nature portfolio

Peer Review File

Oligomerization and a distinct tRNA-binding loop are important regulators of human arginyl-transferase function

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a beautiful study elucidating the structure of human ATE1. ATE1 structure has been elusive for decades, and this group is one of those that recently made a breakthrough in solving the structure of yeast ATE1. The present study is the important next step, since mammalian ATE1 is essential for embryogenesis and is implicated in a large number of key biological processes. The present work represents a highly significant finding for those working in the rapidly growing field of protein arginylation.

The paper is of very high quality, and I have no improvements to suggest to the data and the experiments. My comments revolve largely around the writing, and some gaps in discussion, reference to prior work, and putting this study into the context in the field.

Throughout the abstract and the introduction, the authors present a bit of a biased view on arginylation that de-emphasizes a body of prior studies and it some cases includes incorrect citations of the prior findings. I feel that this somewhat contentious writing is not necessary, and a lot of the issues could be fixed if the text in those places could be shortened and rearranged (and corrected) to straighten out the story. There are also some citations I would emphasize more in the results. I listed those instances in the comments 1-5 below.

My other group of comments relates to putting the very interesting findings of the paper into context of the prior findings and into the overall big picture in the field. These are summarized in comments 6-9 below.

1. The first sentence of the abstract “The arginyl-transferase ATE1 is a tRNA-dependent enzyme that covalently attaches an29 arginine molecule to a protein substrate to promote its degradation” is already a bit polarizing. Other functions of arginylation not related promoting degradation have been demonstrated. Can you remove “to promote its degradation” from this sentence to avoid controversies?

2. Same issue in the first paragraph of the introductions. Both degradatory and non-degradatory roles of ATE1 should be acknowledged, and a more balanced overlook on the field should be presented. Personally, I don’t understand why an extensive discussion of the N-degron pathway should be included at all, the authors could just say that ATE1 transfers Arg to the N-terminus of the acidic residues, and that this has been implicated in a multitude of biological processes including, prominently, in protein

degradation. In reality, there are many more targets identified that are not believed to degrade after arginylation compared to those that do. I think the manuscript starts in a bit of a wrong tone.

3. Along the same lines, the very small paragraph that does eventually discuss non-degradation roles of arginylation, has factual errors. Actin arginylation has been implicated in cell migration (by Kashina group), not the myocardium. In fact, it is the Ate1- dependent RGS degradation has been implicated in the myocardium development (by Kwon group). Additionally, arginylation of calreticulin has been implicated in stress response through non-degradatory mechanisms (originally by Hallak group), and this work is not cited at all. All these references should be set straight. There have also been global screens for non-degradation substrates of Ate1, which do not necessarily need to be cited, but which do demonstrate that the non-degradatory substrates of ATE1 are not isolated exceptions but potentially a global trend. In fact, the authors don't really need a comprehensive overview of all this literature, just an acknowledgement that the effects of Ate1 in vivo are highly variable in their biological consequences. My suggestion is to rearrange the entire first part of the introduction to present this in a more neutral way.

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5. The authors should probably also mention the paper by Wang et al 2014 (on side chain arginylation) in the place where they talk about NMR and arginylation by ATE1 being predominantly N-terminal. In this paper, the authors reached the same conclusion by doing NMR on in vitro arginylated peptides, and it has been proposed in that paper that side chain arginylation occurs largely on proteins versus peptides and in the absence of the available N-terminal acidic residue. The present data seems in good agreement with that earlier study. This is just a suggestion, though, since I am aware the two studies are by no mean identical in their design.

6. The authors mention double arginylation detected on peptides derived from the actin N-terminus, but it is not clear where does this double arginylation occur. Is it possible to derive this information from the data? And, is there any idea of how this happens and where? This is a finding that to my knowledge has not been reported before, and it would be interesting to see the authors' thoughts on this.

7. The result that human ATE1 forms a dimer in its active state is extremely interesting and important. While I understand that analyzing other ATE1 enzymes to see if they also dimerize is outside the scope of the present study, I would be curious if the authors could speculate whether other human ATE1 isoforms also form dimers, and whether this happens in, e.g., mouse, and other evolutionarily lower species. I am sure some of these predictions, at least for ATE1 isoforms, and maybe for mouse, should be possible based on sequence homology.

