nature research

Peer Review Information

Journal: Structural and Molecular Biology
Manuscript Title: The SARS-CoV-2 spike reversibly samples an open-trimer conformation exposing novel epitopes
Corresponding author name(s): Professor Susan Marqusee

Reviewer Comments & Decisions:

Decision Letter, initial version:

14th Oct 2021

Dear Dr. Marqusee,

Thank you again for submitting your manuscript "The SARS-CoV-2 spike reversibly samples an open-trimer conformation exposing novel epitopes". I apologize for the delay while we awaited comments (copied below) from the 2 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

I hope you will be pleased to see that both reviewers are quite positive about the potential interest of the findings and the quality of the work. Each requests that additional information or analyses be provided in the text or supplementary information to facilitate critical evaluation of the data, that some more speculative aspects of the Discussion be toned down, and that some discussion of what gives rise to the conformational change in the spike trimer be included. Reviewer #1 raises some technical queries that need to be clarified, and suggests alternative approaches to further support the open conformation model of state B. Editorially, we agree that these suggestions would strengthen the study, and ask that they be included in a revised manuscript.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no

similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please use the link below to submit your revised manuscript and related files:

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This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

With kind regards,

Beth

Beth Moorefield, Ph.D.
Senior Editor
Nature Structural & Molecular Biology

Referee expertise:

Referee #1: Viral neutralization/escape mechanisms

Referee #2: Viral protein dynamics/HDX-MS

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Costello & Shoemaker et al. use hydrogen deuterium exchange mass spectrometry (HDX-MS) to characterize an engineered form of the SARS-CoV-2 spike trimer. This S-2P spike construct involved a diproline modification of the S protein that discourages adoption of the post-fusion conformation, along with knock out of a proteolytic cleavage site, and addition of a trimerization domain in place of the transmembrane anchor. Similar modifications such as the proline substitutions in the S2 subunit were included in the design of most COVID-19 vaccines, though in many cases sequences including the full-length protein rather than truncated ectodomain were used in vaccines. The authors report the existence of a conformational "state" that is distinct from the well-characterized "prefusion" trimer. Their HDX-MS analysis indicates that this alternative conformation (termed "state B") exhibits more exposed inter-protomer interfaces. They report that factors such as temperature, ACE2 receptor binding, changes in S sequence (for example swapping the B.1.1.7 S1 subunit sequence, or adding additional stabilizing proline substitutions, or disulfide bonds) and an S2 subunit-binding antibody modulates the propensity of the S trimer to adopt this alternate state.

Overall the study is carried out carefully, and analysis of the complex bimodal mass spectral envelopes is performed in a manner that enabled the authors to deduce the relative populations of prefusion state A and "open" state B trimers under the condition being examined. Notably, they did not observe dynamic interconversion on the time-scale normally considered when characterizing protein

conformational change and dynamics. Instead over the course of hours and days, when incubated for example at 4°C vs 37°C, they observed a gradual shift in relative populations.

The significance of the reported results is reasonably high, because something close to this construct is in extremely wide use both clinically and in research. It is, however, not clear that the state they characterize also exists in the functional S protein on virus. Though the authors infer that the state B may correspond to an on-pathway intermediate that S adopts on its way to adopting the stable post-fusion conformation, the fact that their S construct has the double proline modification, an altered S1/S2 cleavage site and a foldon trimerization domain in place of the transmembrane anchor, make any inference about where the observed state sits along a fusion-relevant pathway highly speculative.

The reason the results are significant in my view is that the state B appears to expose epitopes that were assumed to be inaccessible based upon the prefusion S trimer structures. Thus if the form of S being generated or introduced into vaccinated individuals exposes these epitopes they may lead to antibodies that against those sites which may be non-neutralizing or less desirable than the receptor binding domain for example. It also provides valuable data for research on antibodies, since the temperature and storage conditions of the trimer used can affect antibody interactions and affinity.

Main issues -

1. While the authors have included a lot of the raw data in the submitted package, in the manuscript and supplemental info, actual presentation of data is not provided for a number of key experiments such as the disulfide-locked example (other than being summarized in the text). More complete presentation of experimental data in supplementary information are warranted.

