nature research

# Peer Review File

**Manuscript Title:** Cryo-EM structure of the inhibited (10S) form of myosin II

## Editorial Notes:

## Reviewer Comments & Author Rebuttals

### Reviewer Reports on the Initial Version:

Referees' comments:

***Referee #1:***

This is a clear milestone and achievement. It is beautifully and clearly presented, and I have only minor comments. It is great to see Craig, whose work on phosphorylation-driven unfurling of 10S myosin opened up this field (Nature, 1983), now taking our understanding to near-atomic atomic resolution. As the authors write in their conclusion, this work corrects and integrates previous structural and solution studies, and I fully agree.

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***Referee #2:***

Myosin II is the major class of motor protein powering cellular contractility in both muscle and non-muscle cells. Myosin II adopts either a folded autoinhibited state as a soluble dimer or an extended, active state through oligomerization of dimers into filaments. In the autoinhibited state, the ATPase activity of the motor domains are suppressed through extensive intramolecular interactions between their head and tail domains. Phosphorylation at S19 on the ELCs triggers the transition from the autoinhibited state to the extended state by potentially disrupting intramolecular interactions.

While the autoinhibited state is of fundamental importance, due to its substantial conformational flexibility and modest biochemical stability in vitro, decades of investigation have only yielded structures at resolution around 20 Å. Therefore, our mechanistic understanding of autoinhibition is still limited to the level of domain-domain interactions, and a high-resolution structure of the autoinhibited state is much needed to enable detailed understanding of the autoinhibition and the activation mechanism of myosin II. This remains a widely acknowledged long-term goal of the field.

The manuscript by Yang et al. provides a cryo-EM reconstruction of the turkey gizzard smooth muscle myosin II at high resolution. By rigid body fitting, homology modelling and real space refinement, the authors generate a 3D model for the entire auto-inhibited myosin II structure except the distal tail region. Based on the model, the authors provide a comprehensive analysis of the potential intramolecular interactions at the sub-nanometer level. The sub-nanometer-level organization of the autoinhibited state is revealed and the activation mechanism upon S19 phosphorylation is suggested. The structure also provides mechanistic insight into disease-causing mutations. This study represents a major breakthrough in our understanding of the mechanism of myosin II autoinhibition and activation. Given the fundamental importance of myosin II in cell biology, physiology and disease, and as a model molecular motor, this study is of broad general interest.

Overall, we are highly enthusiastic about the impact of this study. We believe that it represents a major fundamental advance, and it will ultimately be suitable for publication in Nature. However, the manuscript suffers from some issues in data presentation that could be improved for accessibility to a broad readership. Furthermore, there are some matters about cryo-EM data processing and interpretation with atomic models that could be improved or clarified. We thus believe that the paper should undergo minor revisions prior to acceptance.

Major points:

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range, well-established computational methods like Molecular Dynamics Flexible Fitting or Rosetta can provide highly reliable, if not definitive, information at the atomic level. Employing these methodologies would likely improve the atomic model and substantially strengthen the authors' claims at the residue level.

3. In Fig. 4 the models of the highly flexible N terminus of the RLC in the phosphorylated and unphosphorylated states appear to be solely derived from previous molecular dynamics simulations and are thus highly speculative. While the authors make this clear in the text, it would be appropriate to include appropriate caveats in the figure legend, where the model is currently described as definitive rather than speculative.

Minor points (improving clarity of presentation at authors discretion):

1. It is admirable that the authors choose to present their raw data maps in the main figures. However, they use a lot of valuable real estate on showing the map and the model-map fit. In our opinion, the panels of Fig. 2a and b are redundant. We would recommend removing 2b, and adding several panels showing detail views of the locations of disease mutations. The residues in these panels should be labelled.

2. The residues displayed in the panels of Fig. 3 should be labelled.

3. The labelling of the cartoons in in Figs. 2d and 3a are small and crowded. It would be helpful to make these panels larger, and increase the relative size of labels if possible, for better readability.

**Author Rebuttals to Initial Comments:**

**Note:** Author rebuttals in blue

We are very grateful to the referees for their thoughtful comments and constructive suggestions, which we think have helped to improve the manuscript and our analysis substantially. Our responses are in blue below and substantive changes are shown in blue in the manuscript. We have also made the following important changes to comply with Nature editorial requirements.