8. The figures are stunning, and I couldn't help wishing that some of the panels were larger, especially those for structures and images. I understand that there is a constraint in fitting all these data into the limited figure size, but I just wanted to mention it in case it is possible, e.g., to reduce white spaces or increase the number of figures in the manuscript. Normally structures are presented in separate panels much larger than the current ones.

9. One of those figures where I wished for more detail was the ATE1-tRNA complex. There is a recent study that Ate1 can utilize structures corresponding to the tRNA stem conjugated to Arg, how does this finding reconcile with the structure shown in this paper? From the figure it seems in agreement, but a brief discussion would be nice.

Reviewer #2 (Remarks to the Author):

Lan et al's study aims at elucidating the structural mechanism of human arginyl-transferase ATE1. While the yeast enzyme has been structurally characterized, the human protein sequence and domain organization is different and warrants a detailed characterization. Accordingly, the authors, using cryo-EM and a well-designed set of biochemical, biophysical and cellular experiments, demonstrate that the mechanism of human ATE1 regulation substantially differs from that of the yeast protein. The authors' experiments provide strong support for the mechanism, in which dimerized (inactive) ATE1 is activated upon arginyl-tRNA binding to individual dissociated subunits. The manuscript is well written. I have only a few minor criticisms; I recommend the publication of this paper after a minor revision.

1. Interpretation of the experiment with the R56E mutant is questionable (lines 230-232). The authors argue that this mutant is not properly folded ("rather than" it lost its "substrate coordination function"), but the loss of folding does not rule out that R56 is involved in substrate coordination. The results are therefore inconclusive, and this must be properly explained in the manuscript.

2. The next inconclusive experiment is the observation that the melting temperature of hsATE1 decreases in the presence of 20 mM arginine amide. The authors propose that this is due to (a) dimer dissociation and (b) binding of arginine amide in the active site as a product (i.e. specific binding). Although these outcomes are possible, these results do not directly report on dimer dissociation, and may involve local protein misfolding due to non-specific interactions with arginine amide. Indeed, since arginine amide is only a minimal mimic of the product, its binding to the active site can be very poor in

the absence of other amino acids of the substrate peptide. This section of the manuscript must be updated accordingly.

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5. The authors say that "future studies" are needed to understand tRNA specificity, but it is not clear why they can't do that based on their structures. If the resolution of the tRNA complex does not allow any interpretation of tRNA specificity due to insufficient resolution, they should make that clear for the readers.

We thank the Editor and Reviewers for the insightful and constructive comments, which helped revise the presentation of our findings and strengthen the manuscript. The individual comments/critiques from all reviewers have been considered and incorporated into the revised text and further described in the point-by-point response below.

Reviewer #1：

This is a beautiful study elucidating the structure of human ATE1. ATE1 structure has been elusive for decades, and this group is one of those that recently made a breakthrough in solving the structure of yeast ATE1. The present study is the important next step, since mammalian ATE1 is essential for embryogenesis and is implicated in a large number of key biological processes. The present work represents a highly significant finding for those working in the rapidly growing field of protein arginylation.

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We thank the reviewer for the positive feedback and have revised the text accordingly.

Reviewer #1, Comment 1: The first sentence of the abstract “The arginyl-transferase ATE1 is a tRNA-dependent enzyme that covalently attaches an29 arginine molecule to a protein substrate to promote its degradation” is already a bit polarizing. Other functions of arginylation not related promoting degradation have been demonstrated. Can you remove “to promote its degradation” from this sentence to avoid controversies?

> **Author’s response:** as suggested, we have revised the abstract and removed “to promote its degradation”.

Reviewer #1, Comment 2: Same issue in the first paragraph of the introductions. Both degradatory and non-degradatory roles of ATE1 should be acknowledged, and a more balanced overlook on the field should be presented. Personally, I don’t understand why an extensive discussion of the N-degron pathway should be included at all, the authors could just say that ATE1 transfers Arg to the N-terminus of the acidic residues, and that this has been

implicated in a multitude of biological processes including, prominently, in protein degradation. In reality, there are many more targets identified that are not believed to degrade after arginylation compared to those that do. I think the manuscript starts in a bit of a wrong tone.

Author's response: as suggested, we have revised the first part of the introduction, removed the description of the N-degron pathway, and set the manuscript to a more neutral tone.