2. The state B is not well-characterized with complementary methods that would help test the proposed open conformation model. The only additional data was a gel filtration chromatogram in supplementary materials that stated both are trimers, but size exclusion chromatography with multiangle light scattering, the standard approach for measuring molecular weight in conjunction with SEC was not used. The relative homogeneity or heterogeneity of the state was also not characterized nor was some form of aggregated state ruled out (except for what was inferred from a reversibility experiment). HDX-MS alone is quite limiting for composing structural models. Electron microscopy, native gel electrophoresis or analytical ultracentrifugation would be informative. These may not be able to characterize a distinct state but they can rule out other possibilities such as aggregation or dissociation.

3. I did not see any discussion of what may give rise to the conformational change; is it due to weakened hydrophobic interactions at interface for example that might be consistent with low temperature promoting state B? This type of mechanistic information would give insight into how one can rationally stabilize trimers to maintain state A for example; the disulfide locked case may be effective, but did not provide insight into what structurally was producing the conformational instability of the prefusion trimer. Is the propensity to transition to this alternative state a consequence of the 2P mutation and proline isomerization for example?

4. Likewise, the description of the HexaPro version of the trimer was observational but did not offer much insight into how the additional prolines may have contributed to the slower kinetics of state B adoption.

5. Too much is assumed about energetics and relative free energies of the state A and B. For example S-2P and the HexaPro data were said to be "consistent with two low energy conformations" despite the significantly different kinetics. The casual way that energetics and conformational as well as energetic landscapes are invoked does not add to the study because they aren't backed up by quantitative analysis that could inform those aspects of the systems. Also, at times "conformational landscape" and "energetic landscape" are used seemingly interchangeably, but there did not seem to be sufficient information to link these in this study.

Minor issues -

1. Fig1C and supplementary Fig 2 are very hard to make sense of. Too many overlapping protein segments with similar colorations, etc. If the point is that the HDX-MS protection trends are in agreement with "secondary structure" and "buried elements" this could be shown in other ways. It would be helpful for the data to be presented in uptake plots or 'chiclet' plots (such as in fig 3) rather than just heat map on the structure. A butterfly plot (see for example Lim et al., Nature Communications 2017 https://doi.org/10.1038/ncomms14339) is one way that could also help summarize the data and show differences between state A and B.

2. In the plots of mass spectra undeuterated/t0 and totally deuterated data should be included

3. Continuous vs pulse experiments should be better labeled in the text and figures to avoid confusion

4. Fig 5 and 6 use of arrows is confusing - do the arrows indicate the population fraction or energy level?

5. Fig S5 - uptake of the foldon isn't a great indicator that the construct is trimeric, should include data from other parts of the trimer that maintain interprotomer interactions as well.

6. Analysis - it's unclear why they chose the peptides they're using for defining the populations of state A/B; what do the other bimodal peptides show?

7. The methods section for protein purification is too limited in referring to past studies without any brief summary of the methodology. On that note, how many biological replicates were performed and how different were the starting materials from different preps? Always the same ratios to start?

8. Is the B.1.1.7 variant more susceptible to neutralization by 3A3 and similar antibodies? Also why only swap the S1 subunit? Were any mutations in S2 deemed to have negligible impact?

9. Two sections in the Discussion are in my view too speculative: First the section on the role of state B in spike function. Too little discussion was provided about the limitations of translating how the S-2P engineered trimer behaves over to how spike functions on virus. Second, the discussion about "druggable sites" was highly speculative.

10 The use of the term "solvent accessibility" to describe the state B could be misunderstood for "solvent accessible surface area" which is not what HDX-MS actually probes. It is probably best to avoid such terminology in reference to HDX.

Reviewer #2:
Remarks to the Author:
This is a provocative paper showing that the 2P version of the SARS-CoV-2 spike adopts two conformations: the canonical A conformation, and another the authors term B. The two conformations interchange slowly and their balance changes as a function of temperature. This result would explain the wide anecdotal observation that there can be problems storing spike at 4 C.

In addition, the authors find that mutations can change the balance of conformations. This is shown not only for the artificial HexaPro mutations, but also for natural mutations in the B.1.1.7 (Alpha) variant spike. All these concepts are well explained.

Overall, we first admit the caveat we are not experts in HDX-MS, and the technical aspects need to be evaluated by reviewers more expert in that technique. But assuming the work is technically sound, this is definitely a result that seems worthy of publication in this type of journal. The real biological and evolutionary significance of the observations made here probably remain a bit unclear, but they are potentially substantial if the exposed epitopes can be targeted, and if the results from the Alpha variant spike generalize to other variants. Answering those questions would require further work, but we think that work is beyond the scope of the current study which is already a valuable standalone study.

MAJOR COMMENTS:

- Some comment on whether some of the results could be artifacts of the 2P mutations would be helpful.