The submitted version was 1.36 pages too long. We have reduced the length primarily by moving Fig. 4, its legend, and the text referring to it to Extended Data Fig. 8. We have also made minor word edits throughout the manuscript to further save space, without any change in meaning. We have moved Extended Data Table 1 to Supplementary Information Table 1, per Nature requirements. We have added 4 panels to Extended Data Fig. 7 to demonstrate how actin binding is inhibited, in addition to inhibition of converter domain movements already shown. This is in response to ref. 1, point 6. We have also included additional images in Extended Data Fig. 2, showing the quality of the map in response to Ref. 2. Finally, we have added a dedication to the memory of two of our former mentors/collaborators who did much to advance the field of myosin II structure. We hope this is acceptable for this paper.

Referees' comments:

Referee #1:

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studies, and I fully agree.

1. Cartoon movies are a little oversaturated. 379485_0_video_3467841_qctrpl has a glitch with the scale bar.

We thank the referee for pointing this out, but we don't have an explanation. The colors in the movies are the same as those used to produce the figures, although with a black background in the movies. They are default colors that come from Chimera. To us, they show the features clearly, and don't appear oversaturated, and as far as we know cannot be toned down in Chimera. We have asked colleagues whether they find the colors OK and they have not found any problem. This is hard to explain. We considered the possibility that different players might be the problem. However, QuickTime, Windows Media Player, VLC media player and Movies TV all worked similarly. Possibly different displays show the colors differently.

The scale bar shows 10 Å and, in our own replay of the movie, does not appear to have a glitch, appearing just as it does in the Chimera display. Again, our colleagues did not notice any issues.

2. Abstract: "activity is highly inhibited" is a bit vague; please clarify that assembly and actin activation are both inhibited.

We had kept this terse to limit the abstract to Nature length. We have now elaborated (LL 30-31), and included our 2019 reference on 3D reconstruction of 10S myosin (Yang et al., 2019), which discusses these different aspects of inhibition.

3. The writing gives the impression that the formation of 10S myosin is primarily an energy conservation strategy. I suggest that the authors spend a few introductory lines setting out the importance of the dynamic, regulated formation and dissolution of myosin thick filaments across a range of eukaryotic systems, including muscle, and posing the question of how far the IHM in 10S may or may not resemble/relate to that in super relaxed thick filaments. Currently there is no mention of the thick filament IHM until L84, and no mention of the relevance of the current results beyond smooth muscle until L455 (Methods).

We agree with the referee that these are important points. The reason for minimizing their discussion was to keep the manuscript within the length constraints of Nature (we still failed!). However, we did mention the thick filament IHM in the third sentence of the introduction, and the key significance of 10S in nonmuscle cells was in the abstract. To further highlight the points raised by the referee, we have now added a clause on activation of 10S to form filaments in the abstract (LL 32-33) and a sentence describing the equilibrium between 10S and filaments in the introduction (LL 51-52, 54-55). We now also point to the importance of the IHM in thick filaments, where it contributes to the relaxed state (LL 49-50). To avoid digressing too far from the focus of the paper, we have not brought up the super-relaxed state, but reference our earlier reconstruction paper (Yang 2019) where the extra space allowed us to discuss these points more fully.

4. L97, Head-tail junction "not been possible to study by x-ray crystallography": Please clarify why.

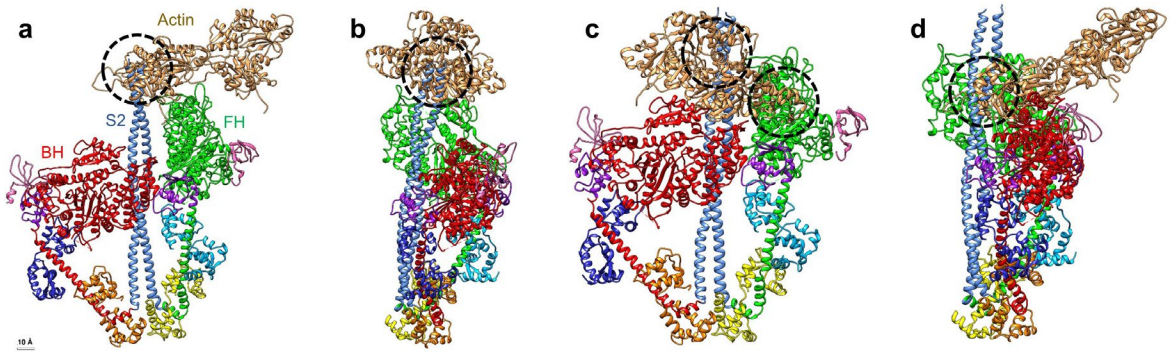
We have now added the explanation (previously omitted to save space). LL. 96-97.

