nature portfolio

Peer Review File

A proteolytic AAA+ machine poised to unfold protein substrates

Corresponding Author: Professor Joseph Davis

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Ghanbarpour et al report a high-resolution structure of ClpX bound to a tagged substrate protein, which allowed them to visualize axial channel residues in ClpX in contact with the C-terminal degradation tag on the substrate and to see details about the disposition of the six subunits (A-F) within the ClpX hexameric ring when a protein substrate is bound. The data are high quality and provide details about engagement of ClpX channel loops with the extended polypeptide tag, contacts between the folded domain of the substrate protein and the surface residues surrounding the channel opening in the ClpX ring, and structural rearrangements between ClpX subunits within the hexamer and between ClpX docking loops and ClpP. This work builds on earlier structural studies that showed peptides bound to the axial loops of the ClpX hexamer and (X. Fei et al., 2020, eLife 9:e52774; Ghanbapour et al 2023, PNAS 120:e2219044120), which provided a model of how cycles of engagement and disengagement could exert the pulling force needed to unfold bound substrate proteins.

The authors draw a number of very specific conclusions based on their analysis of this latest structure compared to previously published structures. Many are valuable in confirming earlier models, as with the description of the bulky channel residues of ClpX that efficiently grab the substrate and hold it during power strokes, which was suggested in the report by Fei et al., eLife 2020;9:e52774. The different orientations of the axial pore-1, pore-2, and pore-3 loops in the different subunits give a dynamic picture of a machine working to pull the substrate protein from a chain end to unravel it and then translocate it along the channel. All of this gives a further concrete confirmation of the model of the unfolding process that has emerged from many earlier elegant biochemical, biophysical, and structural studies. Other conclusions (below) are less well supported or need to be made more clearly.

Questions and issues that need to be addressed:

1. The authors report that, "Our structure also resolved a peripheral collar of contacts between the six RKH loops of ClpX and native DHFR." They suggest these contacts help prevent disengagement of the substrate during "denaturation attempts." Because the folded DHFR domain is pulled against the channel opening with no perturbation of the folded domain beyond an alteration in conformation of Arg159, the C-terminal residue of native DHFR, they conclude that, "The absence of slack would ensure that force from a power stroke is directly applied to the native structure."

One or two conclusions are not substantial and cannot with confidence be applied to the action of the native form of the ClpX complex. For example, the conclusion that ClpX does not "unfold" the bound substrate is unclear. What do the authors mean by unfolding in this context? It is confusing that the authors would conclude that these or similar contacts would not affect the unfolding pathway based on the observed binding to a protein that is highly resistant to unfolding. MTX-stabilized DHFR is known to be highly resistant to unfolding from previously published work (Lee et al 2001, Mol Cell 7, 627). Whether the observations here would apply to a less stable form of DHFR (without MTX) or to any other potential substrate with less stable tertiary structure. is not known. Two, the N-terminal domains of ClpX are missing in the truncated form of ClpX and thus their effects on folding and stability of a bound substrate protein cannot be extrapolated from any data presented here. These matters should be addressed by the authors.

2. They authors further state, "As newly highlighted in our structure, packing between the native DHFR domain and the ClpX determines the angle between the degron tail and the terminal DHFR structural element." They provide a specific measure

of the angle (120°). They should be more specific in defining the angle. Is the 120° deviation from the direction of the terminal beta strand or from the plane of the beta strand? If the former, what is the angle between the tail and the plane of the beta strand? the issue is important, because Matouschek (Lee et al 2001, Mol Cell 7, 627) showed that the effectiveness of the pulling force in unraveling a protein is highly dependent on the secondary structure on which it applied, and others have shown that the angle at which the force is applied is equally as important. The authors missed an opportunity to relate their observations to this basic issue regarding ClpX action. Figure 2b should be modified (or another image added) to better show the geometry of attachment of the degron tail with respect to the terminal structure of DHFR.

3. There is another conceptual problem regarding interpretation of the manner in which the folded DHFR structure packs against the axial opening in ClpX and speculation about how pulling forces would be effective in unraveling DHFR. As mentioned above, the form of DHFR used here is resistant to unfolding, and it is not entirely clear that the orientation of a degradable form of DHFR without MTX bound would be the same as what is present here. It seems quite likely that the stable complex that is visualized might be occur precisely because DHFR is drawn against the surface of ClpX, resists being pulled apart., and settles into a most favorable conformation. With a deformable form of DHFR, any number of orientations of DHFR could result in sufficient pulling to disrupt the structure of DHFR. Is there any evidence that DHFR without a degron tag can bind to ClpX? Or pack against the axial channel even transiently?

4. The degron extension used here is a branched chain and the issue is glossed over by the authors and need to be addressed. The authors do not report on the ability of ClpXP to degrade this substrate in the absence of MTX. Implicit in this study is the premise that what this structure represents is a productive (i.e. degradable) complex between this substrate and ClpXP. Providing that data would give the results and conclusions here more robustness. There are 34 C-terminal residues of the DHFR construct that are not modeled in the structure but are there, presumably occupying some volume within the channel or between ClpX and ClpP and possibly interacting with the ClpP proximal part of ClpX ring or with ClpP. While not much can be said about the chain itself, its presence should be noted, and an assessment of its effect provided.

5. Referring to the displacement of subunit F (from down to up, closer to the level of subunit B), the authors state, "These engaged subunits may be specialized for substrate denaturation." This idea is buttressed by the observation of classes of complexes in which the DHFR structure is unresolved above the pore. The meaning of "specialized" here is not clear and needs some further explication. Do they mean that subunit F (in the "up" position is the one exerting force on bound protein? It's an interesting idea and should be expanded. Could the authors relate that action to the slight dip in ATPase activity seen. A reaction pathway would be helpful to see. One could imagine that a cycle of force application that is futile (resulting in non-optimal release of a grip) might impede one of the kinetically important steps in the ATPase cycle. At the least, the matter deserves more than a cursory discussion at the end.