Reviewer #1, Comment 3: Along the same lines, the very small paragraph that does eventually discuss non-degradation roles of arginylation, has factual errors. Actin arginylation has been implicated in cell migration (by Kashina group), not the myocardium. In fact, it is the Ate1- dependent RGS degradation has been implicated in the myocardium development (by Kwon group). Additionally, arginylation of calreticulin has been implicated in stress response through non-degradatory mechanisms (originally by Hallak group), and this work is not cited at all. All these references should be set straight. There have also been global screens for non-degradation substrates of Ate1, which do not necessarily need to be cited, but which do demonstrate that the non-degradatory substrates of ATE1 are not isolated exceptions but potentially a global trend. In fact, the authors don't really need a comprehensive overview of all this literature, just an acknowledgement that the effects of Ate1 in vivo are highly variable in their biological consequences. My suggestion is to rearrange the entire first part of the introduction to present this in a more neutral way.

Author's response: as suggested, we have revised the introduction to summarize the non-degradation functions of ATE1 and included the proper citations on page 3.

Reviewer #1, Comment 4: In the results, the authors discuss recovering self-arginylated ATE1 after bacterial expression. The data are very nice, and goes beyond what was previously demonstrated, but setting the record straight, self-arginylation of ATE1 has been previously shown. This should be acknowledged. This prior work (Wang et al 2011) is cited in the paper, but not acknowledged in this particular place.

Author's response: as suggested, we have acknowledged that our data agree with prior findings, which are presented on page 5. We have also included citations of published work in those instances.

Reviewer #1, Comment 5: The authors should probably also mention the paper by Wang et al 2014 (on side chain arginylation) in the place where they talk about NMR and arginylation by ATE1 being predominantly N-terminal. In this paper, the authors reached the same conclusion by doing NMR on in vitro arginylated peptides, and it has been proposed in that paper that side chain arginylation occurs largely on proteins versus peptides and in the absence of the available N-terminal acidic residue. The present data seems in good agreement with that earlier study. This is just a suggestion, though, since I am aware the two studies are by no mean identical in their design.

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Reviewer #1, Comment 6: The authors mention double arginylation detected on peptides derived from the actin N-terminus, but it is not clear where does this double arginylation occur. Is it possible to derive this information from the data? And, is there any idea of how this happens and where? This is a finding that to my knowledge has not been reported before, and it would be interesting to see the authors' thoughts on this.

Author's response: It is indeed feasible to identify potential mid-chain arginylation sites using mass spectrometry. However, due to the low abundance of doubly arginylated species, extensive efforts and repeated experiments are required to identify and validate robust and specific sites, which is beyond the scope of the current manuscript.

Reviewer #1, Comment 7: The result that human ATE1 forms a dimer in its active state is extremely interesting and important. While I understand that analyzing other ATE1 enzymes to see if they also dimerize is outside the scope of the present study, I would be curious if the authors could speculate whether other human ATE1 isoforms also form dimers, and whether this happens in, e.g., mouse, and other evolutionarily lower species. I am sure some of these predictions, at least for ATE1 isoforms, and maybe for mouse, should be possible based on sequence homology.

Author's response: we briefly allude to this possibility in the first paragraph of the Discussion. "…*The residues on the dimeric interface are rather conserved in mice and humans, but not in fly or arabidopsis (Supplementary Fig. S1, blue dash boxes)* …" Based on this analysis, we speculate dimerization of ATE1 only occurs in higher organisms, including mice and humans. Additionally, many residues on the dimeric interface, including S278-E280, K285, E305, D308-E309, and G311-E314, are in exon 7b (ATE1-2, also denoted as 1b7b). Although the residues on the dimeric interface are mostly conserved or highly similar between exons 7b and 7a, we cannot rule out a potential disruption of the dimeric interface in isoforms containing exon 7a. Future experiments will be needed to fully address this question. We have expanded the discussion on page 12 to include this analysis.

Reviewer #1, Comment 8: The figures are stunning, and I couldn't help wishing that some of the panels were larger, especially those for structures and images. I understand that there is a constraint in fitting all these data into the limited figure size, but I just wanted to mention it in case it is possible, e.g., to reduce white spaces or increase the number of figures in the manuscript. Normally structures are presented in separate panels much larger than the current ones. **Comment 9:** One of those figures where I wished for more detail was the ATE1-tRNA complex. There is a recent study that Ate1 can utilize structures corresponding to the tRNA stem conjugated to Arg, how does this finding reconcile with the structure shown in this paper? From the figure it seems in agreement, but a brief discussion would be nice.

