COLOR CODE :
RED = the text of the original manuscript, now to be revised.
YELLOW background = ToDoList as suggested by the reviewer.
GREEN = our reply to the reviewer comments.

Comments and Suggestions for Authors

Initial observation: the Figure numbers have changed and also the Reference numbers. In order to keep tracking in a simple way here we give those numbers of the original version, so they coincide with any numbers of the reviewer comments.

1) RsmE is a methyltransferase (MTase) and was first discovered in Escherichia coli.

>>Provide appropriate reference after above line.

(Basturea et al.)

We follow the good piece of advice of our reviewer and cited the correct reference here. To this end we moved the old reference Ref6 to this place, and cited it as new Ref1 also in the place of old Ref6, which was: **Basturea, G.N.; Rudd, K.E.; Deutscher, M.P. Identification and characterization of RsmE, the founding member of a new RNA base methyltransferase family. RNA 2006, 12, 426-434.** As a result this old reference REF6 was kept but with the new REF ID number Ref1 though.

2) Compared to this N-methylation, C-methylation 88 requires a totally different mechanism since the Arrhenius (or activation potential) energy 89 barrier lies much higher than for m3U1498.

>>Justify and explain the above fact about barrier energy.

We are grateful for the detection of this “faux pas”. At the time of writing up, there was another work about inorganic chemistry based on catalytic mechanism by in vitro experiments where tunneling the activation energy barrier is necessary by metallo-organic catalysis. Here in the live science field of cell-based biomolecules, all reactions are enzyme driven and mentioning barriers is pointless since they are proteins in the cells. So we dropped the mislead aspect here.

OLD TEXT :

Compared to this N-methylation, C-methylation requires a totally different mechanism since the Arrhenius (or activation potential) energy barrier lies much higher than for m^3U1498.

NEW TEXT:

Of note, compared to our target enzyme for N-methylation, C-methylation requires a totally different mechanism for m^3U1498.

3) A deeper understanding of the biochemical steps during the catalytic methylation 91 cycle of RsmE is still lacking. Hence, we propose a computational approach to analyze the 92 exit mechanism of SAH by molecular dynamics simulations.

>>Provide appropriate reference for MD simulation to justify its use and significance. Ref like:

Again, we acknowledge the excellent improvements of our work and added the proposed reference along with other references here.

Wasif Baig, M., Pederzoli, M., Kývala, M., Cwiklik, L., & Pittner, J. (2021). Theoretical investigation of the effect of alkylation and bromination on intersystem crossing in BODIPY-based photosensitizers. *The Journal of Physical Chemistry B*, *125*(42), 11617-11627.

We added the aforementioned reference in addition to four others at this stage. One out of the four, however was the old reference REF46 which we kept but with a new REF ID number though. In particular the methylation reactions on rRNA in bacteria were summarized with an in-depth view in 2015 by Sergeeva et al. and cited. The old reference Ref43 by P. Sergiev et al. was cited here too. It is the following: [Singh, N., Dueñas-González, A., Lyko, F., & Medina-Franco, J. L. (2009). Molecular modeling and molecular dynamics studies of hydralazine with human DNA methyltransferase 1. ChemMedChem, 4(5), 792–799.].

As a result all four references were ordered with decreasing publication years as documented here:

[Wasif Baig, M., Pederzoli, M., Kývala, M., Cwiklik, L., & Pittner, J. (2021). Theoretical investigation of the effect of alkylation and bromination on intersystem crossing in BODIPY-based photosensitizers. The Journal of Physical Chemistry B, 125(42), 11617-11627],

[Sergeeva, O. V., Bogdanov, A. A., & Sergiev, P. V. (2015). What do we know about ribosomal RNA methylation in Escherichia coli? In Biochimie (Vol. 117, pp. 110–118). Elsevier BV. https://doi.org/10.1016/j.biochi.2014.11.019],

[Mokmak, W., Tongsima, S., & Jenwitheesuk, E. (2009). Molecular dynamics simulation of a human thiopurine S-methyltransferase complexed with 6-mercaptopurine model. Bioinformation, 4(2), 59–62.],

[old43] = [Sergiev, P., Aleksashin, N., Chugunova, A. et al. Structural and evolutionary insights into ribosomal RNA methylation. Nat Chem Biol 14, 226–235 (2018) https://doi.org/10.1038/nchembio.2569].

4) To further investigate the catalytic mechanism of RsmE, the structural model of 5VM8 was taken as a reference because it yielded the most consistent results and unveiled the most continuous electron density map.

>>Kindly provide details after above text on Page 6 how electron density were obtained.

We agree again and added the missing description.