6. The presence of seven docking clefts in ClpP and only six IGF loops in ClpX leaves one cleft unoccupied. The authors observe that the unoccupied cleft is situated between subunits E and F when no substrate is present in the complex, but, when substrate is present, the shift of subunit F to the "up" position is accompanied by a shift of the IGF loop of subunit E so that the unoccupied cleft is now between subunits D and E. The gapped cleft is between D and E as it was in their structure with SspB/SsrA bound, unlike the unoccupied states where the gapped cleft is between E and F. Presumably, the shift in the IGF loop is made to accommodate the ring distortion that occurs when subunit F moves "up" without weakening the interaction between ClpX and ClpP, but the authors do not discuss the switch and simply relate the phenomenon. Some suggestion regarding the underlying cause and possible advantages of this conformational change is warranted.

The conformational changes in ClpX seen here and in the structure with SspB and SsrA-GFP are attributed to the presence of substrate in the complex, but the authors do not explain why similar changes were not seen in the previous structure with an unspecified substrate bound (Fei et al., eLife 2020;9:e52774). If the differences reflect the use of the ATP hydrolysis deficient mutant of ClpX in the earlier study, that should be mentioned and perhaps discussed in this report.

7. The experimental procedures could be written more clearly in places. One has to study the sequences of the two tags to understand what DHFR-C15-SsrA is? It is the extended SsrA polypeptide linked to the cysteine at position 15 in the C-terminal extension that was added to DHFR, I assume. Where does the rest of that peptide end up? Is it degraded? What is the evidence? The text suggests that a continuous peptide extension from the C-terminus of DHFR is what is present in the ClpX channel. Are any of the residue side chains visible? Is the density of sufficient quality throughout the chain? The substrate density appears to be less well defined than the earlier structures with GFP-SsrA. A cryo-EM density map of the polypeptide needs to be provided; it should have been part of Figure S3 or a more extended image provided in Figure 1b.

One experimental detail, the maleimide-SsrA species presumably has the reactive group attached to the N-terminus, but that should be stated explicitly for those wishing to reproduce the experiment.

8. The authors refer to the extension as C15-ssrA. The chain is modeled as a C chain, but in fact it is branched at the point where the SsrA peptide is attached, a matter that should be made more explicit in the description of the structure. Is the branch modeled in the structure? The polypeptide extension of DHFR past C15 is not visible. Is it there or was it degraded? If it is there, how does that impact the positioning of the visible part of the polypeptide in the channel? Does it or how does it affect the pore loops of ClpX?

Reviewer #2

(Remarks to the Author)

This study from Ghanbarpour, Sauer, and Davis presents the cryo-EM structure of E. coli ClpXP complex in a stalled substrate-engaged state, summarized in this brief 7-paragraph manuscript. The authors found that by incubating ClpX with an ssrA-tagged DHFR substrate stabilized with MTX, translocation proceeded until the globular domain of the substrate became lodged against the ATPase domain, resulting in a stable substrate-engaged conformation. Analysis of the data with CryoDRGN showed some variability in the substrate density, but the ATPase domains were conformationally static. Overall, the structures revealed key interactions between the substrate and the axial pore loops of the ClpX that are largely consistent with those previously observed. As described by the authors, numerous other structures of E.coli ClpXP with substrate bound have been determined previously (Fei, Bell et al. 2020, Fei, Bell et al. 2020, Ripstein, Vahidi et al. 2020, Ghanbarpour, Cohen et al. 2023, Ghanbarpour, Fei et al. 2023). I imagine the impetus for this study was to identify new conformational states of the machinery by imaging actively hydrolyzing ClpX as it tries to unfold and translocate a stabilized substrate and leveraging cryoDRGN analyses. While the experimental setup novel, as there are no other ClpX cryoEM studies targeting the wild type ATPase in the presence of ATP, the findings constitute what I consider to be an incremental advance in the field. The authors observe that the orientation of the substrate relative to ClpX positions the ATPase domains to optimally transfer their unfolding forces on the folded substrate, although this hypothesis is not supported with other biochemical or biophysical data. Further, the observed arrangement (with F subunit at the top instead of bottom) was also observed in prior studies from the group, where it was also postulated that the ClpX conformation was associated with force application. I'm unclear how much of a scientific advance is required to be considered for publication in Nature Communications, but based on my reading of the papers from the journal, this study seems too limited in scope for consideration in its current state.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
This manuscript from Ghanbarpour et al. describes cryo-EM structures of wild type E. coli ClpXP engaged to a native substrate. The authors' findings include two reconstructions of ClpXP in a "substrate engaged" state and one reconstruction of ClpXP in a "translocation" state. Native substrate targeted in this study, DHFR, contained a C-terminal branched or linear ssrA-tag, and stabilization with a small molecule methotrexate (MTX) enabled the authors to trap the complex in the process of translocation/unfolding. These structures reveal key interactions between the substrate and the axial pore loops of the ClpX, as well as an atypical positioning of the ClpX pore-1 loop in the hexameric spiral. Notably, the use of wild-type ClpX and ATP reveals a unique structural configuration of hClpX with three ATP and three ADP in the nucleotide binding sites across all three cryo-EM structures. The most surprising observation presented in this manuscript is presence of three ATP and three ADP nucleotides in the nucleotide binding pockets, which is inconsistent with the canonical 4ATP/2ADP or 5ATP/1ADP occupancies observed in previously in AAA+ structures. Additionally, the seam subunit adopts an "up" conformation. Based on these structural findings, the authors infer that the "up" conformation could be an intermediate during substrate unfolding and translocation or a pre-powerstroke organization associated with the large-step mechanism of translocation.

