21</sup> The pathway of (c)mnm<sup>5</sup>(s<sup>2</sup>)U34 synthesis is complex and involves many proteins acting in series. However, the modification at the C5-position and C2-position occur independently. The first step of the C5 modification always involves the MnmE/MnmG (MnmEG) complex. Depending on the substrate of MnmEG an unmodified uridine is converted either into nm<sup>5</sup>U (5-aminomethyluridine) using ammonium as a substrate or cmnm<sup>5</sup>U (5-carboxymethylaminomethyluridine) using glycine as a substrate (Figure 1). MnmE is highly conserved in bacteria<sup>22</sup> and was recently also discovered in two different *Archaea*.<sup>22,23</sup> Furthermore, homologues of MnmE and MnmG are found in most eukaryotes including yeast and humans. In eukaryotes, the transcripts of *mnmE* and *mnmG* contain a 5' mitochondrial targeting signal.<sup>22</sup> Moreover, experiments have confirmed the mitochondrial localization of the yeast and human counterparts of MnmE<sup>24,25</sup> as well as MnmG.<sup>26</sup> In yeast, the MnmE and MnmG homologues are called MSS1 and MTO1, respectively, and were shown to be responsible for the cmnm<sup>5</sup>U34 modification of some mitochondrial tRNAs.<sup>26</sup> Curiously, in human mitochondria the MnmE and MnmG homologues, GTPBP3 and MTO1, incorporate taurine instead of glycine into tRNA, leading to 5-taurinomethyluridine ( $\tau$ m<sup>5</sup>U)<sup>27–29</sup> (Figure 1).

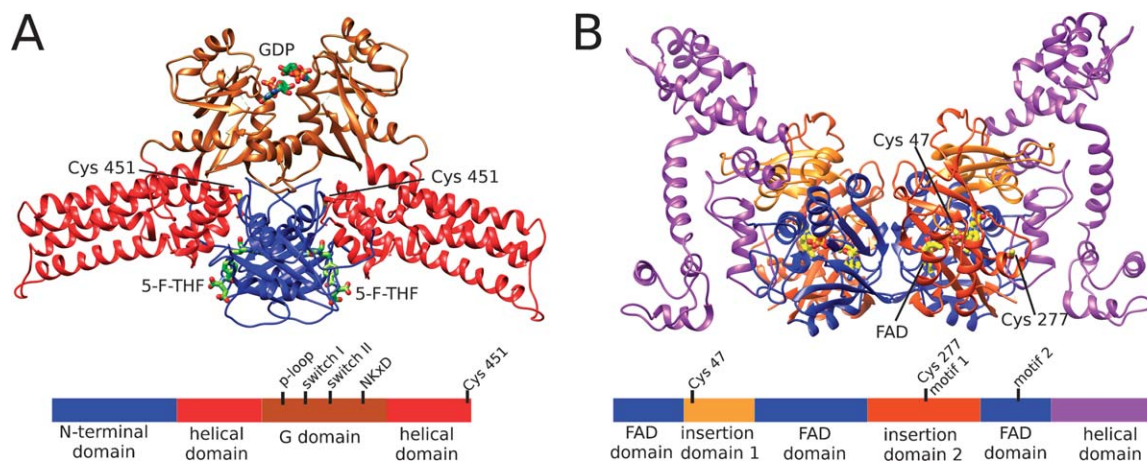


**FIGURE 1** The tRNA wobble modifications introduced by the MnmEG complex and its eukaryal homologues. In addition to FAD, 5,10-CH<sub>2</sub>-THF and potentially also NADH, the bacterial MnmEG complex uses either glycine or ammonium to convert uridine into 5-carboxymethylaminomethyluridine or 5-aminomethyluridine, respectively. In yeast 5-carboxymethylaminomethyluridine is formed by the yMSS1/yMTO1 complex. In contrast, the homologous human complex, hGTPBP3/hMTO1 utilizes taurine to convert uridine into 5-taurinomethyluridine. The modifications from the canonical base are highlighted in red.

In this review we will first focus on MnmE's G domain and the conformational changes occurring during the GTPase cycle. Subsequently, we will focus on the interaction with MnmG and discuss the complex interplay of these two partners during tRNA modification. Finally, we will highlight some examples on the role of the MnmEG complex and their tRNA modification in human diseases.

### MnmE IS A MULTI-DOMAIN HAS-GTPase ACTIVATED BY POTASSIUM

MnmE is a homodimeric protein with 50 – 55 kDa subunits.<sup>30</sup> Crystal structures have been reported of MnmE from *Thermotoga maritima* (TmMnmE), *Chlorobium tepidum* (CtMnmE) and *Nostoc* (NoMnmE).<sup>31,32</sup> All current structures confirm the



**FIGURE 2** Cartoon representation and domain architecture of (A) MnmE and (B) MnmG. MnmE consists of an N-terminal domain (blue), a helical domain (red) and a G domain (brown). MnmG consists of an FAD-binding domain (blue), two insertion domains (orange and red–orange) and a helical domain (purple). The position of the different motifs and catalytic cysteines are marked as bars on the domain architectures.

dimeric nature of MnmE, where each subunit consists of three discernible domains: an N-terminal domain required for dimerization, an all helical domain and a G domain, which in the primary structure is inserted in the helical domain (Figure 2A).