NEW TEXT:

In particular, the underlying electron density map was obtained from a separate omit map file called 2Fo-Fc. It has been made available for research in the structural biology field along with the corresponding PDB entry 5VM8. As a most valuable asset 2F0-Fc omit maps offer information regarding how accurately the model accounts for the experimental data. The omit map was fetched from the PDB server and visualization carried out under PyMol.

5) The 194 carboxyl group of SAM faces towards the binding site exit without making any other 195 contact with RsmE, whereas its amino group is attracted by the backbone oxygen of 196 Gly195.

>>Please mention here whether hydrogen bonding is present between these groups or not.

The missing aspects of how the contact is established between amino group Gly195 side chain, wee modified the text accordingly:

OLD TEXT:

The carboxyl group of SAM faces towards the binding site exit without making any other contact with RsmE, whereas its amino group is attracted by the backbone oxygen of Gly195.

NEW TEXT:

The carboxyl group of SAM faces towards the binding site exit without making any other contact with RsmE, whereas its amino group is forming hydrogen bonds with the backbone oxygen atom of the peptide bond on Gly195.

6) This interaction dissimilarity between SAM and SAH side 228 chains is due to the inward displacement of the sulfur atom that allows the methyl group 229 to swing over during the ongoing methyl transfer process.

>>Compute free energy changes for displacement of sulfur atom and swinging motion of methyl group.

We acknowledge the thoughtful inquiry of our reviewer. Four measures were undertaken to eliminated this mischief.

1) **The following explanation was added. It provides a deeper understanding because the Figure 6 is now embedded in a text providing mechanistic insight with full details.**

NEW TEXT (inserted above Figure 6)

"The sulfur atom displacement (or shift) of the three ligands can be fully understood when considering the following facts: two ligands can be lumped together since the sulfide S-atom is methylated becoming a cation. They are accommodated in the binding cleft in the same way, because the surrounding spatial requirements of the cavity is the same. In stark contrast the S-unmethylated ligand is smaller and approaches a closer deeper occupation zone at the binding site. Since the sulfur atom sits in the middle of a sidechain on the scaffold, it becomes evident that the binding conformations vary from one to the other group. For better viewing we extracted the active conformations (Figure 6). Our molecular dynamics simulations reflect this side chain displacement (or shift) at room temperature. "

2) **Moreover, all expressions throughout the entire manuscript were searched for the word "swing" in order to clean up the word confounding.**

The original text before the original Figure 6 reads :

" This interaction dissimilarity between SAM and SAH side chains is due to the inward displacement of the sulfur atom that allows the methyl group to swing over during the ongoing methyl transfer process, as displayed in Figure 6 panel A."

Here the word "to swing" is not correctly used. There is no conformational freedom for the H3-group sitting on a dialkyl-sulfide substructure, yielding a "trialkyl sulfide cation" (trialkyl-sulfonium ion). For any -C-C- alkyl group there is a known rotation at room temperature since those side chains have conformational freedom thanks to -C-C- alkyl group rotation.

OLD TEXT

This interaction dissimilarity between SAM and SAH side chains is due to the inward displacement of the sulfur atom that allows the methyl group to swing over during the ongoing methyl transfer process, as displayed in Figure 6 panel A.

NEW TEXT

This interaction dissimilarity between SAM and SAH side chains is due to the inward displacement of the sulfur atom that allows the methyl group to shift over during the ongoing methyl transfer process, as displayed in Figure 6 panel A.

3)

As seen on Ammoniak NH3 in the gas phase there is a energy barriers to pyramidal inversion for the N atom with sp3 hybridization. In (quantum chemical) theory there is one, too for aforementioned "trialkyl sulfide cation" (trialkyl-sulfonium ion). But this inversion would only mean that there is a chiral invertion if any, or a prochiral center, counting with

the lone pairs (s, p and d orbitals of S). This is not of relevance under cell-based (physiological) conditions. This is noteworthy in organic chemistry and in vitro reactions though.

Literature on the N – inversion: Scior, T.; Roth, H., J. Molecular modeling study of the antiseptic hexetidine: Comparison of conformation, basic character, and protonation. 1993. PZ (Pharmazeutische Zeitung) Wissenschaft 138(1): 13-15.