5. L140: Two different positions of S2 are established, and the proposal is made that the S2 prefers the mesa but is displaced from it in the 10S state by the tail segment. This seems like it might be relevant to the mechanism by which molecules leave the thick filament and take up the 10S structure in solution? And predicts that HMM should adopt the thick filament type of IHM?

We agree that this would predict that HMM should adopt the thick filament type of IHM. However, after submitting the paper we discovered that this experiment had actually been done. Burgess et al. (2007) compared smooth muscle myosin and HMM by negative staining and made a difference image from which they concluded that within the ~20 Å resolution of the technique, S2 is in the same position in HMM and 10S myosin (their Fig. 6 and their discussion on p. 1173), running across the edge of the BH, just as seen in our reconstruction, and also interacting with the tip of the FH, as we find. This suggests that the difference is actually between the position of S2 in the thick filament and that in the single molecule (both HMM and 10S). A different location, and the bending of S2, in thick filaments may result from the differing molecular constraints in myosin molecules incorporated into a filament and undergoing intermolecular interactions with other molecules in the polymer (as well as intramolecular interactions within the IHM), compared with only intramolecular interactions in the single molecule. We have amended this discussion point on LL 138-141.

6. L189: In the interesting discussion of the mechanism of inhibition, it is pointed out that the tail blocks the actin binding site on FH, and that this is part of the inhibition mechanism. Does this imply that the FH of dephosphorylated HMM should be able to bind actin?

No. Seg1 would also play a role in this inhibition (as stated on LL 184-185, 193). In fact, when an HMM version of the 10S structure (i.e. lacking segs 2 and 3, but including seg1) is attached to actin in the rigor configuration via the FH, S2 clashes with actin. See the figure below (we have also now included this figure in Extended Data Fig. 7h-k, now referenced on LL 185). The model would thus predict that binding of HMM to actin via the FH would be inhibited. Similarly, if HMM is attached via the BH, there are multiple clashes, of both the FH and S2 with actin (consistent with the early Wendt model of the IHM in which actin-binding by the BH is blocked). This modelling assumes that S2 in HMM is in the same position as in the 10S structure, as discussed in #5 above. The binding of the 10S whole myosin molecule to actin would be further exacerbated by the additional segments of tail, consistent with the finding that in molecules trapped in the folded state by crosslinking, neither head binds to actin, even under rigor conditions (Olney 1996).



**Inhibition of actin-binding by HMM in the IHM configuration. a, b.** Binding via FH. S2 clashes (dashed circle) with actin (2 monomers shown). **b.** Rotated 90° around vertical axis with respect to **a. c, d.** Binding via BH. S2 and FH clash with actin. **d.** Rotated 90° around vertical axis with respect to **c.** BH and FH were attached to actin by superposing their MDs on the MD (not shown) of mammalian actomyosin in the rigor state (PDB 5H53), as described in Extended Data Fig. 3.

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In a loose sense 10S has to be a substrate for MLCK. If not, then it could not be phosphorylated. How this occurs is not known. Experiments in the Ikebe lab show that myosin II can be phosphorylated by MLCK under "10S conditions" (low salt, presence of Mg.ATP) – so MLCK does phosphorylate myosin in the conditions where myosin forms the 10S structure. But whether MLCK directly phosphorylates the 10S conformation of myosin is not clear. It is possible that MLCK phosphorylates the low amount of 6S in equilibrium with 10S, shifting the equilibrium to 6S until all molecules are phosphorylated. An alternative possibility is that the molecule retains the overall folded, 10S structure, but "breathes", allowing periodic access to the RLCs on the two heads. We proposed a possible similar mechanism in our earlier, negative staining paper on the 3D structure of 10S myosin (Yang et al., 2019), which we now refer to in Extended Data Fig. 8. The Ikebe lab finds that 10S myosin is phosphorylated significantly more slowly than S1 and HMM, but as a practical matter, myosin in 10S conditions is phosphorylated.