The N-terminal domain of MnmE consists of a five-stranded mixed  $\beta$ -sheet and three  $\alpha$ -helices and is a major contributor to the dimerization interface of the MnmE homodimers. The overall structure of this domain resembles the tetrahydrofolate (THF) binding domain of *N,N*-dimethylglycine oxidase (DMGO), and subsequent binding studies also show that EcMnmE is able to bind THF derivatives with an affinity in the low micromolar to sub-micromolar range.<sup>4,31</sup> Structures either soaked (for TmMnmE and CtMnmE) or co-crystallized (NoMnmE) with 5-formyl-THF show that two symmetry-related 5-formyl-THF molecules bind on the dimer interface of the N-terminal domains. The  $\alpha$ -helical domain consists of three to six small  $\alpha$ -helices and four long helices forming a 4-helix bundle. The C-terminal residues of this domain, which are not part of a helix, come in relatively close proximity to the formyl group of the 5-formyl-THF group bound to the N-terminal domains. These C-terminal amino acids form a highly conserved FC(V/I/L)GK motif, of which the cysteine residue (Cys451, EcMnmE numbering) was found to be important for the tRNA modification reaction *in vivo* (see further).<sup>33</sup> The G domain of MnmE closely resembles the canonical G domain, as e.g., observed in Ras, with 6  $\beta$ -strands and 5  $\alpha$ -helices.<sup>31</sup> Also, at least 4 of the 5 sequence motifs that typify G proteins are conserved: the GxxxxGK(S/T) motif in the P loop, a conserved threonine in switch I, the DxxG motif

in switch II and the NKxD motif responsible for specificity toward a guanine nucleobase (Figure 3A).<sup>1</sup> In the different crystal structures both switch regions are highly flexible and switch II was not observable in the electron density.

Early on it was realized that the G domain of MnmE contains a number of peculiar biochemical features compared to the “classical” molecular switches as exemplified by Ras. These latter GTP binding proteins typically show a very low intrinsic GTPase activity combined with high affinities for the substrate GTP and product GDP, resulting in very low off rates of GDP. As such these proteins require auxiliary GTPase activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs) to catalyze hydrolysis and nucleotide release in order to be able to proceed through the GTPase cycle.<sup>34</sup> In contrast, the G domain of *E. coli* MnmE (EcMnmE) displays a rather low affinity for guanine nucleotides, with reported affinities for GDP in the range of 0.6  $\mu$ M–4.1  $\mu$ M and for stable GTP analogues (GppNHp or GTP $\gamma$ S) in the range of 1.5  $\mu$ M–6  $\mu$ M.<sup>31,35</sup> The dissociation rates ( $k_{\text{off}}$ ) of GDP have been found to be correspondingly high, with reported values varying from 2.2  $\text{s}^{-1}$  to 3.3  $\text{s}^{-1}$ .<sup>31,36</sup> Also the EcMnmE mediated GTP hydrolysis rate is relatively high compared to classical G protein switches. In the absence of potassium ions and certain other monovalent ions (but in the presence of  $\text{Na}^+$ ), steady state measurements of GTP hydrolysis yield a  $k_{\text{cat}}$  value of 0.0055  $\text{s}^{-1}$ .<sup>37</sup> However, in the presence of certain monovalent ions the GTPase activity is increased significantly, with an effect depending on the ionic radius in the order  $\text{K}^+ \geq \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+$ .<sup>38,39</sup> In the presence of 150 mM  $\text{K}^+$  the  $k_{\text{cat}}$  of GTP hydrolysis is increased nearly 25-fold to 0.13  $\text{s}^{-1}$  (Figure 3B). Such concentrations



of  $K^+$  are close to reported concentrations of 100 – 200 mM in the cytoplasm of *E. coli*.<sup>40</sup> The high nucleotide dissociation rates, in combination with the high GTP turnover, would make the presence of GEF and GAP molecules obsolete. Interestingly, in the human homologue, GTPBP3, a  $k_{cat}$  value for GTP turnover was found that is about 100-fold lower than the corre-

sponding value in EcMnmE, while the binding affinities for the nucleotides are in the same range.<sup>29</sup> This leaves open the possibility that in these cases an extra regulation through GAP proteins is still required. Since the discovery of MnmE's potassium activation, a similar mechanism has been found for other GTPases, including YqeH, FeoB, RbgA, and EngA.<sup>41–44</sup>