The cryo-EM data collection, analysis, and modeling has been performed expertly, and these structures contribute important insights into the interactions between a wild type AAA+ motor and a native substrate. However, the analysis and interpretation of the nucleotide densities and their binding pockets warrants further consideration and discussion. How confident are the authors that nucleotide states can be assigned definitively? Given that single particle cryo-EM is an ensemble analysis, is it possible that there is a mixture of ATP and ADP in all the binding pockets, and that there is a greater probability of hydrolysis the longer the ATP is in the pocket (i.e. a gradient of ATP and ADP nucleotide densities in the pockets). There is no mention of the arginine fingers or other components of the nucleotide binding pocket and how they differ when ATP or ADP is present, and how these compare to prior structures.

The authors present a large-step model as a possible mechanism for translocation for contextualizing the new conformation. However, such a model would require the substrate to dissociate from all other pore loop interactions, correct? Given the extent of interaction between the pore loops and the substrate polypeptide, this seems unlikely. Further, could pore loop interactions from a single subunit provide the sufficient "grip" to drive translocation? I agree with the authors that the 2-residue translocation-per-ATP model is inconsistent with prior biophysical studies and that there must be an alternative translocation mechanism – perhaps hydrolysis isn't required for each processive motion of the motor.

Additional points that should be addressed:

- What is the rationale behind designing a DHFR with a branched degron tail or DHFR with N-terminal H6-TEV tag and a linear C-terminal degron? It's unclear what specific questions the authors aim to address.

- The statement "The linear degron tail immediately C-terminal to DHFR followed a similar trajectory to that of the branched degron substrate, indicating that the ClpX axial channel can readily transition between conformations able to accommodate single or multiple substrate chains" is a bit of an overstatement. Is there evidence that ClpX couldn't use identical trajectories for both linear and branched substrates, particularly if contact is only made with a single substrate polypeptide?

- In Figure 1B, Arg 159 is mentioned to adopt alternate conformations. Please clearly show this with a side-by-side comparison of the structures (it is also not shown in Figure 3 despite the callout).

- The authors include a table of ClpXP structures adopting in various state but do not elaborate on these conformations. It would be helpful for readers if the distinct structural features defining the “recognition”, “engaged”, “intermediate” and “unfolding/translocation/degradation” states conformations were described to provide context for the new structures. In Figure 4, the authors state that “The IGF loop of chain E5 moves into a binding cleft that is unoccupied in structures similar to that in panel A. These movements correlate with Fseam upward movement, as shown in panels C and D”. Do all the structures with Fseam in an “up” position listed in table 2 have an empty cleft between D4 and E5 as shown in Figure 4B? Is the “up” conformation of Fseam specifically associated with an “engaged” state (depicted in Figure 4D); if so, why would the ClpXP structure in absence of MTX also adopt a similar Fseam conformation?

- Density for the surrounding residues, rather than just the zoned nucleotide density, should be shown for Supplementary Figure 15 to provide a sense of the quality of the density in the binding pocket.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed my concerns, I find the manuscript appropriate for publication.

Point-by-point responses

Overview. We thank both reviewers for their comments. Our responses are indented and highlighted in light grey. We significantly revised the manuscript to include two new cryo-EM structures and additional biochemical experiments. One new structure is a fully engaged complex of ClpXP and MTX-bound DHFR with a linear C-terminal ssrA degron and an N-terminal affinity tag. Strikingly, native DHFR in this complex assumed an orientation rotated by ~180° compared to branched-degron DHFR in our original complex. This change in DHFR orientation illustrates how ClpX is able to adapt to different protein substrates, in part because of the flexibility of its RKH loops, and helps explain the ability of ClpXP to degrade a wide variety of different degron-tagged substrates.

We also determined a structure of ClpXP with branched-degron DHFR in the absence of MTX. Native DHFR was not observed in this MTX-free structure, probably because it had been unfolded and denatured DHFR was being translocated through the ClpX channel. Notably, however, the seam subunit of ClpX assumed the same 'up' conformation in this structure that we observed in both fully engaged DHFR•MTX structures. This result is incompatible with our previous suggestion that the 'seam subunit up' conformation is specialized for substrate unfolding and instead suggests that it is part of the normal catalytic cycle and employed both during substrate unfolding and translocation. In addition, all three ClpX structures in the revised paper contain three ATP-bound and three ADP-bound subunits. This configuration of nucleotide-bound subunits has not been previously observed for ClpX, perhaps due to the common use of Walker-B mutations or ATPγS in prior studies.

The text, analysis, and discussion in the revised manuscript have been expanded to discuss the implications of our new results as well as those from the original paper. For example, we have expanded our discussion of these new structures' relationship to the commonly referenced 'hand-over-hand' vs. 'large step' models for substrate translocation.

Reviewer 1:

1. The authors report that, "Our structure also resolved a peripheral collar of contacts between the six RKH loops of ClpX and native DHFR." They suggest these contacts help prevent disengagement of the substrate during "denaturation attempts." Because the folded DHFR domain is pulled against the channel opening with no perturbation of the folded domain beyond an alteration in conformation of Arg159, the C-terminal residue of native DHFR, they conclude that, "The absence of slack would ensure that force from a power stroke is directly applied to the native structure."

As noted in the Overview above, we determined a second fully engaged ClpXP•DHFR•MTX structure in which DHFR assumes a different orientation compared to our original structure. In both structures, the native body of DHFR was pulled tightly against ClpX in the sense that density between native DHFR and the degron tail was continuous, whereas this would not have been expected if several residues of the degron were disordered. Our use of '*absence of slack*' was intended to imply that any attempt to translocate the extended degron in a β-strand conformation would apply an unfolding force to native DHFR. We would appreciate suggestions about clearer ways of trying to communicate this point.