The referee raises the very interesting question of whether binding of the BH RLC phosphorylation domain to seg3 might inhibit binding of MLCK. We agree that this is a reasonable suggestion, and came to a similar conclusion based on our earlier negative stain reconstruction (Yang et al., 2019). Our improved fitting of the BH RLC N-terminus to the cryo-reconstruction (see below) enables us to say a bit more. The best fit is now shown in Extended Data Fig. 8i. It shows the N-terminal helix and linker in contact with seg3, through interactions involving K11, K12 and R13. From modeling, it looks like the MLCK binding region might be exposed enough for MLCK to bind (data not shown)—but the engagement of K11-R13 with seg3 could hinder binding because these residues are also thought to be involved in MLCK substrate recognition. So our current model supports the referee's suggestion. We now comment on this at the end of ED Fig. 8 legend.

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Myosin II is the major class of motor protein powering cellular contractility in both muscle and non-muscle cells. Myosin II adopts either a folded autoinhibited state as a soluble dimer or an extended, active state through oligomerization of dimers into filaments. In the autoinhibited state, the ATPase

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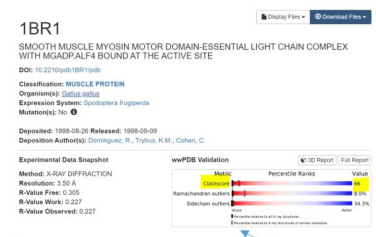
A soft mask (5-pixel extension, 6-pixel soft cosine edge), enclosing the entire 10S structure, was applied during 3D refinement and post-processing. The overall gold standard resolution (FSC = 0.143) was calculated using this soft mask in the post-processing step. We have modified the Methods to include this information. We now quote the 4.3 Å resolution as the global resolution in the abstract (LL. 34-35) to indicate that the resolution varies, and we quote the resolution range in the Results (LL. 66). We note that the majority of the structure (two MDs, the BH RD and parts of all three tail segments = ~2/3 the total mass altogether) are in the resolution range 4.1-5.7 Å, according to the local resolution map, which

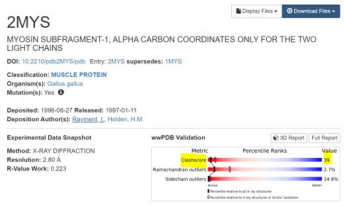
could help to account for the overall 4.3 Å number. A similar difference between global and local resolution was seen in a recent muscle publication on thick filament structure (Hu et al., Sci. Adv. 2016; 2 : e1600058), where the global resolution was 6.2 Å, with the tails (about one half the total mass) at 5.5 Å while the heads (the other half of the mass) were at 12-21 Å resolution.

2. A key advance provided by the manuscript is a reliable atomistic model of the 10S myosin II. As the authors note, "because of the limited resolution of the reconstruction we consider these putative interactions potential rather than definitive". This level of interpretation undermines the impact of the study. In particular, the authors' choice to simply rigid-body dock existing atomic models, followed by real-space refinement, does not take full advantage of the information content of their maps, and can in fact lead to issues like clashes, as indicated by the high ClashScore they report. In the 4-8 Angstrom resolution range, well-established computational methods like Molecular Dynamics Flexible Fitting or Rosetta can provide highly reliable, if not definitive, information at the atomic level. Employing these methodologies would likely improve the atomic model and substantially strengthen the authors' claims at the residue level.

We thank the referees for these suggestions, both of which we have now tried. Unfortunately, neither improved the overall structure. Following Molecular Dynamics Flexible Fitting using various gscales (0.3, 1, 3, and 5), real space refinement in Phenix degraded the stereochemical quality of our original structure. This included the clash score, which went from 38 to 43, and poorer geometric parameters (bond angles, outliers, etc.). It is possible that our model already had a good fit and that is why MDFF did not improve it. The best Rosetta structure was similar, improving the clash score to 27 after refinement, but with poorer geometric parameters. We tried improving the geometry but the clash scores again became worse than we had obtained originally. We think we understand a part of the reason that our clash score was not closer to ideal. The two X-ray crystal structures used to construct the 1i84 IHM model (primarily 1br1 (3.5 Å resolution), with a contribution from 2mys (2.8 Å resolution) to the RLC) were both obtained in the 1990s, and both show quite poor clash scores themselves (66 and 39, respectively; **A** and **B** in the figure), presumably due to the less sophisticated software available at the time. (It was not possible for us to try to improve these structures ourselves as the original X-ray data are unavailable.) The original IHM (1i84; Wendt et al., 2001) had similarly poor scores (**C**). Our starting model, a refined version of 1i84 (Liu et al., 2003), which used those X-ray structures, also had poor scores (Clashscore 71; **D**). Our final refined structure (6xe9) actually improved on all of these clash scores considerably (score 38) and also on the Ramachandran and sidechain outlier scores (**E**).