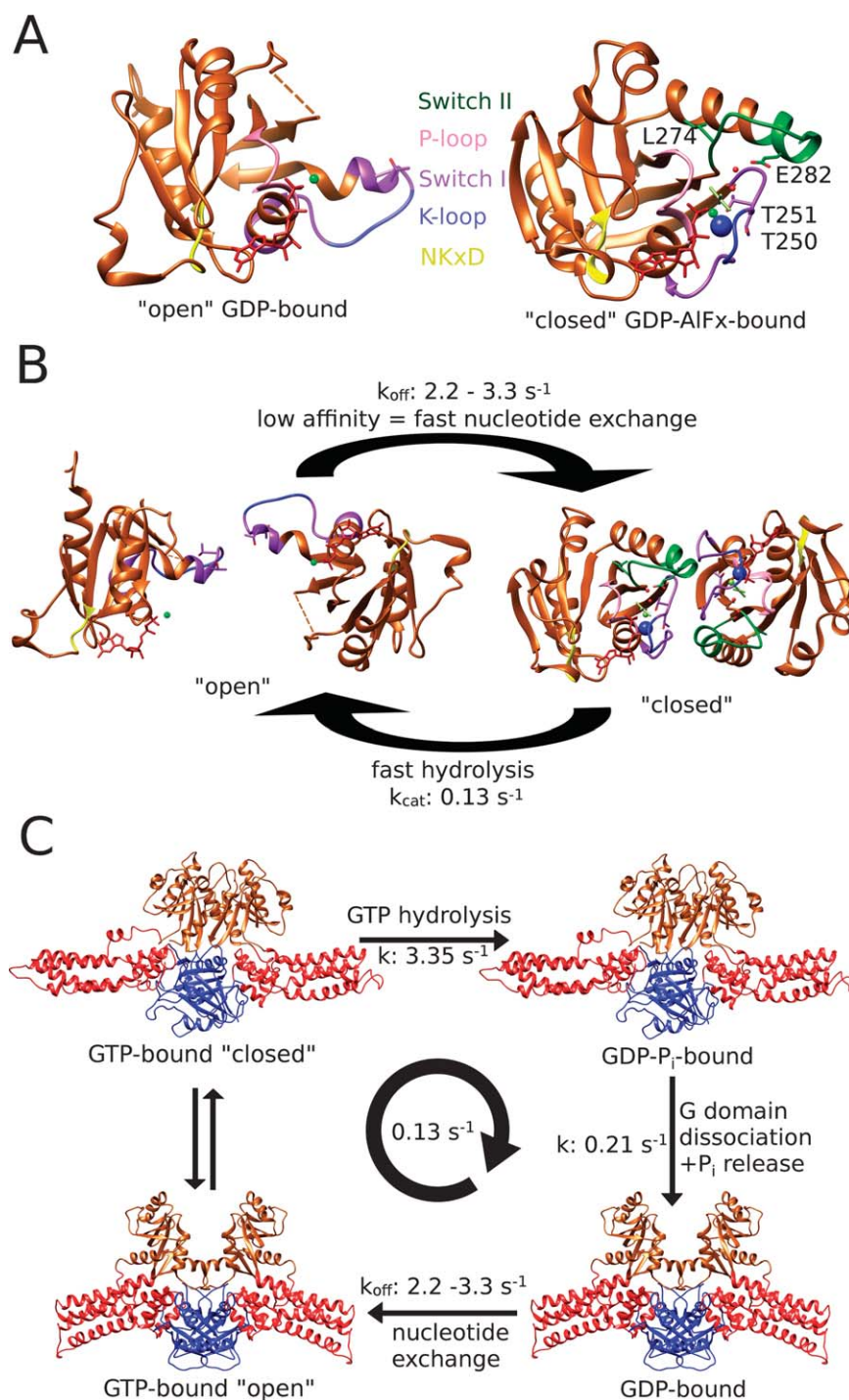


FIGURE 3.

Moreover, certain ATPases, such as GroEL, Hsc70, and YchF, also display increased ATPase activity in the presence of  $K^+$  ions.<sup>45–47</sup>

Further insight into the mechanism of GTP hydrolysis by the G domains of MnmE has been provided by crystal structures of the isolated G domain. The G domains of MnmE can be expressed on their own and retain a GTPase activity that is comparable to the full length protein.<sup>30,39</sup> When run on a size exclusion chromatography these G domains behave as monomeric proteins as expected from the crystal structures of full length MnmE. However, in the presence of both  $K^+$  and the transition state analogue GDP- $AlF_x$  a shift in elution profile toward a G domain dimer is observed. The crystal structure of the isolated G domain of EcMnmE in complex with  $Mg^{2+}$ ,  $K^+$ , and GDP- $AlF_x$  (GDP- $AlF_4^-$  in this case) correspondingly shows that the protein forms a tight dimer (buried interface of 1790 Å<sup>2</sup>).<sup>39</sup> Comparison of the latter structure with the structure of the G domain embedded in the full length protein reveals large conformational changes throughout the G domain (Figures 3A and 3B). The most notable reordering involves a structuring of the switch regions, which now form most of the inter-subunit interactions in the dimer interface. In this conformation switch I and switch II of one subunit mainly interact with switch II and switch I of the other subunit, respectively. As expected from other G proteins in their “on state”, the  $AlF_4^-$  group (mimicking the  $\gamma$ -phosphate in the transition state) interacts with the main chain amine of T250 and T251, coming from the switch I GTTRD motif, and with the main chain amine of G273, provided by the switch II DxxG motif. However, in contrast to what is expected from “classical” Ras-like G proteins, the region of switch I preceding

these two threonine residues is involved in the binding of a  $K^+$  ion required for activity and G domain dimerization. This part of the switch I region has therefore been dubbed the K-loop. Further scrutiny revealed that this  $K^+$  ion is located in a similar position in the active site of MnmE as the catalytic arginine finger in the Ras-RasGAP complex,<sup>48</sup> suggesting a similar catalytic role of  $K^+$  in stabilizing the excess of negative charges accumulating in the transition state.

Apart from the above mentioned arginine finger, supplied by the GAP protein, Ras uses an endogenous glutamine residue to orient and/or activate a water molecule to perform a nucleophilic attack on the  $\gamma$ -phosphate of GTP.<sup>49</sup> This glutamine residue (Q61 in Ras) is situated adjacent to the DxxG motif in switch II. However, sequence analysis revealed that this glutamine residue is not present in MnmE and is replaced by a hydrophobic amino acid (Leu274 in EcMnmE). This substitution is typical for the so-called HAS-GTPases (Hydrophobic Amino acid Substituted for catalytic glutamine GTPases), including e.g. Obg, FeoB, EngA and dynamin-related GTPases.<sup>41,50–52</sup> The structure of the EcMnmE G domain dimer reveals an alternative mechanism in this HAS GTPase, coupling the activation of the nucleophilic water molecule to dimerization and  $K^+$  binding. Indeed, one of most marked conformational changes induced by  $K^+$ -mediated dimerization is the ordering of switch II leading to an extension of helix  $\alpha 2$  and bringing residue Glu282 into the active site pocket. This glutamate residue is oriented appropriately to activate the nucleophilic water via a second bridging water molecule (Figures 3A and 3B). Correspondingly, mutation of Glu282 to alanine reduced the GTPase activity about 2000-fold.<sup>39</sup>

**FIGURE 3** Conformational changes of MnmE and its G domain in different nucleotide-bound states. A: Close up on a single G domain to illustrate the conformational changes that occur upon GDP- $AlF_x$  and  $K^+$  binding and switching into the “closed” state. This change involves rearrangements and structuring of switch I (purple) and switch II (green). Structuring of switch II orients E282 in the active site where it is proposed to activate the nucleophilic water via a bridging water molecule (red dots). A potassium ion (blue sphere), that acts as a GTPase activating element is coordinated via the K-loop (blue) which is part of switch I. The P loop and the NKxD motif are shown in pink and yellow, respectively. The  $Mg^{2+}$  ion is shown as a green sphere and GDP or GDP- $AlF_x$  (here GDP- $AlF_4^-$ ) are shown in stick representation with the GDP part in red and  $AlF_4^-$  in lime green. B: The transition from the “open” to the “closed” state is achieved via a fast nucleotide exchange to the GTP-bound state in the presence of  $K^+$  ions and is accompanied by the dimerization of the G domains. This in turn brings the catalytic machinery in position for a fast GTP hydrolysis, leading to dissociation of the G domains. The associated rate constants are indicated. C: During the GTPase cycle the G domains (brown) and helical domains (red) of MnmE adopt at least two different conformations with respect to the N-terminal domain (blue). In the GDP bound state the G domains are in the “open” conformation and the helical domains point downward (as observed in the crystal structure). In the transition state of GTP hydrolysis, and presumably also in the GDP- $P_i$ -bound state, the G domains are in the “closed” conformation and the helical domains make an upward movement. In the GTP-bound state MnmE is in an equilibrium between the “open” and “closed” states. The rate constants associated with each step are indicated on the arrows and the overall turnover rate ( $k_{cat}$ ) is shown in the center.