4)

OLD TEXT:

Figure 6. Crystal SAM and docked SAM/SAH conformations in the RsmE binding site of Neisseria gonorrhoeae. In A. the surface of the RsmE binding site is shaded by H-bonding potential. The methyl position of the crystal or docked SAM is circled to indicate how the sulfur position in SAH lies further inwards than in either crystal complex or docked SAM model (orange arrow). Residues labels are only for position reference. B. H-bond or other hydrophobic interactions of SAM and SAH contacting the binding site residues of N. gonorrhoeae RsmE are depicted in green dotted lines. The residues depicted in color magenta interact with the homocysteine tail of docked SAH. C. Superposition of the crystal SAM (ball and stick, gray carbons), the docked SAM (ball and stick, light blue carbons) and the docked SAH (stick, magenta carbons).

NEW TEXT:

Crystal SAM and docked SAM/SAH conformations in the RsmE binding site of *Neisseria gonorrhoeae*. **A.** In this panel the surface of the RsmE binding site is shaded by H-bonding potential. The methyl positions of the crystal and docked SAM are circled and labeled “Methyl” to indicate how the sulfur position in SAH lies further inwards than in either crystal complex or docked SAM model (orange arrow). Residues labels are only for positional reference. **B.** H-bonds and hydrophobic interactions of SAM or SAH with the binding site residues of *N. gonorrhoeae* RsmE are documented by green dotted lines. The magenta-colored residues show interaction with the homocysteine tail of docked SAH. **C.** Superposition of the extracted active conformations for crystallo-graphically observed SAM, the docked SAM and the docked SAH. Style and color coding for the three ligand models: (i) crystal SAM: ball and sticks with gray carbon atoms; (ii) docked SAM: ball and sticks with light blue carbon atoms; and (iii) docked SAH: sticks with magenta-colored carbon atoms.

7) The distance between the SAH adenine ring and the amine group with Leu215, 348 Arg218, and Leu220 residues remained practically stable at 3 Å for the entire simulation.

>>Please explain after above text that how stability is inferred from distance of 3 Angstroms. Justify and explain this fact please.

As with the word “swing” for the S-methyl group, here is another misleading word “ stable “ which associates with stable energies , stability calculations etc. “ Luckily this problem

only occurs once, so the modification is done here. The explanation is given graphically at atomic scale by an illustration in Figure 10.

OLD TEXT

"The distance between the SAH adenine ring and the amine group with Leu215, Arg218, and Leu220 residues remained practically stable at 3 Å for the entire simulation…"

NEW TEXT

The distance between the SAH adenine ring and the amine group with Leu215, Arg218, and Leu220 residues remained practically constant at 3 Å for the entire simulation (Fig. 10), maintaining this part of the molecule very close to the crystal pose, further reinforcing the importance of the role of the polar amino acid.

8) In contrast, however, when it is present, its side 377 chain terminal carboxyl group adopts an orderly and defined back and forth rotation 378 similar to a swing.

>>It should be more appropriate if you can compute free energy changes for above mentioned conformational changes.

With regard to (6) with a mayor conundrum with the word "to swing" here in this subchapter 2.5. the subtitle "SAH exchange between Arg221 and Thr222 is based on swinging side chains" is correct. And the text therein, too. Here "swinging" is used in the correct context of rotatable bonds. For instance: " SAH exchange between Arg221 and Thr222 is based on swinging side chains " or " . In contrast, however, when it is present, its side chain terminal carboxyl group adopts an orderly and defined back and forth rotation similar to a swing. "

Our molecular dynamics simulations reflect that amino acid side chains can freely rotate at room temperature, since here is conformational freedom for single bonds whithout steric hindrance. In more specific terms for amino acids, for any -C-C- alkyl group there is a known rotation at room temperature since those side chains have conformational freedom thanks to -C-C- alkyl group rotatable bonds. And the rotational energy values for methyl or any alkyl group on a scaffold is part of more physico-chemical description which is not the focus here in biochemistry. We are convinced in this case, that our text here could remain unchanged, please.

OLD and NEW TEXT

In panels B and C of Figure 12 the conformations of side chains of Arg221 and Thr222 changed randomly when SAH is absent. In contrast, however, when it is present, its side chain terminal carboxyl group adopts an orderly and defined back and forth rotation similar to a swing. MD analysis detected this differential behavior.

9) while the adenosyl side chain remains 477 unprotonated for an estimated 99 to 99.9 % (pka 4 – pH 7 = 99.9% free base).

>>Fix the typo in above sentence at page 9. It should be pKa not pka.

Thank you again.

OLD TEXT

while the adenosyl side chain remains unprotonated for an estimated 99 to 99.9 % (pka 4 – pH 7 = 99.9% free base).

NEW TEXT

while the adenosyl side chain remains unprotonated for an estimated 99 to 99.9 % (pKa 4 – pH 7 = 99.9% free base).

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Finally, we feel much beholden of the constructive comments helping us to improve the manuscript for successful publication.