A

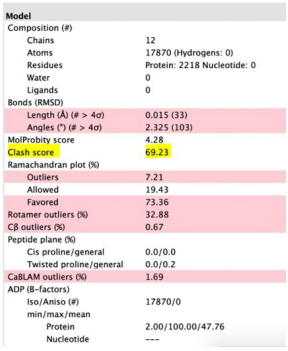



B

X-ray components (used in 1i84) – 1990s


C

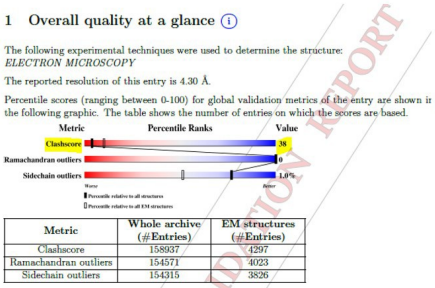
PDB 1i84 (2001) used as starting model for our structure (6xe9)



| Model | |
|---|---|
| Composition (#) | |
| Chains | 12 |
| Atoms | 17870 (Hydrogens: 0) |
| Residues | Protein: 2218 Nucleotide: 0 |
| Water | 0 |
| Ligands | 0 |
| Bonds (RMSD) | |
| Length (Å) (# > 4σ) | 0.015 (33) |
| Angles (°) (# > 4σ) | 2.325 (103) |
| MolProbity score | 4.28 |
| Clash score | 69.23 |
| Ramachandran plot (%) | |
| Outliers | 7.21 |
| Allowed | 19.43 |
| Favored | 73.36 |
| Rotamer outliers (%) | 32.88 |
| Cβ outliers (%) | 0.67 |
| Peptide plane (%) | |
| Cis proline/general | 0.0/0.0 |
| Twisted proline/general | 0.0/0.2 |
| CaBLAM outliers (%) | 1.69 |
| ADP (B-factors) | |
| Iso/Aniso (#) | 17870/0 |
| min/max/mean | |
| Protein | 2.00/100.00/47.76 |
| Nucleotide | --- |

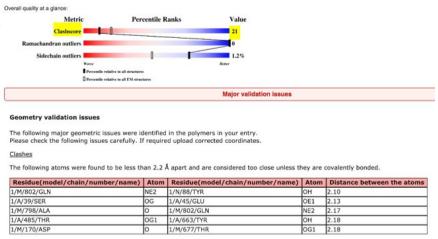
D

Refined version of 1i84 (Liu et al., 2003) (PDB not deposited)