### MnmE IS A PROTOTYPE OF THE FAMILY OF “G PROTEINS ACTIVATED BY NUCLEOTIDE-INDUCED DIMERIZATION”

The structure of the dimeric G domains of EcMnmE bound to GDP-AlF<sub>4</sub><sup>-</sup> and K<sup>+</sup> (see above) would suggest that such a G domain dimerization could be an integral part of the GTPase reaction cycle of MnmE.<sup>39</sup> However, all currently available structures of full length MnmE show the protein dimerized via its N-terminal domains, while the symmetry related G domains are oriented with their nucleotide binding pockets toward each other but not contacting each other, with distances of 25–34 Å between the β-phosphates of the bound nucleotides (“open” state). Superposition of the full length structures furthermore shows very large rotational and translational displacements of the G domains between the structures, indicating a high flexibility of the G domains vis-à-vis the other domains. While the structure of TmMnmE was solved with its G domain in the apo form, the structure of CtMnmE was solved in complex with GDP or the nonhydrolysable GTP analogue GppCp and the one from NoMnmE in complex with GDP. Despite attempts to crystallize CtMnmE and NoMnmE in presence of K<sup>+</sup> and the transition-state analogue GDP-AlF<sub>x</sub>, these structures only contain a GDP molecule in the nucleotide binding pocket, possibly because commonly used molecules in crystallization solutions (e.g., glycerol, PEG) inhibit the interaction of the G domains.<sup>32</sup> A first proof that full length EcMnmE is indeed going through an open-close cycle was obtained via distance measurements by pulse double electron-electron resonance (DEER) spectroscopy.<sup>32</sup> In the nucleotide-free and GDP-bound state, the inter-spin distances that were obtained by this method corresponded to the expected distances from the crystal structures with the G domains in the “open” state. However, in the presence of the transition state analogue GDP-AlF<sub>x</sub> and certain ions large domain movements of up to 20 Å are observed, corresponding to the expected transition from the “open” to the “closed” conformation as observed in the crystal structure of the isolated G domains in presence of GDP-AlF<sub>4</sub><sup>-</sup> and K<sup>+</sup>. The ability of ions to stabilize this closed conformation varied in the order K<sup>+</sup> > Rb<sup>+</sup> > NH<sub>4</sub><sup>+</sup> > Cs<sup>+</sup> ≈ Na<sup>+</sup>, which also correlates with the ability of these ions to stimulate GTP hydrolysis. In the presence of the ground state GTP analogue, GppNHp, an intermediate situation is observed with the distance distribution showing two different populations corresponding to 70% of the population in the “open” conformation and 30% in the “closed” conformation. The open-close transition in full length EcMnmE was subsequently also confirmed by small angle X-ray scattering experiments (SAXS), where scatter data of EcMnmE in the apo-form nearly perfectly correspond to the theoretical scatter profile of the crystal struc-

tures of MnmE in the open state.<sup>53</sup> Binding to GDP-AlF<sub>x</sub> and K<sup>+</sup> leads to clear changes in the scatter profile, indicative of large conformational changes. These scatter curves can however be accounted for by the dimerization of the G domains of MnmE accompanied by a less pronounced “upward” movement of the α-helical domains (Figure 3C). In agreement with the DEER experiments, the SAXS profiles in the presence of GppNHp and K<sup>+</sup> can only be accounted for by an equilibrium between the “open” and “closed” states.

Based on these data and the available high-resolution structures of the G domains bound to GDP-AlF<sub>x</sub>, a mechanism for the GTPase activity of MnmE can be proposed<sup>39</sup> (Figure 3B). Binding of GTP and certain monovalent ions such as K<sup>+</sup> lead to dimerization of the G domains of MnmE. This in turn causes an ordering of the switch I and II regions in a catalytically competent conformation where the switch regions of adjacent G domains interact with each other. K<sup>+</sup> is held in place via the K-loop, located within the switch I region. The bound K<sup>+</sup> ion is ideally located to stabilize the developing negative charges in the transition state of GTP hydrolysis and hence takes up the role of the “arginine finger” in classical Ras-GAPs. Ordering of switch II moreover brings Glu282 into the active site pocket. This glutamate residue is oriented appropriately to activate the nucleophilic water via a second bridging water molecule. Together, these rearrangements assemble all the catalytic machinery necessary for GTP hydrolysis, leading to P<sub>i</sub> release and opening of the G domains (Figure 3). A kinetic study of the EcMnmE-catalyzed GTPase cycle<sup>36</sup> has shown that the single turnover rate of the GTP hydrolysis reaction is 3.35 s<sup>-1</sup>, while the G domains dissociate with a rate of 0.21 s<sup>-1</sup>, the latter being very close to the multiple turnover k<sub>cat</sub> value of GTP hydrolysis. This means that G domain dissociation, or a process closely associated to G domain dissociation (such as P<sub>i</sub> release), is the rate limiting step in the GTPase cycle (Figure 3C). Curiously, in an *in vitro* setting MnmE is able to catalyze GTP hydrolysis not only in the absence of any GEF or GAP protein, but also in the absence of its partner protein MnmG and its physiological substrate tRNA, seemingly leading to the futile consumption of GTP molecules. Therefore, it would be expected that either extra regulatory mechanisms would be in place *in vivo* or that the protein is nearly constantly saturated with tRNA substrates under physiological conditions.