Author's response: We thank the reviewer for the suggestions and have reduced the white spaces and increased panel sizes.

We have also broadened the discussion on page 13 to include the arginyl-tRNA fragments. Our data indicate that arginyl-tRNA fragments containing the acceptor arm can be utilized as arginine donors, consistent with prior findings. We also expect arginyl-tRNA fragments containing both the A- and T-arms will be more efficient than smaller fragments due to the additional contact sites in human ATE1.

Reviewer #2: Lan et al's study aims at elucidating the structural mechanism of human arginyl-transferase ATE1. While the yeast enzyme has been structurally characterized, the human protein sequence and domain organization is different and warrants a detailed characterization. Accordingly, the authors, using cryo-EM and a well-designed set of biochemical, biophysical and cellular experiments, demonstrate that the mechanism of human ATE1 regulation substantially differs from that of the yeast protein. The authors' experiments provide strong support for the mechanism, in which dimerized (inactive) ATE1 is activated upon arginyl-tRNA binding to individual dissociated subunits. The manuscript is well written. I have only a few minor criticisms; I recommend the publication of this paper after a minor revision.

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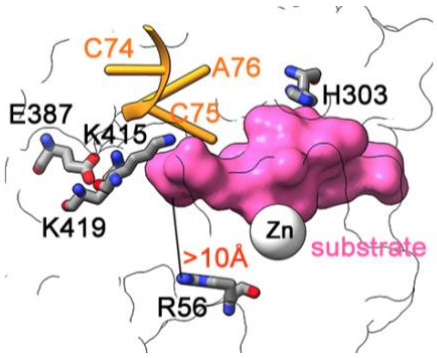

Fig. 1. Additional zoom-in view of the complex (related to main Fig. 4a).

Author's response: In the structural model of ATE1/tRNA/substrate complex, the guanidine groups of both R56 and R57 are over 10 Å away from the D1 residue of the substrate (Fig. 1) and are involved in intra-ATE1 contacts, making them unlikely to coordinate substrate binding. However, given the resolution limitation of our structure and based on reviewer's suggestion, we have softened the claim associated R56E with protein misfolding by removing the phrase, "rather than loss of substrate coordination function" to suggest that R56E disrupts protein folding.

Reviewer #2, Comment 2: The next inconclusive experiment is the observation that the melting temperature of hsATE1 decreases in the presence of 20 mM arginine amide. The authors propose that this is due to (a) dimer dissociation and (b) binding of arginine amide in

the active site as a product (i.e. specific binding). Although these outcomes are possible, these results do not directly report on dimer dissociation, and may involve local protein misfolding due to non-specific interactions with arginine amide. Indeed, since arginine amide is only a minimal mimic of the product, its binding to the active site can be very poor in the absence of other amino acids of the substrate peptide. This section of the manuscript must be updated accordingly.

Author's response: as suggested, in the revised manuscript, we clarify that the observation was made in the absence of tRNA and now include the alternative scenario that arginine amide might affect ATE1 stability through local conformational changes. The original Fig. 4b was also moved to Supplementary Fig. S4a.

Reviewer #2, Comment 3: The idea that the enzyme has a higher affinity for the product (arginylated peptide) than the substrate (peptide) is concerning. Enzymes normally have a higher affinity for substrates, so that they can function in the multiple turnover mode. Otherwise, the enzyme would be inefficient due to product inhibition. Also, the MST experiments suggest that the binding affinity is in the range of 160-530 uM. This is unusually high; most proteins in the cell would not achieve this concentration. I wonder if the experimental conditions for these experiments were not properly designed. Was the binding measured in the presence of the other substrate – arginyl-tRNA? If not, these affinity estimates are misleading and should be re-measured with the tRNA.

Author's response: This is an important point that the reviewer makes. Indeed, the experiments referred to were performed in the absence of the co-substrate arginyl-tRNA because of technical challenges. Despite our numerous attempts to generate the native arginyl-tRNA using either *in vitro* transcribed or endogenously purified tRNA-Arg, we have been unable to obtain sufficient amounts of high-quality reagents for quantitative measurements.