- An analysis of the raw HDX-MS data sets from Raghuvamsi et al and Huang et al to test the hypothesis that they simply missed the biomodal distributions of key peptides as described in the supplement would be great if those datasets are available. Obviously, if they are not available then such an analysis is not possible.

MINOR COMMENTS:

- "UK variant" should be "Alpha variant" or "B.1.1.7 variant."

- The claim that Alpha might have higher infectivity because of faster conversion to B state is speculative. This possibility is still worth mentioning, but should be more clearly framed as speculation.

- What do the authors posit triggers the transition from A to B in the context of real viral infection? Clearly it isn't temperature there.

Author Rebuttal to Initial comments

Point by point response to reviewer's comments: Manuscript NSMB-A45292 "The SARS-CoV-2 spike reversibly samples an open-trimer conformation exposing novel epitopes" Costello, Shoemaker, et al.

Reviewer number 1: Major Comments 1. 'While the authors have included a lot of the raw data in the submitted package, in the manuscript and supplemental info, actual presentation of data is not provided for a number of key experiments such as the disulfide-locked example (other than being summarized in the text). More complete presentation of experimental data in supplementary information are warranted.'

Thank you for pointing out that omission - this was an oversight on our part. We have now added data for the disulfide-locked protein in the Supplemental Figures. In addition, to make these data more accessible to the reader, we split Supplemental Figure 3 into two figures (Supplemental Figure 3 and 4). Supplemental Figure 3 now shows the continuous labeling time course for the representative set of bimodal peptides in all three proteins and Supplemental Figure 4 shows the pulsed-labeling data for the bimodal peptides used in our kinetic analysis. In addition, please note that with this revision we have now included .csv files containing all deuteration data for all relevant continuous labeling experiments shown in Figures 1, 3, and 4 and .csv files containing the difference in deuteration used to generate Figures 3 and 4. We have also provided the raw and extracted spectra from the pulsed-labeling time courses for S2P, HexaPro and the alpha S1 HexaPro that were used to generate Figures 2 and S4 as .csv files.

2. The state B is not well-characterized with complementary methods that would help test the proposed open conformation model. The only additional data was a gel filtration chromatogram in supplementary materials that stated both are trimers, but size exclusion chromatography with multiangle light scattering, the standard approach for measuring molecular weight in conjunction with SEC was not used. The relative homogeneity or heterogeneity of the state was also not characterized nor was some form of aggregated state ruled out (except for what was inferred from a reversibility experiment). HDX-MS alone is quite limiting for composing structural models. Electron microscopy, native gel electrophoresis or analytical ultracentrifugation would be informative. These may not be able to characterize a distinct state but they can rule out other possibilities such as aggregation or dissociation.

As suggested, we have now carried out SEC-MALS on two samples of S-2P, one incubated at 4°C and the other incubated at 37°C, both for 4 days. In addition, after incubation, we, we took an aliquot of each sample and carried out a 1 minute deuterium pulse experiment to determine the A:B ratio of each sample. The sample incubated at 37°C confirmed, as expected, that the protein is predominantly state A; the SEC-MALS data on this sample show one major peak with an observed molecular weight consistent with a glycosylated spike ectodomain trimer. And, as expected, the HDX results for the sample incubated at 4°C reveal a mixed population (~40% state A and 60% state B). The SEC-MALS for this sample shows two major species with similar proportions and observed molecular weights indicating that they are both trimers. These results are presented in the text of the main manuscript and displayed in figure S5.

We did not carry out the more simple experiments suggested, such as native gel electrophoresis. The results would be too complicated to interpret due to the heterogeneous masses arising from the varying degrees of glycosylation. CyroEM is also not an option because, as mentioned in our manuscript, several publications have noted that incubation under conditions that favor state B do not result in samples amenable to CryoEM analysis, most likely due to the conformational heterogeneity of state B. Thus, to date, state B appears refractory to Cryo EM analysis, highlighting the utility of HDX-MS in studying this conformational change. The HDX data also inform us that the basic structure of the individual domains are preserved in state B, a feature unlikely to be resolved by CryoEM, given the presumed flexibility of this structure.

3. I did not see any discussion of what may give rise to the conformational change; is it due to weakened hydrophobic interactions at interface for example that might be consistent with low temperature promoting state B? This type of mechanistic information would give insight into how one can rationally stabilize trimers to maintain state A for example; the disulfide locked case may be effective, but did not provide insight into what structurally was producing the conformational instability of the prefusion trimer. Is the propensity to transition to this alternative state a consequence of the 2P mutation and proline isomerization for example?