The proposed mechanism of MnmE is characteristic for a relatively recently described functional group of G proteins, dubbed G proteins activated by nucleotide-induced dimerization or GADs.<sup>54</sup> Members of this group of G proteins all show a low affinity for nucleotides (μM range) making the involvement of GEFs obsolete. Moreover, the relatively high GTPase activity induced by transient dimerization of the G domains



also bypasses the requirement for GAPs. A unifying principle of these GADs is that they are believed to execute their biological function in the dimeric state, possibly through interaction with an effector protein. The GTPase reaction, leading to disassembly of the G domains, would hence function as a molecular timer for these biological effects. However, to understand the possible function and implications of the GTP-induced conformational changes in MnmE, one first needs to discuss the physiological role of MnmE in tRNA modification, for which it intimately collaborates with its partner protein MnmG.

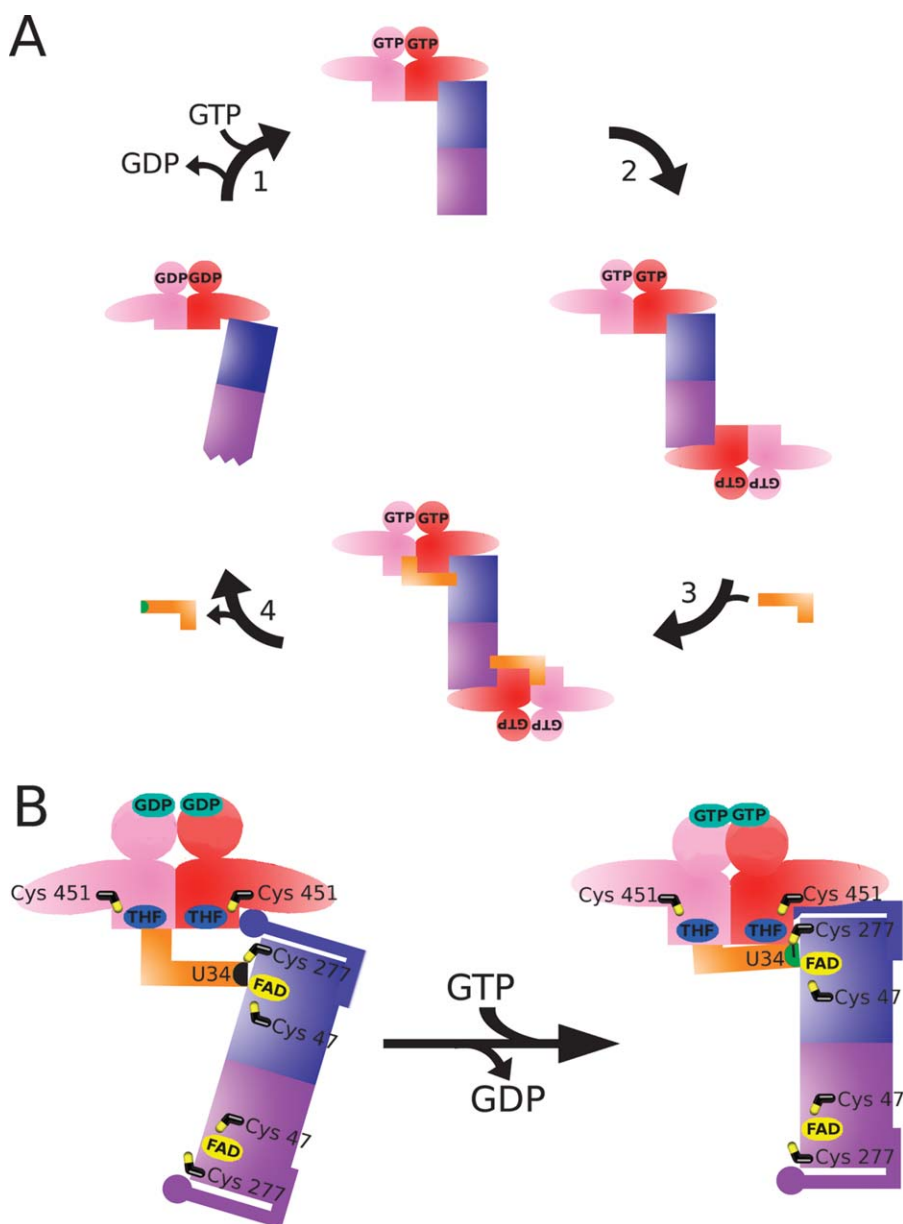
### MnmE AND MnmG COOPERATE IN A COMPLEX WAY TO CATALYZE tRNA MODIFICATION

In 2006 Yim et al. showed, using gel filtration and native PAGE experiments, that a MnmE homodimer can interact with an MnmG homodimer to form an  $\alpha_2\beta_2$  complex.<sup>2</sup> Knock out mutants of either MnmE or MnmG result in the same type of s<sup>2</sup>U hypomodified tRNA, leading to the conclusion that MnmE and MnmG form a functionally intertwined complex where the two proteins collaborate in catalyzing the modification reaction, rather than independently catalyzing different steps of the modification reaction.<sup>2</sup> Authors from the same group moreover showed that in bacteria the result of the modification reaction depends on both the identity of the substrate tRNA and the growth conditions.<sup>4,55</sup> More specifically it was shown that under conditions of exponential growth preferentially glycine is used as one of the substrates, leading to incorporation of a carboxymethylaminomethyl (cmnm) at position C5 of U34, while under conditions of high cell densities ammonium is used preferentially as a substrate leading to incorporation of an aminomethyl group (nm) (see Ref. 56 for a review on the different pathways of wobble uridine modification).