E

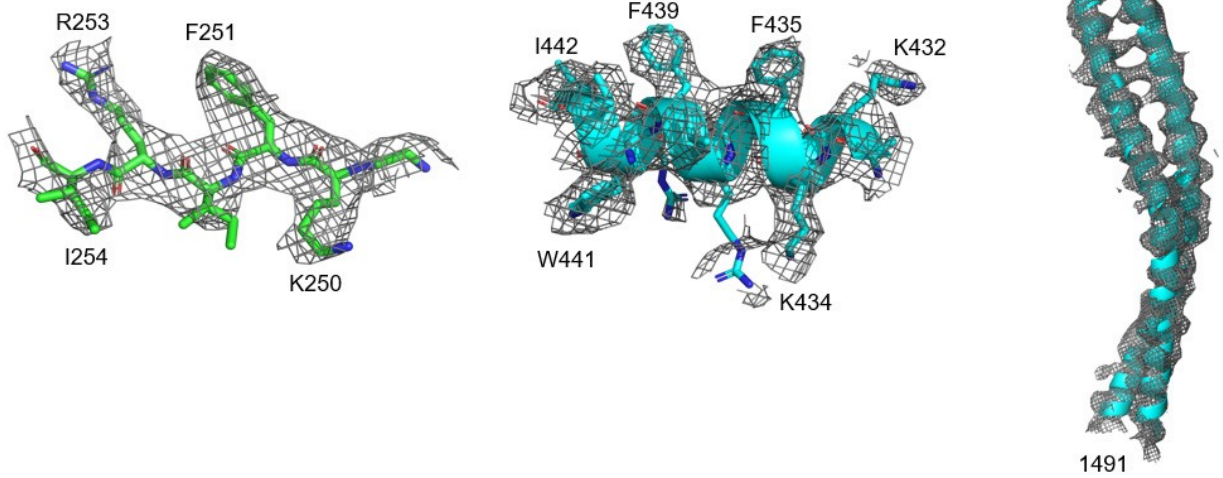
Our structure as submitted – 6xe9




F

PDB obtained after map autosharpening and refinement. This structure has been deposited as the revised version of 6xe9

Although our implementation of the referees' suggestions did not improve our structure beyond this, they prompted us to try other approaches to obtaining further detail from our map. Autosharpening in Phenix produced a significantly improved map, in which aromatic sidechains became visible in the core (highest resolution) regions of the heads, and the main chain helical path of the α-helices in these regions and in seg2 became better resolved (Figure below; and now included in Extended Data Fig. 2). When the model was refined against the map, the fitting parameters improved considerably, with the




clashscore going from 38 to 21 (only 5 clashes reported by wwPDB, compared with 30 in the original deposition), and other parameters also improving (**F**). We have submitted this improved PDB to the

database in place of the original. We have also amended our statement concerning "these putative interactions potential rather than definitive" to distinguish the higher resolution regions from those with lower resolution (LL. 148). Our statement now reads: "Because of the limited resolution of the reconstruction, we consider them potential rather than definitive, at least in the lower resolution regions." We hope this captures the point.

3. In Fig. 4 the models of the highly flexible N terminus of the RLC in the phosphorylated and unphosphorylated states appear to be solely derived from previous molecular dynamics simulations and are thus highly speculative. While the authors make this clear in the text, it would be appropriate to include appropriate caveats in the figure legend, where the model is currently described as definitive rather than speculative.

Thank you for this suggestion. We have now moved Fig. 4 to Extended Data Fig. 8 to help reduce the manuscript to Nature length. We now include the caveats mentioned by the referees. We have also added new information (Extended Data Fig. 8i) based on the sharpened map that appears to support the MD simulations.

Minor points (improving clarity of presentation at authors discretion):

1. It is admirable that the authors choose to present their raw data maps in the main figures. However, they use a lot of valuable real estate on showing the map and the model-map fit. In our opinion, the panels of Fig. 2a and b are redundant. We would recommend removing 2b, and adding several panels showing detail views of the locations of disease mutations. The residues in these panels should be labelled.

Thank you for this suggestion. We have removed Fig. 2b. However, given the space limitations, we were forced to show only a summary of the mutation data in the main text and to give the details in Extended Data Fig. 9, where there was space to show the details with labels.

2. The residues displayed in the panels of Fig. 3 should be labelled.

We originally considered this, but the figure would then be crowded with numbers, and the labels would have to be too small. There is more space in Supplementary Table 1, corresponding to this figure, where we do label the residues. We cross-reference this table in the legend to Fig. 3.

3. The labelling of the cartoons in in Figs. 2d and 3a are small and crowded. It would be helpful to make these panels larger, and increase the relative size of labels if possible, for better readability.

Thank you for this critique. For Fig. 3a, we now focus the cartoon just on the region of the reconstruction, allowing it to be enlarged, with larger lettering. We have now rearranged Fig. 2 vertically, with panel (b) removed, so that the cartoon and labeling can be enlarged.

**Reviewer Reports on the First Revision:**

Referees' comments:

***Referee #1:***

I have reviewed the revisions made by the authors in response to my comments and those of the other reviewers and am happy now to recommend publication.

***Referee #2:***

In their revised manuscript, Craig and colleagues have thoroughly and substantively responded to all of the points we raised during the previous round of review. While it is clear our suggestions for improving the structural analysis did not pan out, the Phenix auto-sharpened maps did result in a substantially improved structure from which more definitive side-chain level interpretation is justified in well-resolved regions. Additionally, the phosphorylation-activation mechanism has been better contextualized. Finally, the overall presentation and clarity of the display items and text has been considerably improved.

We recommend acceptance for publication without further delay.

**Author Rebuttals to First Revision:**

N/A