One or two conclusions are not substantial and cannot with confidence be applied to the action of the native form of the ClpX complex. For example, the conclusion that ClpX does not "unfold" the bound substrate is unclear. What do the authors mean by unfolding in this context? It is confusing that the authors would conclude that these or similar contacts would not affect the

unfolding pathway based on the observed binding to a protein that is highly resistant to unfolding. MTX-stabilized DHFR is known to be highly resistant to unfolding from previously published work (Lee et al 2001, Mol Cell 7, 627). Whether the observations here would apply to a less stable form of DHFR (without MTX) or to any other potential substrate with less stable tertiary structure. is not known.

We agree that our structures do not exclude the possibility that binding alone (*i.e.,* in the absence of ATP-dependent pulling) would not be sufficient to unfold some substrates. We removed this speculation from the revised manuscript.

Two, the N-terminal domains of ClpX are missing in the truncated form of ClpX and thus their effects on folding and stability of a bound substrate protein cannot be extrapolated from any data presented here. These matters should be addressed by the authors.

We added a new experiment showing that full-length ClpX and ClpX$^{\Delta N}$ support almost complete ClpP degradation of DHFR within 15 min in the absence of MTX (Supplementary Figure 2). This result is in agreement with prior studies showing that full-length ClpXP degrades human titin^{I27}-ssrA with a V_{max} of 0.25 min^{-1}, whereas ClpX$^{\Delta N}$/ClpP degrades the same substrate with a V_{max} of 0.24 min^{-1} (Martin et al., 2005). Indeed, we have never seen substantial differences in the kinetics of substrate degradation by full-length ClpXP or ClpX$^{\Delta N}$/ClpP unless the substrate or an adaptor needed to bind to the N-terminal domain.

2. They authors further state, "As newly highlighted in our structure, packing between the native DHFR domain and the ClpX determines the angle between the degron tail and the terminal DHFR structural element." They provide a specific measure of the angle (120°). They should be more specific in defining the angle. Is the 120° deviation from the direction of the terminal beta strand or from the plane of the beta strand? If the former, what is the angle between the tail and the plane of the beta strand? the issue is important, because Matouschek (Lee et al 2001, Mol Cell 7, 627) showed that the effectiveness of the pulling force in unraveling a protein is highly dependent on the secondary structure on which it applied, and others have shown that the angle at which the force is applied is equally as important. The authors missed an opportunity to relate their observations to this basic issue regarding ClpX action. Figure 2b should be modified (or another image added) to better show the geometry of attachment of the degron tail with respect to the terminal structure of DHFR.

We agree that any angle between the C-terminal β-strand of DHFR and the degron in the ClpX channel would need to be more precisely defined. Moreover, this angle (however described) clearly changes in our new ClpXP structure containing DHFR with a linear degron (see figure below). In the revised manuscript, we removed any discussion of exact angles and expanded discussion of how different engagement conformations may impact the efficacy of a power stroke in locally or globally denaturing a substrate.

Comparison of substrate/degron angles between His6-TEV-DHFR-ssrA (green) and DHFR-branched-degron-ssrA (cyan), where atomic models are aligned by the native substrate. Red circle highlights the interface between the terminal beta strand of the substrate and the beginning of the degron present in the ClpX axial channel, which was omitted for clarity.

3. There is another conceptual problem regarding interpretation of the manner in which the folded DHFR structure packs against the axial opening in ClpX and speculation about how pulling forces would be effective in unraveling DHFR. As mentioned above, the form of DHFR used here is resistant to unfolding, and it is not entirely clear that the orientation of a degradable form of DHFR without MTX bound would be the same as what is present here. It seems quite likely that the stable complex that is visualized might be occur precisely because DHFR is drawn against the surface of ClpX, resists being pulled apart., and settles into a most favorable conformation. With a deformable form of DHFR, any number of orientations of DHFR could result in sufficient pulling to disrupt the structure of DHFR. Is there any evidence that DHFR without a degron tag can bind to ClpX? Or pack against the axial channel even transiently?

We agree and do not expect the orientation of different native substrates to remain constant with respect to ClpX, as shown by the two fully engaged DHFR•MTX structures presented in the revised manuscript. We hope that this is clear in the revised manuscript. With regard to the possibility of degron-independent interactions between ClpX and DHFR, we did not observe degradation of DHFR lacking an ssrA tag (Supplementary Figure 2) and imagine that binding would be too weak to be detected in the absence of a degron.

4. The degron extension used here is a branched chain and the issue is glossed over by the authors and need to be addressed.

In the revised manuscript, we show schematic structures of the branched-degron substrate and a second DHFR substrate bearing a linear degron terminating in the ssrA tag (Supplementary Figure 1). We also revised the text to make it clear that both substrates were used.

The authors do not report on the ability of ClpXP to degrade this substrate in the absence of MTX. Implicit in this study is the premise that what this structure represents is a productive (i.e. degradable) complex between this substrate and ClpXP. Providing that data would give the results and conclusions here more robustness.

In Supplementary Figure 2, we show that the branched-degron substrate is degraded by full-length ClpXP and by ClpX$^{\Delta N}$/ClpP in the absence but not the presence of MTX.

There are 34 C-terminal residues of the DHFR construct that are not modeled in the structure but are there, presumably occupying some volume within the channel or between ClpX and ClpP and possibly interacting with the ClpP proximal part of ClpX ring or with ClpP. While not much can be said about the chain itself, its presence should be noted, and an assessment of its effect provided.

In the revised manuscript, we emphasize the position of the cysteine that served as the branch point (see Figure 1; cysteine highlighted in yellow). We also note that this region of the ClpP portal is wide enough to accommodate the remainder of the branched peptide chain. Unfortunately, the density in this region of the map did not allow us to model the branch.