Thank you for encouraging us to add this discussion, which we have done in pages 17/18. We believe the conformational change can be thought of as a sort of 'cold-denaturation' of the trimer interface due to a higher heat capacity of state B compared to state A. This explanation is consistent with an exposed hydrophobic interface in the trimer interface. The issue of the role of the two prolines is interesting. The conversion between state A and state B is unlikely to be controlled by proline isomerization. First, the conversion is much slower than expected for proline isomerization. Additionally, the dramatic effect of temperature on the population would not be expected from proline isomerization.

4. Likewise, the description of the HexaPro version of the trimer was observational but did not offer much insight into how the additional prolines may have contributed to the slower kinetics of state B adoption.

Similarly, we do not believe that the slower kinetics and increase in the relative stability of the prefusion state of HexaPro are a result of proline isomerization. Rather, as stated in Hsieh et al. Science 2020 (DOI: 10.1126/science.abd0826), the proline mutations were designed to cap helices or stabilize loops in the prefusion conformation. Our observations of an increase in the relative stability of the prefusion state compared to the expanded trimer, are consistent with this design and suggest these changes do not provide the same stabilization to the expanded trimer. Additionally, the increased kinetic barrier may arise in part via this simple ground-state stabilization. We have now added this discussion to the manuscript.

5. Too much is assumed about energetics and relative free energies of the state A and B. For example S-2P and the HexaPro data were said to be “consistent with two low energy conformations” despite the significantly different kinetics. The casual way that energetics and conformational as well as energetic landscapes are invoked does not add to the study because they aren’t backed up by quantitative analysis that could inform those aspects of the systems. Also, at times “conformational landscape” and “energetic landscape” are used seemingly interchangeably, but there did not seem to be sufficient information to link these in this study.

Thank you for giving us the opportunity to clarify our statements. This should have said ‘consistent with two conformations with a small free energy difference’. Our conclusion that the two conformations are likely similar in energy comes directly from our data. We observe detectable (>5%) levels of both state A and state B in nearly all conditions tested (the disulfide locked variants excluded). Assuming that we are only observing two states and that they have reached equilibrium, the free energy difference between these two states cannot be more than 2 kcal/mol. We have now limited our discussion of this and clarified this in the text. For consistency, we have modified the text to only use the term conformational landscape - which we think accurately reflects our studies of the system.

Minor Comments

1. Fig1C and supplementary Fig 2 are very hard to make sense of. Too many overlapping protein segments with similar colorations, etc. If the point is that the HDX-MS protection trends are in agreement with “secondary structure” and “buried elements” this could be shown in other ways. It would be helpful for the data to be presented in uptake plots or 'chiclet' plots (such as in fig 3) rather than just heat map on the structure. A butterfly plot (see for example Lim et al., Nature Communications 2017 https://doi.org/10.1038/ncomms14339) is one way that could also help summarize the data and show differences between state A and B.

Thank you for making this suggestion. While ribbon diagrams are nice to look at, the point of the HDX protection patterns are not easily apparent in this format. Therefore, Figure 1C has been replaced with Figure 2, which is a clearer summary of the data in one-dimensional format. This new figure illustrates our continuous exchange HDX data using a Wood’s plot format. The data are separated by domain with both secondary structures and solvent accessibility (approximated using a model of the full length prefusion structure) shown. Given the number of peptides, the degree of peptide overlap, and the differences in peptide length, we believe this is a better presentation of the data than a butterfly plot or uptake plots, but we have also included the uptake data as a .csv file allowing for the generation of these plots by the reader if desired.

2. In the plots of mass spectra undeuterated/t0 and totally deuterated data should be included

Thank you for pointing this oversight out. We have added undeuterated spectra for all peptides in supplemental figures 3 and 4. We have not added undeuterated spectra for main text figures 3, 4, and 6 as these spectra are provided to illustrate the relative populations of the two states and we believe undeuterated spectra are not needed for this interpretation. We have included them in the supplementary material for reference. We are unable to add fully deuterated spectra for all data sets, as we only collected a single replicate of fully deuterated S2P, primarily to characterize the levels of back exchange in our experimental design, as shown in figure S1.

3. Continuous vs pulse experiments should be better labeled in the text and figures to avoid confusion

We thank the reviewer for this suggestion. We have made both these changes throughout the text and figures.