As suggested, we remeasured the affinity in the presence of the A+T arms of tRNA. The new data is included in Fig. 4c. In the presence of tRNA mimetics, the product peptide binds ATE1 significantly tighter (11 μM). While we fully expect that the substrate peptide also binds tighter in the presence of arginyl-tRNA, or the A+T arms containing the arginyl-group, developing a system that accurately reports quantitative binding measurements is associated with inherent challenges (for example, measuring substrate binding, while preventing subsequent catalysis and dissociation without adversely affecting binding). We have revised the text on page 10 to include the new data.

Reviewer #2, Comment 4: How does the structure of the ATE1*tRNA complex explain the specificity for arginyl-tRNA over other tRNAs? What specific residues/base-pairs of the tRNA determine the specificity and how are they recognized? These could be the interaction sites with ATE1 – even if the detailed side-chain interactions can't be seen at this resolution, the interpretation of the contact sites (on the tRNA) can give the clues as to how these elements of the tRNA differ from other tRNAs. **Comment 5:** The authors say that "future studies" are

needed to understand tRNA specificity, but it is not clear why they can't do that based on their structures. If the resolution of the tRNA complex does not allow any interpretation of tRNA specificity due to insufficient resolution, they should make that clear for the readers.

Author's response: Interpretion of our model indicates that residues Y21-Y25, S58, K60, Y298-Q299, I302-H303, D305, E314, R317, F318, Y367, Y369, F408, I410, K415, and K419 of ATE1 are likely involved in substrate binding. These residues help to coordinate interactions with the D1-D2 regions of the substrate peptide and C75-A76 of the tRNA. These interactions, or the precise pocket formed by them, might help to explain the selectivity toward the arginyl moiety over other aminoacyl groups. Additionally, residues on the αC helix of ATE1 contact the major groove of the acceptor arm of tRNA, whereas helix αC_{loop} (aa 191-220) in *hs*ATE1 form a secondary contacting site with D- and T-arms of tRNA. This information could pinpoint regions of ATE1 that convey the selectivity toward different aminoacylated tRNAs.

As this reviewer suggests, the current resolution of our structure does not allow for full interpretation of the molecular interactions contributed by individual side-chains and bases in our ternary complex. While we can speculate some interactions, based primarily on proximity, these limitations prevent us from identifying the polar and hydrophobic contacts, hydrogen bonds, and salt bridges that are likely critical for binding and catalysis. As such, we have limited our analysis and description of the structure. As this reviewer suggests, we have included a statement indicating that the resolution of our structure does not allow for the interpretation of aminoacyl-tRNA specificity on page 9.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed all my comments to my satisfaction. I recommend publication as is.

Reviewer #2 (Remarks to the Author):

Authors did a good job addressing my criticisms. I recommend the manuscript for publication.

Reviewer #3 (Remarks to the Author):

I am reviewing the work purely from the standpoint of MDFF. To start, given the resolution it is good choice of method, however, structural verification of the MDFF model can definitely improve.

Understandably map-model metrics like EMringer and Q-scores will not work, so CCs are fine to start out with.

I suggest that the authors should look into RMSF analysis of the fitted ensemble and match it against the local resolution of the density values (see the analysis here: https://elifesciences.org/articles/16105). A linear correlation in the RMSF (with and without MDFF) vs local resolution will indicate minimal overfitting of the model.

If the CCs and RMSFs are computable with half maps, and quality of the map-model consistency drops minimally (let's say remains respectable by statistical standards), that is also a good indicator of the model.

Also, how the Molprobity score improved from the rigid body model will serve as a reasonable indicator of the physical relevance of the model.

Introducing divalent ions in the simulations and tracking their final position relative to the RNA will offer key insights about the overall electrostatic properties of the model. Normally nucleic acids house several tightly bound divalent (Mg or Ca) ions, that are not visible at the medium resolution. The strength of MD refinement (particularly in explicit solvent) is that ions can be released in the box without coupling to the density and they can find some key sites on the macromolecule, indirectly assisting the refinement.
Some of the first Ribosome models by Schulten and Frank had benefitted from this trick. If relevant here, I suggest the authors to experiment with the ionic setup.

Finally, it is not clear whether the coupling to the density is using C-alpha, backbone or all-heavy atoms. The choice should be justified in view of the resolution of the map. Coupling too many atoms will lead to overfitting.

We thank the Reviewers for the positive feedback. The additional comments/critiques have been considered and incorporated into the revised text and addressed in the point-by-point response below.