5. Referring to the displacement of subunit F (from down to up, closer to the level of subunit B), the authors state, "These engaged subunits may be specialized for substrate denaturation." This idea is buttressed by the observation of classes of complexes in which the DHFR structure is unresolved above the pore. The meaning of "specialized" here is not clear and needs some further explication. Do they mean that subunit F (in the "up" position is the one exerting force on bound protein? It's an interesting idea and should be expanded. Could the authors relate that action to the slight dip in ATPase activity seen. A reaction pathway would be helpful to see. One could imagine that a cycle of force application that is futile (resulting in non-optimal release of a grip) might impede one of the kinetically important steps in the ATPase cycle. At the least, the matter deserves more than a cursory discussion at the end.

As noted in the Overview at the beginning of this point-by-point response, we no longer think that the 'F-up' conformation is specialized for substrate denaturation but rather is an intermediate in both substrate unfolding and translocation. Moreover, we realized that ClpX subunits in different structures were not consistently named with respect to the spiral of pore-1 loop interactions with substrate polypeptide in the axial channel. In 12 of 15 ClpXP structures, subunit F is the seam subunit that connects the top and bottom of the spiral, with F "up" in 4/12 structures. In 3 of 15 structures, subunit A is the seam subunit, and in each of these 3 instances it is "up". In Table 2 of the revised manuscript, we now summarize different ClpXP structures in which the 'seam' subunit is either in the up or down conformation and also characterize these structures in terms of function (substrate free, recognition, substrate engaged, translocation, etc.) and nucleotide occupancy. We also note that structures with three ATP-bound and three ADP-bound subunits (as observed for all of the new structures presented in this paper) are not predicted intermediates in current translocation models for AAA+ proteases and remodeling machines.

6. The presence of seven docking clefts in ClpP and only six IGF loops in ClpX leaves one cleft unoccupied. The authors observe that the unoccupied cleft is situated between subunits E and F when no substrate is present in the complex, but, when substrate is present, the shift of subunit F to the "up" position is accompanied by a shift of the IGF loop of subunit E so that the unoccupied cleft is now between subunits D and E. The gapped cleft is between D and E as it was in their structure with SspB/SsrA bound, unlike the unoccupied states where the gapped cleft is between

E and F. Presumably, the shift in the IGF loop is made to accommodate the ring distortion that occurs when subunit F moves "up" without weakening the interaction between ClpX and ClpP, but the authors do not discuss the switch and simply relate the phenomenon. Some suggestion regarding the underlying cause and possible advantages of this conformational change is warranted.

We agree that the cleft swap occurs to accommodate movement of the 'seam' subunit. At a minimum, these motions seem to be tightly coupled, as a detailed cryoDRGN analysis did not reveal 'mixed' particles with the 'seam' subunit up and an unoccupied cleft characteristic of the 'seam' subunit down conformation. Inspection of structures is consistent with this possibility, but we are unable to determine rigorously whether a mixed conformation is impossible or simply improbable from an energetic perspective. Thus, we have elected to simply state our observation, and leave it to future studies to interrogate the biophysical basis of this coupling.

The conformational changes in ClpX seen here and in the structure with SspB and SsrA-GFP are attributed to the presence of substrate in the complex, but the authors do not explain why similar changes were not seen in the previous structure with an unspecified substrate bound (Fei et al., eLife 2020;9:e52774). If the differences reflect the use of the ATP hydrolysis deficient mutant of ClpX in the earlier study, that should be mentioned and perhaps discussed in this report.

Our original manuscript incorrectly assumed that the 'seam up' conformation was a consequence of having an engaged native substrate as the naming different conventions led us to overlook structures such as 6WSG and 6VFS that have the 'seam' subunits up without a fully engaged substrate. We now discuss this issue in the revised manuscript and include Table 2, which consolidates these disparate naming schemes. We also discuss the possibility that the observed nucleotide occupancy in many prior structures may reflect the use of the slowly hydrolyzed ATPγS analog and/or Walker-B ATPase mutations.

With regard to the unspecified substrates bound in Fei et al., as the reviewer noted, the previously determined 6PP6 and 6PP8 structures had substrate above the channel, but the identities of these substrates (or ensembles of substrates) were unknown and the resolution was too low to allow model building. Because of low resolution of the protein above the channel in the 6PP6/6PP8 structures, we were unable to determine if this protein was a substrate or an adaptor and could not verify that density for this protein connected to the clear polypeptide in the axial channel of ClpX.

7. The experimental procedures could be written more clearly in places. One has to study the sequences of the two tags to understand what DHFR-C15-SsrA is? It is the extended SsrA polypeptide linked to the cysteine at position 15 in the C-terminal extension that was added to DHFR, I assume. Where does the rest of that peptide end up? Is it degraded? What is the evidence? The text suggests that a continuous peptide extension from the C-terminus of DHFR is what is present in the ClpX channel. Are any of the residue side chains visible? Is the density of sufficient quality throughout the chain? The substrate density appears to be less well defined than the earlier structures with GFP-SsrA. A cryo-EM density map of the polypeptide needs to be provided; it should have been part of Figure S3 or a more extended image provided in Figure 1b.

One experimental detail, the maleimide-SsrA species presumably has the reactive group attached to the N-terminus, but that should be stated explicitly for those wishing to reproduce the experiment.

As noted above, Supplementary Figure 1 now shows cartoon structures of the branched and linear degron substrates and hopefully makes it clear that the maleimide group was at the N-terminus of the peptide containing the ssrA tag. The text was also revised to clarify these points.

We don't know exactly where disordered parts of the branched degron are in the structure, but linkage to the ordered part suggests that they are in the ClpP portal and top of the ClpP degradation chamber. We saw no trimming of branched-degron DHFR•MTX in the ClpXP degradation experiments shown in Supplementary Figure 2, but we can't rule out that a few terminal amino acids of the branched degron might have been cleaved.