MnmG is a dimeric protein of about 70 kDa subunits that binds the cofactors FAD and NADH (Figure 2B).<sup>3</sup> Structural information on the MnmG protein is available for representatives of *E. coli*, *Chlorobium tepidum* and *Aquifex aeolicus*.<sup>3,57,58</sup> These crystal structures show that each MnmG subunit consists of a typical FAD-binding domain, a small and larger domain inserted into the fold of the FAD-binding domain and a C-terminal all-helical domain. A large positively charged patch on the surface of this protein already suggested that within the MnmEG complex, the latter would be mainly responsible for tRNA binding. This hypothesis was later confirmed via a gel retardation experiment.<sup>57</sup> Moreover, in addition to the conserved cysteine residue of MnmE (part of

the C-terminal “FC(V/I/L)GK” motif) that is implicated in tRNA modification, also two catalytically indispensable cysteine residues were identified in MnmG (Cys47 and Cys277 using *E. coli* numbering).<sup>57</sup> While Cys47 is stacked against the isoalloxazine ring of FAD, Cys277 is located on a flexible loop about 10-15 Å away from the first cysteine. A first successful *in vitro* reconstitution of the MnmEG-catalyzed tRNA modification reaction was achieved using total small RNA purified from a  $\Delta mnmE$  *E. coli* knock-out strain with [2-H<sup>3</sup>] glycine, 5-formyl-THF, FAD and NADH as substrates in the presence of GTP and KCl.<sup>37</sup> This assay was based on a previous proposal that 5-formyl-THF would be the donor of the first carbon of the carboxymethylaminomethyl group.<sup>31</sup> However, it was subsequently shown that the reaction could equally well proceed without externally added tetrahydrofolate derivative as carbon donor, indicating that a methyl donor is co-purified with one of the two enzymes.<sup>4</sup> Based on additional experimental indications, and according to analogy with other uridine methylating enzymes such as TrmFO and ThyA,<sup>59-64</sup> 5,10-methylene-THF can be put forward as the most likely candidate for the donor of the first methyl group in the MnmEG-catalyzed reaction. According to these findings an alternative mechanism for the MnmEG-catalyzed tRNA modification reaction was proposed (see Refs. 55,56,65 for details; Figure 4). In the first step 5,10-methylene-THF (bound to MnmE) would be converted to a reactive iminium ion. In a next step this activated THF intermediate would react with either ammonium or with the amino group of glycine (leading to respectively a nm<sup>5</sup>U or cmnm<sup>5</sup>U modified wobble uridine as a final modification). This MnmE-bound THF intermediate is suggested to be dehydrogenated by MnmG-bound FAD. Subsequently, this THF-bound iminium group is transferred from THF to the C5 atom of U34 concomitant with nucleophilic attack of MnmG-Cys277 on the C6 atom of U34 forming a covalent MnmG-tRNA adduct. This intermediate is reduced by FADH<sub>2</sub>. Finally, deprotonation of C5 leads to cleavage of the C6-Cys277 covalent bond and completion of the modification reaction cycle. However, lacking further detailed analysis this mechanism still remains largely speculative for the moment. It will be especially intriguing to find out how a complex and large substrate as a tRNA molecule is modified by an enzyme complex consisting of two proteins, using potentially three cofactors where FAD (and probably NADH) is bound to MnmG and 5,10-methylene-THF to MnmE. Moreover, three cysteines are required for the reaction to occur, again with one of these residues located on MnmE and the other two on MnmG, and where one of the latter cysteines forms a covalent adduct with the substrate at least during certain steps of the reaction cycle.





**FIGURE 4** Model of the MnmEG complex during the tRNA modification cycle (MnmE is depicted in red and pink; MnmG in blue and purple; tRNA in orange). A: While MnmE is in the GDP-bound state, MnmEG forms an asymmetric  $\alpha_2\beta_2$  complex. Binding of MnmG to MnmE induces dimerization of the G domains. (Step 1) Binding of GTP to the MnmEG complex leads to tightening of the “closed” conformation of the G domains of MnmE with a concomitant upward movement of its helical domains, which in turn leads to a conformational change in MnmG. (Step 2) This change allows the binding of a second MnmE dimer to MnmG leading to an  $\alpha_4\beta_2$  complex. (Step 3) In the following step one or two tRNA molecules bind to the complex mainly via interactions with MnmG. (Step 4) Upon GTP hydrolysis and  $P_i$  release the tRNA becomes modified (indicated in green) and is released from the complex, which returns into the  $\alpha_2\beta_2$  form. (B) Close-up on the proposed changes in the MnmE-MnmG-tRNA interaction upon GTP binding. For clarity only one MnmE dimer is shown bound to MnmG in the GTP-bound state. The conformational changes could bring the active sites of MnmE and MnmG in closer proximity, which is required for the concerted action of the two cofactors (FAD and 5,10-CH<sub>2</sub>-THF) and three cysteine residues in the tRNA U34 modification reaction. The cofactors are depicted with ovals (GDP: green; 5,10-CH<sub>2</sub>-THF: blue; FAD: yellow) while the catalytic cysteines are shown as sticks.

## GTP HYDROLYSIS INDUCES MAJOR CHANGES IN THE MnmEG COMPLEX AND ORCHESTRATES THE tRNA MODIFICATION REACTION

Insights into the mechanism of tRNA modification could be provided by high resolution structural information regarding the MnmEG complex, potentially showing how the THF binding site and FAD binding site of MnmE and MnmG, respectively, collaborate. However, until now attempts to crystallize an MnmEG complex have proven unsuccessful to the best of our knowledge. A low resolution model of an  $\alpha_2\beta_2$  MnmEG complex, representing MnmE in the nucleotide-free form, was obtained via small angle X-ray scattering (SAXS) and rigid protein docking.<sup>53</sup> Unexpectedly, this model suggests that MnmE and MnmG interact in an asymmetric “head-to-tail” fashion, where one MnmE dimer is binding via the N-terminal domain and the helical domain of one subunit to the C-terminal domain of one subunit of the MnmG dimer (Figure 4). This arrangement thus leaves one subunit vacant on MnmE as well as one on MnmG. Although in this model the THF and FAD binding sites on MnmE and MnmG are oriented toward each other, both cofactors are still separated by 30 Å. Moreover, the G domains of MnmE are separated from the MnmE/MnmG interface by a distance of >25 Å, and more than 60 Å separates them from the FAD binding site on MnmG. This model hence raises questions on the role of GTP binding and/or hydrolysis in the tRNA modification reaction.