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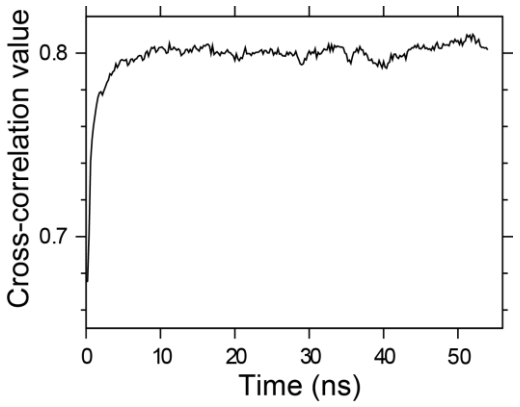
Comment 1: I am reviewing the work purely from the standpoint of MDFF. To start, given the resolution it is good choice of method, however, structural verification of the MDFF model can definitely improve.

Author's response: We thank the reviewer for acknowledging that MDFF is the proper method for this resolution and appreciate the feedback on improving the structural verification of the model further. We have now conducted additional analyses and generated a new Supplementary Figure 3 that details the structural validation process of our MDFF model.

Comment 2: Understandably map-model metrics like EMringer and Q-scores will not work, so CCs are fine to start out with.

Author's response: We now include the time evolution of cc value during MDFF as Supplementary Figure S3b on page 19:

".... A frame from the last 10 ns of the trajectory with a cc value of 0.81 was selected (Supplementary Fig. S3b) and subsequently minimized using Geometry Minimization in phenix."



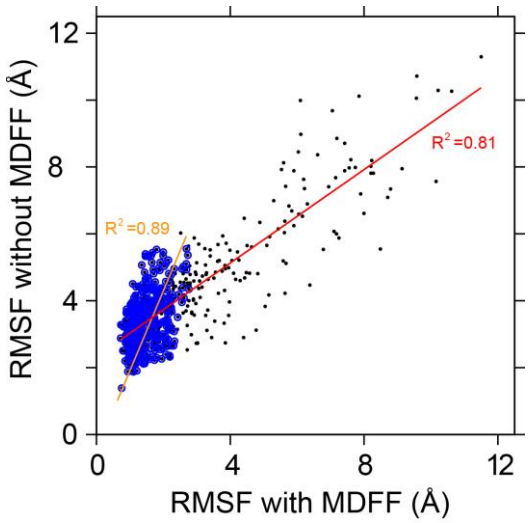
Supplementary Figure S3b. Time evolution of cross-correlation value between structure model and cryoEM map.

Comment 3: I suggest that the authors should look into RMSF analysis of the fitted ensemble and match it against the local resolution of the density values (see the analysis here: https://elifesciences.org/articles/16105). A linear correlation in the RMSF (with and without MDFF) vs local resolution will indicate minimal overfitting of the model.

Author's response: We thanked the reviewer for this suggestion and have performed additional simulations to examine the structure model from MDFF by comparing the dynamics of ensembles with and without MDFF to validate that there is minimal overfitting of the model. In the original methodology development reference (https://elifesciences.org/articles/16105), the low-resolution maps were derived by low-pass filtering of the high-resolution experimental

maps. The availability of high-resolution maps and local resolutions enables the comparison of RMSF with local resolution. In our case, since our cryoEM map is at medium resolution, such a comparison is not possible. Therefore, we only included the RMSF analysis in the revised method section on page 19.

"…Using this model as the starting structure, an additional 200 ns MD simulations were performed with MDFF (gscale = 0.3) and without MDFF (gscale = 0). Root-mean-square-fluctuation (RMSF) of individual residues was used for comparing the ensemble with and without MDFF[48]."

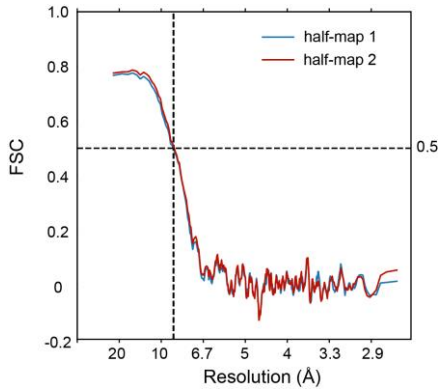


Supplementary Figure S3a. Root-mean-square-fluctuation (RMSF) analysis of the ensemble with and without molecular dynamics flexible fitting (MDFF) for all residues in the full-length structure (black dots) and residues within cryoEM map (blue dots). Both comparisons show strong linear relationships with R2 of 0.81 (red line) and 0.89 (orange line), respectively.