Side-chain density for the linear portion of the branched polypeptide in the channel was good for residues contacted by the pore-1 loops of ClpX (Supplementary Figure 9B) and less good for residues closer to the ClpP portal. Do note that this is the first time we see degron extended all the way to the ClpP chamber, and that the density for the degron within the ClpX axial channel is well defined.

8. The authors refer to the extension as C15-ssrA. The chain is modeled as a Cα chain, but in fact it is branched at the point where the SsrA peptide is attached, a matter that should be made more explicit in the description of the structure. Is the branch modeled in the structure? The polypeptide extension of DHFR past C15 is not visible. Is it there or was it degraded? If it is there, how does that impact the positioning of the visible part of the polypeptide in the channel? Does it or how does it affect the pore loops of ClpX?

We thank the reviewer for careful reading of our manuscript, and believe that the newly included figures and descriptions of this branched substrate clarify our manuscript. Please see our responses to points 4 and 7 for comments on these remaining questions.

Reviewer #2 (Remarks to the Author):

This study from Ghanbarpour, Sauer, and Davis presents the cryo-EM structure of E. coli ClpXP complex in a stalled substrate-engaged state, summarized in this brief 7-paragraph manuscript. The authors found that by incubating ClpX with an ssrA-tagged DHFR substrate stabilized with MTX, translocation proceeded until the globular domain of the substrate became lodged against the ATPase domain, resulting in a stable substrate-engaged conformation. Analysis of the data with CryoDRGN showed some variability in the substrate density, but the ATPase domains were conformationally static. Overall, the structures revealed key interactions between the substrate and the axial pore loops of the ClpX that are largely consistent with those previously observed. As described by the authors, numerous other structures of E.coli ClpXP with substrate bound have been determined previously (Fei, Bell et al. 2020, Fei, Bell et al. 2020, Ripstein, Vahidi et al. 2020, Ghanbarpour, Cohen et al. 2023, Ghanbarpour, Fei et al. 2023). I imagine the impetus for this study was to identify new conformational states of the machinery by imaging actively hydrolyzing ClpX as it tries to unfold and translocate a stabilized substrate and leveraging cryoDRGN analyses. While the experimental setup novel, as there are no other ClpX cryoEM studies targeting the wild type ATPase in the presence of ATP, the findings constitute what I consider to be an incremental advance in the field. The authors observe that the orientation of the substrate relative to ClpX positions the ATPase domains to optimally transfer their unfolding forces on the folded substrate, although this hypothesis is not supported with other biochemical or biophysical data. Further, the observed arrangement (with F subunit at the top instead of bottom) was also observed in prior studies from the group, where it was also postulated that the

ClpX conformation was associated with force application. I'm unclear how much of a scientific advance is required to be considered for publication in Nature Communications, but based on my reading of the papers from the journal, this study seems too limited in scope for consideration in its current state.

We respectfully disagree. ClpX unfolding of native substrates is critical to the biological functions of ClpX and ClpXP and no previous structures have captured the native portion of a protein substrate at sufficient resolution to model the complex and interactions between the folded substrate and ClpX. In the revised manuscript, we present a second structure of a fully engaged DHFR substrate that illustrates how flexibility of the RKH loops of ClpX allow it to adapt to different conformations of a bound substrate, with obvious implications for the ability of ClpXP to degrade an amazingly diverse set of cellular substrate. Structures, like the ones presented here, will be crucial for understanding the molecular mechanism of substrate denaturation and have garnered great interest not only within the AAA+ protease community but also among researchers studying other AAA+ remodeling machines that utilize ATP hydrolysis to exert mechanical force on substrates. In this paper, we determined three structures in which ClpX had a heretofore unseen configuration of three ATP-bound and three ADP-bound subunits, which was seen in two different fully engaged complexes as well as a translocation complex. Notably, a 3-ATP/3-ADP configuration of subunits for ClpX or any other AAA+ machine has not to our knowledge been proposed as an intermediate in hand-over-hand or alternative models of protein translocation by AAA+ machines.