Classical G proteins act as conformational switches oscillating between an inactive GDP-bound state and an active GTP-bound state.<sup>1,54</sup> Hence, as long as the protein is bound to GTP the switch is in the “ON” state and downstream effects are being triggered. Surprisingly, however, EcMnmE mutants that are deficient in GTP hydrolysis but are still capable of binding GTP with almost wild-type affinities are unable to complement for the mnm<sup>5</sup>U modification of tRNA in a *mnmE* deletion strain.<sup>33</sup> This is a clear indication that the GTP-bound state is not an active state, but rather that active GTP turnover is needed for the *in vivo* tRNA modification activity of MnmE. This finding was further corroborated by showing that in the presence of K<sup>+</sup> ions only GTP can support tRNA modification in an *in vitro* assay, but not GDP nor stable GTP analogues or transition state analogues (GppCP and GDP-AlF<sub>x</sub>).<sup>37</sup> Likewise, mutations on MnmE that disrupt either GTP hydrolysis or interfere with the closing of the G domains are deficient in *in vitro* tRNA modification. The long distance between the GTP binding site in MnmE and the active sites involved in tRNA modification makes it highly unlikely that GTP is directly involved in the modification reaction. Rather, the open/close transition of the G domains upon GTP binding and hydrolysis

seems to be relayed throughout the entire MnmEG complex. This “conformational communication” from MnmE to MnmG is illustrated by the effect of the nucleotide-state of MnmE on its affinity for MnmG.<sup>37</sup> The reciprocity of this communication is shown by the observation that binding of MnmG to MnmE induces closing of the MnmE G domains, thereby stimulating its GTP hydrolysis rate 6-fold.<sup>37,66</sup> Although in the absence of high resolution crystal structures of the MnmEG complex in different nucleotide-bound states the exact nature of these conformational states remains elusive, one can speculate that they are required to tune the tRNA modification reaction by bringing active sites on MnmE and MnmG in close proximity during certain steps of the reaction (Figure 4B).<sup>37</sup>

Finally, the most recent SAXS studies have revealed another degree of complexity regarding the functional cycle of the MnmEG complex (Figure 4A).<sup>53</sup> In addition to MnmE’s conformational changes upon GTP binding, we also observed a change in stoichiometry of the MnmEG complex depending on the bound nucleotide. In the GDP-bound state MnmE and MnmG form an  $\alpha_2\beta_2$  complex (i.e., one MnmE dimer bound to one MnmG dimer), whereas in the K<sup>+</sup> and GTP-bound state MnmE and MnmG form an  $\alpha_4\beta_2$  complex (i.e.,  $\alpha_2\beta_2\alpha_2$  where one MnmG dimer is flanked on each side by one MnmE dimer).<sup>53</sup> The latter oligomeric state can be obtained stably by replacing GTP with GDP-AlF<sub>x</sub>. Time-resolved SAXS has moreover shown that the GTP-induced formation of this  $\alpha_4\beta_2$  complex occurs on a time scale fast enough to be physiologically relevant (<20 ms), while its disassembly is directly coupled to the rate of GTP hydrolysis.<sup>53</sup> Again, this suggests that the interconversion between the  $\alpha_2\beta_2$  and  $\alpha_4\beta_2$  states is an integral part of the MnmEG catalytic cycle, although the exact implications for the tRNA modification reaction await further elucidation.

## MUTATIONS IN GTPBP3 AND MTO1 LINK TO SEVERE MITOCHONDRIAL DISEASES

The human homologues of MnmE and MnmG, called GTPBP3 and MTO1, are nuclear-encoded proteins that are targeted to mitochondria where they play a role in the efficiency of the mitochondrial translation process via modification of mitochondrial tRNA molecules.<sup>25,67</sup> The mitochondrial genome contains 37 genes, 22 encoding tRNA, 2 encoding rRNA and 13 encoding proteins of the respiratory chain complexes and ATP synthase. For the synthesis of the latter the organelle uses its own protein synthesis machinery. Like in the cytoplasm, the efficiency of mitochondrial translation is affected by tRNA modifications. As such, mutations in these tRNA genes or in the (nuclear-encoded) modification proteins

lead to impaired oxidative phosphorylation, which ultimately affects organs with high energy demand such as the brain and muscle.<sup>68,69</sup> GTPBP3 and MTO1 catalyze the formation of 5-taurinomethyluridine ( $\tau\text{m}^5\text{U}_{34}$ ) at the wobble position of mitochondrial tRNA<sup>Leu</sup>(UUR), tRNA<sup>Gln</sup>, tRNA<sup>Trp</sup>, tRNA<sup>Lys</sup> and tRNA<sup>Glu</sup>. Correspondingly, point mutations in mitochondrial tRNA<sup>Leu</sup>(UUR) and tRNA<sup>Lys</sup> are associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy associated with ragged-red fibers (MERRF), respectively.<sup>70–73</sup> While these mutations are present in the D loop and anticodon stem of tRNA<sup>Leu</sup>(UUR) and in the T loop of tRNA<sup>Lys</sup>, they lead to the absence of the wobble  $\tau\text{m}^5\text{U}$  modification, probably in turn resulting in the clinical manifestation.