In the main text on page 9, we added:
"*…For structural validation, we extended MD simulations for an additional 200 ns with and without MDFF. RMSF analysis of the ensemble with and without MDFF demonstrates a strong linear relationship (Supplementary Fig. S3a), indicating minimal overfitting of the model…*"

Comment 4: If the CCs and RMSFs are computable with half maps, and quality of the map-model consistency drops minimally (let's say remains respectable by statistical standards), that is also a good indicator of the model.

Author's response: We now include the FSC curves of the refined model versus half-maps in Supplementary Figure S3c on page 19.



Supplementary Figure S3c. FSC curves of the refined model versus half-map 1 (blue) or half-map 2 (red).

Comment 5: Finally, it is not clear whether the coupling to the density is using C-alpha, backbone or all-heavy atoms. The choice should be justified in view of the resolution of the map. Coupling too many atoms will lead to overfitting.

Author's response: We thank the reviewer for pointing out this critical detail. Due to the medium-resolution of the cryoEM map, we chose coupling only to the backbone atoms. The methods on page 19 have been revised accordingly.

"…Input files for MDFF were prepared using the mdff package in VMD 1.9.6[68] with the scaling factor (gscale=0.3) coupling to backbone atoms and sampled for 55 ns using NAMD3[45] on two NV-link NVidia A100 GPU cards…"

Comment 6: Also, how the Molprobity score improved from the rigid body model will serve as a reasonable indicator of the physical relevance of the model.

Author's response: The initial model built by fitting apo-ATE1 and tRNA (PDB 7UXA) into the map showed a MolProbity score of 7.90. The final Molprobity score of the complex is 2.75 (SI Table 1), suggesting that the refinements improved the model.

Comment 7: Introducing divalent ions in the simulations and tracking their final position relative to the RNA will offer key insights about the overall electrostatic properties of the model. Normally nucleic acids house several tightly-bound divalent (Mg or Ca) ions, that are not visible at the medium resolution. The strength of MD refinement (particularly in explicit solvent) is that ions can be released in the box without coupling to the density and they can find some key sites on the macromolecule, indirectly assisting the refinement. Some of the first Ribosome models by Schulten and Frank had benefitted from this trick. If relevant here, I suggest the authors to experiment with the ionic setup.

Author's response: Since Mg^{2+} was not examined in the current experimental condition, we did not simulate the system in the presence of Mg^{2+} in this study. Previous MD simulation studies on tRNA suggested that Mg^{2+} played a role in stabilizing the tertiary structure of D- and T-loops (Auffinger et al., 1999). Interestingly, the highly positively charged αC_{loop} of human ATE1 (main Figure 3d) also stabilizes the tertiary interaction between D- and T-loops. We thanked the reviewer for this insight and will examine the potential effects of divalent ions in tRNA recognition by both human and yeast ATE1s in future studies.

Reference

Auffinger, P., Louise-May, S., and Westhof, E. (1999). Molecular dynamics simulations of solvated yeast tRNA(Asp). Biophys J *76*.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

I think the authors have amply addressed my comments.

If they chose to, they can add 1-2 sentences in the Discussions stating the relevance of the work in the context of the popular theme of integrative modeling and ensemble generation (https://www.sciencedirect.com/science/article/pii/S2590238521004495 and some citations therein). Otherwise, all good!

We thank the Reviewer for the positive feedback. The additional comments/critiques have been considered and addressed in the point-by-point response below.

Reviewer #3 (Remarks to the Author):
I think the authors have amply addressed my comments.
If they chose to, they can add 1-2 sentences in the Discussions stating the relevance of the work in the context of the popular theme of integrative modeling and ensemble generation (https://www.sciencedirect.com/science/article/pii/S2590238521004495 and some citations therein). Otherwise all good !

Author's response: We thank the reviewer for suggesting the discussion point. However, we believe that a 1-2 sentence discussion would not provide enough depth for a thorough analysis, which we think would be more informative for the readers. We will consider a stand-alone analysis in a follow-up study to fully discuss contemporary integrative modeling and ensemble generation methods for multiple tRNA-containing complexes.