REVIEWER COMMENTS

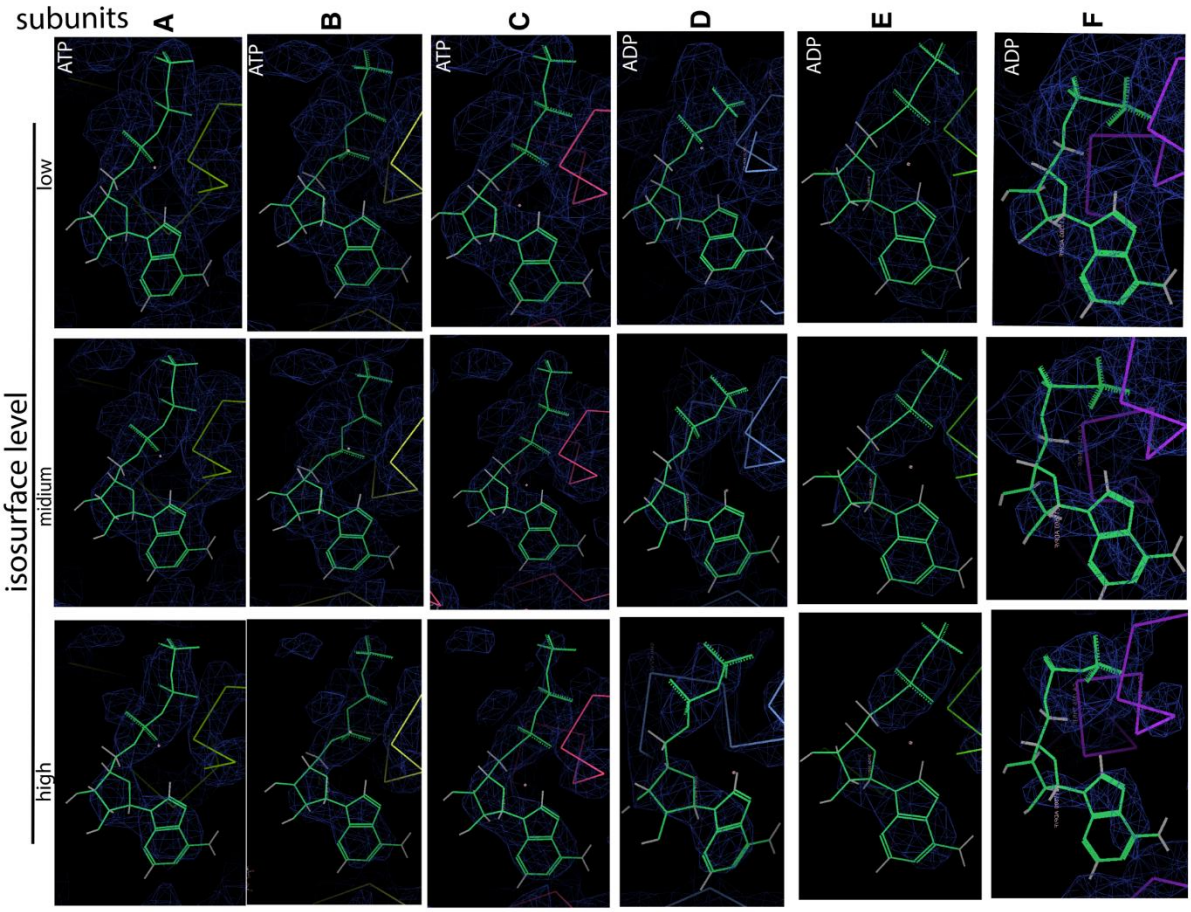
We thank the reviewer for their comments. We have addressed all the points raised by reviewer #2 in our revised manuscript, as detailed below.

Reviewer #2 (Remarks to the Author):
This manuscript from Ghanbarpour et al. describes cryo-EM structures of wild type E. coli ClpXP engaged to a native substrate. The authors' findings include two reconstructions of ClpXP in a "substrate engaged" state and one reconstruction of ClpXP in a "translocation" state. Native substrate targeted in this study, DHFR, contained a C-terminal branched or linear ssrA-tag, and stabilization with a small molecule methotrexate (MTX) enabled the authors to trap the complex in the process of translocation/unfolding. These structures reveal key interactions between the substrate and the axial pore loops of the ClpX, as well as an atypical positioning of the ClpX pore-1 loop in the hexameric spiral. Notably, the use of wild-type ClpX and ATP reveals a unique structural configuration of ClpX with three ATP and three ADP in the nucleotide binding sites across all three cryo-EM structures. The most surprising observation presented in this manuscript is presence of three ATP and three ADP nucleotides in the nucleotide binding pockets, which is inconsistent with the canonical 4ATP/2ADP or 5ATP/1ADP occupancies observed in previously in AAA+ structures. Additionally, the seam subunit adopts an "up" conformation. Based on these structural findings, the authors infer that the "up" conformation could be an intermediate during substrate unfolding and translocation or a pre-powerstroke organization associated with the large-step mechanism of translocation.

The cryo-EM data collection, analysis, and modeling has been performed expertly, and these structures contribute important insights into the interactions between a wild type AAA+ motor and a native substrate. However, the analysis and interpretation of the nucleotide densities and their binding pockets warrants further consideration and discussion.

How confident are the authors that nucleotide states can be assigned definitively? Given that single particle cryo-EM is an ensemble analysis, is it possible that there is a mixture of ATP and ADP in all the binding pockets, and that there is a greater probability of hydrolysis the longer the ATP is in the pocket (i.e. a gradient of ATP and ADP nucleotide densities in the pockets). There is no mention of the arginine fingers or other components of the nucleotide binding pocket and how they differ when ATP or ADP is present, and how these compare to prior structures.

> *We thank the reviewer for their comment. We have compared the densities of each nucleotide at different isosurface levels and have not observed additional density in the ADP binding pockets that could account for a γ-phosphate, as depicted below. Regarding the arginine fingers, we observed that Arg-307 interacts strongly with the ATP γ-phosphate in the subunits containing ATP and is disengaged and more disordered in the subunits containing ADP. Note that while we recognize that the density in this region of the map for subunit F is poor, ADP is most consistent with the density we observe. In sum, although we cannot rule out that these particle stacks include a minority of particles with ATP bound in the pockets we have assigned as ADP, this analysis strongly implies that the majority of the particles bear ADP in these subunits.*
>
> *To guide readers, we have modified Supplementary Figure 15 to now include density for the surrounding residues at the ATP/ADP binding sites.*



The authors present a large-step model as a possible mechanism for translocation for contextualizing the new conformation. However, such a model would require the substrate to dissociate from all other pore loop interactions, correct? Given the extent of interaction between the pore loops and the substrate polypeptide, this seems unlikely. Further, could pore loop interactions from a single subunit provide the sufficient “grip” to drive translocation? I agree with the authors that the 2-residue translocation-per-ATP model is inconsistent with prior biophysical studies and that there must be an alternative translocation mechanism – perhaps hydrolysis isn’t required for each processive motion of the motor.

We agree with the reviewer that the large step model necessitates the dissociation of substrates from all pore loops. We included the following explanation in the manuscript to ensure the reader is aware of different aspects of both models: “Notably however, unlike the ‘hand-over-hand’ model, the ‘large step’ requires coordinated 2 substrate release by pore loops of subunits A1-E5”.