Subsequently, mutations in the genes coding for MTO1 and GTPBP3 have been linked to the occurrence of hypertrophic cardiomyopathy (HCM), lactic acidosis and encephalopathy.<sup>68,69,74</sup> Whole-exome next-generation sequencing of HCM patients revealed several point mutations (Gly59Ala, Thr308Ala, Thr436Ile, Ala453Thr, and Arg502His) and also a frame shift mutation (at Arg645) in MTO1 that link to HCM and lactic acidosis (note that an amino acid numbering is used here that includes the mitochondrial targeting signal).<sup>68,69</sup> Mutations in GTPBP3 leading to HCM, lactic acidosis and encephalopathy were identified in 11 patients.<sup>74</sup> Six of these patients displayed neurological symptoms (epileptic seizures, intellectual disability, developmental delay, feeding difficulties, muscle hypotonia, fatigue, and visual impairment). These clinical mutations included ten point mutations (Arg3Leu, Glu142Lys, Glu159Val, Ala162Pro, Ala222Gly, Glu225Lys, Pro257His, Ala322Pro, Asp337His, and Glu459Lys), two frame shift mutations (at Gln11 and Pro430) and two deletions (Asp223-Ser270, Gly312-Val319). Homology modelling of the human  $\alpha_2\beta_2$  GTPBP3/MTO1 complex (based on the SAXS model of *E. coli* MnmEG and using the experimental structures of CtMnmE (pdb 3GEE) and AaMnmG (pdb 2ZXI); unpublished results), allows us to speculate on the influence of some of these mutations on the MnmEG functioning.

In MTO1, the frameshift mutation at Arg645 leads to the insertion of a stop codon after seven amino acids, resulting in the deletion of the last 73 amino acid residues.<sup>69</sup> These C-terminal amino acids adopt a rather flexible  $\alpha$ -helical structure that has been shown in EcMnmG to be crucial for the interaction with EcMnmE.<sup>3,37</sup> Thr436 is part of a highly conserved sequence motif in MnmG orthologues (motif 2) involved in binding of the cofactor FAD, and mutation to Ile most probably affects this interaction. Thr308 is located on a loop at the entrance of the FAD binding pocket (motif 1). This loop is highly flexible in most MnmG structures that have been solved so far<sup>3,57,58</sup> and harbors the highly conserved catalytic cysteine

residue (Cys277 in EcMnmG) that has been proposed to form a covalent adduct with the C6 atom of U34 (see above). Arg502 is part of a patch of positively charged residues on the surface of all MnmG orthologues that has been shown to be important for interaction with substrate tRNA,<sup>3,57</sup> and its mutation might thus interfere with tRNA binding. The effects of the Gly59Ala and Ala453Thr mutations are so far harder to explain from the current structural models and they might partially also affect the overall structure of the protein.

In GTPBP3, Pro257, Ala322, and Asp337 are located in the G domain. While Pro257 is part of the P loop, interacting with the phosphates of the guanine nucleotide, Ala322 is part of the switch II region that forms the dimerization interface between the G domains in the closed state.<sup>39</sup> The Asp337His mutation probably indirectly, via disruption of a salt bridge with Lys375, affects the binding of the guanine nucleotide. All other point mutations in GTPBP3 (Arg3Leu, Glu142Lys, Glu159Val, Ala162Pro, Ala222Gly, Glu225Lys, and Glu459Lys) are located in the  $\alpha$ -helical domain. Interestingly, the affected residues are all located close to the MnmE-MnmG (or GTPBP3-MTO1) interaction surface that was proposed based on SAXS modeling.<sup>53</sup>

Finally, in recent years, MnmE and MnmG have also been identified as important regulators and determinants of bacterial virulence. In *Streptococcus pyogenes*, a knock-out of either *mnmG* or *mnmE* resulted in significantly reduced levels of multiple virulence factors, ascribed to a specifically reduced translation efficiency of the transcriptional activator RopB.<sup>75</sup> Also in *Salmonella* Typhimurium and *Aeromonas hydrophila*, two causative agents of gastroenteritis and diarrhea, MnmG regulates the expression of virulence genes on a translational level.<sup>76–78</sup> When both MnmE and MnmG are deleted in *S. Typhimurium* the bacterial infection is completely abolished.<sup>79</sup> In the opportunistic pathogen *Pseudomonas aeruginosa* MnmG regulates quorum sensing via the transcription factor RhlR, which in turn coordinates virulence gene expression.<sup>80</sup> Whether the roles of MnmG and MnmE in the control of bacterial virulence are linked to their tRNA-modification activity, and how MnmE and MnmG are integrated in the complex network that regulates virulence and quorum sensing, is currently unknown.

## CONCLUSIONS

MnmE is a member of the multi-domain HAS GTPases. Rather than being regulated by GEF and GAP proteins it functions via a mechanism where GTP hydrolysis is activated by binding of a potassium ion in the active site, concomitant with dimerization of the G domains. As such, the protein cycles between a GTP-bound “closed” state and a GDP-bound “open” state. These conformational changes are most probably

used to tune the interaction with its partner protein MnmG and to drive a very complex tRNA modification reaction of wobble uridine (U34). Instead of acting as a switch it thus needs active GTP hydrolysis to conduct its physiological function.

However, in absence of high resolution structures of the MnmEG complex, ideally bound to substrate tRNA, the details concerning the nature of these conformational changes and the consequences for the tRNA modification reaction remain elusive. Further mechanistic studies will also be required to shed light on the role of the highly conserved cysteines in MnmE and MnmG and on the details of the chemical steps leading to the final  $\text{cmnm}^5\text{U}$ ,  $\text{nm}^5\text{U}$ , or  $\text{tm}^5\text{U}$  modification. Moreover, the regulation of the MnmEG complex still raises important questions. The current model suggests that MnmE would consume two molecules of GTP per tRNA modification cycle. However, *in vitro* MnmE hydrolyses GTP relatively efficiently even in the absence of MnmG and/or tRNA. This raises the question onto how the GTP hydrolysis is regulated *in vivo* as to not unnecessarily consume GTP. Finally, recent data support a model where MnmE and MnmG interact in an asymmetric way leaving one subunit free in both proteins, which suggests a form of negative cooperativity between the subunits within the MnmE and MnmG homodimers. In the GTP-bound state a transient higher oligomer is formed with two MnmE dimers binding to one MnmG dimer. Neither the implications for the mechanism, nor the consequences with respect to the number of GTP molecules consumed per modified tRNA are clear at this moment. Resolving these open questions promises to be an exciting endeavour.

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