With respect to the likelihood of this model, it is notable that the substrate in the axial channel is held by nonspecific interactions between the main chain of the pore loops and the substrate's polypeptide chain, making its dissociation through large step translocation driven by ATP hydrolysis plausible. The nonspecific nature of these interactions could allow new contacts to form continuously as the substrate slides through the channel. Furthermore, the downward movement of the F subunit may weaken the interactions between the substrate and the pore loops of subunits A and E, which are adjacent to the moving subunits. That being said, we have aimed to simply present the two models noting the implications of each, and we concur that further experiments will be needed to conclusively distinguish between them. We have aimed to capture this sentiment in the final sentence of our discussion: “As translocation step size and the number of substrate-pore loop contacts maintained during a power stroke are the primary discriminating features between the ‘hand-over-hand’ and the

'large step' models, biophysical experiments aimed at assessing these features hold the greatest promise in discriminating between these or additional models."

Additional points that should be addressed:
- What is the rationale behind designing a DHFR with a branched degron tail or DHFR with N-terminal H6-TEV tag and a linear C-terminal degron? It's unclear what specific questions the authors aim to address.

We have added an explanation in the Methods section to clarify our rationale for designing the branched degron: "We used this branched-degron DHFR substrate in hopes of visualizing how ClpX accommodates multiple polypeptides in its axial channel. However, the branch was not well-ordered and could not be modeled in the structure."

During the review process, to determine whether the observed engaged structure was resulted from the use of a branched-degron substrate, we generated another construct containing only the linear portion of the degron tag with the ssrA tag. Since ssrA-tag needs to be positioned at the C-terminus of the substrate for proper degron recognition by ClpX, we inserted a H6-TEV tag at the N-terminus of the substrate for purification purposes. This information has been included in the newly revised methods section.

- The statement "The linear degron tail immediately C-terminal to DHFR followed a similar trajectory to that of the branched degron substrate, indicating that the ClpX axial channel can readily transition between conformations able to accommodate single or multiple substrate chains" is a bit of an overstatement. Is there evidence that ClpX couldn't use identical trajectories for both linear and branched substrates, particularly if contact is only made with a single substrate polypeptide?

We thank the reviewer for their comment. We have removed this statement from the manuscript.

- In Figure 1B, Arg 159 is mentioned to adopt alternate conformations. Please clearly show this with a side-by-side comparison of the structures (it is also not shown in Figure 3 despite the callout).

We appreciate the reviewer's comment and, as explained below have elected to remove this relatively subtle point from the manuscript. Specifically, our structures show that 1) the folded domain of DHFR is differently oriented relative to ClpX in the branched and linear-degron structures; and 2) that if one were to align DHFR in these structures, the relative conformation between Arg158 and Arg159 would be similar between the two structures (and different than that observed in the crystal structure of DHFR). We believe that the different orientation of DHFR between the structures is a much more important point to articulate, and that attempting to explain this subtle point about Arg159 will distract from readers from this more important finding. Thus, we have simply removed this sentence and instead highlight the new orientation of DHFR relative to ClpX we observed in the linear-degron structure and the potential implications of these different orientations in substrate unfolding.

- The authors include a table of ClpXP structures adopting in various state but do not elaborate on these conformations. It would be helpful for readers if the distinct structural features defining the "recognition", "engaged", "intermediate" and "unfolding/translocation/degradation" states conformations were described to provide context for the new structures.

We have added a brief description of the structural features of these states in the Table 2 caption.

*ClpX states were defined as follows. In the **recognition state**, the axial channel of ClpX is closed and is interacting with the C-terminus of the degron tag, which is above*

*this point of closure. In contrast, structures of the **intermediate**, **engaged,** and **translocation states** all exhibit an open axial channel of ClpX, with either the degron tag or the unfolded substrate threaded through the opening. In the **intermediate state**, the degron tag is resolved, but neither the flexible linker connecting this degron to the folded substrate, nor the folded substrate itself are resolved. In the **engaged state** (reported here), the folded domain of the substrate is resolved and observed to make contact with the axial channel. In **translocation state**, the substrate is unresolved and sidechains of the substrate polypeptide in the axial channel cannot be assigned. Unlike the intermediate state, in the translocation state, the substrate is inferred to have been unfolded.*

In Figure 4, the authors state that “The IGF loop of chain E5 moves into a binding cleft that is unoccupied in structures similar to that in panel A. These movements correlate with Fseam upward movement, as shown in panels C and D”. Do all the structures with Fseam in an “up” position listed in table 2 have an empty cleft between D4 and E5 as shown in Figure 4B? Is the “up” conformation of Fseam specifically associated with an “engaged” state (depicted in Figure 4D); if so, why would the ClpXP structure in absence of MTX also adopt a similar Fseam conformation?

We thank the reviewer for their comment. We would like to reiterate our previous response as listed below:

Based on the newly determined structures included in our revision, we no longer believe that the ‘F-up’ conformation is specialized for substrate denaturation. Instead, we consider it an intermediate in both substrate unfolding and translocation. Additionally, we recognized that the ClpX subunits in different structures were not consistently named concerning the spiral of pore-1 loop interactions with the substrate polypeptide in the axial channel. In 12 out of 15 ClpXP structures, subunit F serves as the seam subunit connecting the top and bottom of the spiral, with F being “up” in 4 of these 12 structures. In 3 of the 15 structures, subunit A is the seam subunit, and in each case, it is “up.” In Table 2 of the revised manuscript, we summarize the various ClpXP structures in which the ‘seam’ subunit is either in the up or down conformation and also describe these structures in terms of function (e.g., substrate-free, recognition, intermediate, substrate-engaged, translocation) and nucleotide occupancy.

- Density for the surrounding residues, rather than just the zoned nucleotide density, should be shown for Supplementary Figure 15 to provide a sense of the quality of the density in the binding pocket.

We appreciate the reviewer's comment and have now included this information in Supplementary Figure 15.