4. Fig 5 and 6 use of arrows is confusing - do the arrows indicate the population fraction or energy level?

We assume the reviewer is asking about the arrows in Figure 6, which were meant to indicate changes in the relative free energy of both states. We now see that this can easily be misinterpreted as a change in the populations, thus leading to confusion. We have updated our figure by removing arrows and including a clearer text-based description of the changes in populations.

5. Fig S5 - uptake of the foldon isn't a great indicator that the construct is trimeric, should include data from other parts of the trimer that maintain interprotomer interactions as well.

We do not have any data in the interface for us to conclude anything about interprotomer contacts that may be maintained in the expanded trimer. The bimodal peptides in the interface all indicate less protection for state B. The unimodal peptides in the interface are highly deuterated, consistent with low protection in both state A and state B. For this reason, our cartoon model for the open trimer depicts the foldon as the primary trimerization region. As noted above both the Sec-MALS data and the lack of changes in HDX protection in the foldon, (now shown in the same figure), indicate that the construct remains trimeric.

6. Analysis - it's unclear why they chose the peptides they're using for defining the populations of state A/B; what do the other bimodal peptides show?

We have now clarified this in the manuscript (page 8-9). As noted, the one-minute pulse experiment and bimodal peptide analysis is used to define the relative population of state A/B. Instead of doing this with different pulse times, we selected a one-minute pulse because many of the bimodal peptides are clearly separable at this time (many bimodal peptides do not produce separable bimodal spectra at all time points, as shown in figure S3). To simplify analyses, we chose a peptide from two distinct regions that

show bimodal behavior with high signal to noise and distinguishable bimodal distributions after a 1-minute pulse (regions 878-903 at the bottom of the S2 domain and 978-1001 at the top of the S2 domain).

7. The methods section for protein purification is too limited in referring to past studies without any brief summary of the methodology. On that note, how many biological replicates were performed and how different were the starting materials from different preps? Always the same ratios to start?

We have now addressed these issues in our revised methods. Although not true biological replicates comparing different preparations of a single construct, we have carried out HDX on an S-2P and HexaPro construct provided to us by different labs. In both, we see the same bimodal peptides and essentially identical HDX behavior. The observed kinetics of interconversion for these are consistent with our reported data (we have not carried out the same extensive kinetic studies on these samples). The initial ratio for different samples is dependent on the preparation conditions and the duration of storage at 4oC. To control for this, prior to all pulse-labeling experiments we incubate the sample at 37oC overnight, which results in conditions that are predominantly state A. We have clarified this in the manuscript.

8. Is the B.1.1.7 variant more susceptible to neutralization by 3A3 and similar antibodies? Also why only swap the S1 subunit? Were any mutations in S2 deemed to have negligible impact?

We have limited information about the neutralization of B.1.1.7 with 3A3 (reference 28), which indicates that that B.1.1.7 is slightly more potent in the pseudovirus assay. We are hesitant to over interpret the implication of this less than 2-fold difference. The B.1.1.7 construct used in our experiments only contains mutations in the S1 subunit for purely practical reasons - this is the construct that was readily expressed and purified. This construct was originally designed for other studies looking at potential S1 effects on structure. For our studies, however, it allows us to observe changes away from the trimeric interface allosterically affecting the conversion to state B.

9. Two sections in the Discussion are in my view too speculative: First the section on the role of state B in spike function. Too little discussion was provided about the limitations of translating how the S-2P engineered trimer behaves over to how spike functions on virus. Second, the discussion about “druggable sites” was highly speculative.

We have modified the discussion to make it very clear where we are speculating about the role of state B in spike function and potential applications for drug targeting and to clearly state that our studies are not on the membrane and therefore we can not make any conclusions about state B or any rates of conversion on the membrane.

10. The use of the term “solvent accessibility” to describe the state B could be misunderstood for “solvent accessible surface area” which is not what HDX-MS actually probes. It is probably best to avoid such terminology in reference to HDX.

We agree that this can be confusing for the reader and we do not want our manuscript to lead to incorrect assumptions about what conclusions can be drawn from HDX data. We have now clarified this. Discussion of potential changes in solvent accessible surface area are limited to our structural model based on the entirety of our data—temperature sensitivity, binding of ACE2 and 3A3, and HDX protection, which together suggest a model where there are large changes in solvent accessible surface area upon formation of state B. In our revisions, we have tried to make it clear what can be inferred directly from changes in HDX protection, and what we are inferring from our structural model.

Reviewer #2
Major Comments:
Some comment on whether some of the results could be artifacts of the 2P mutations would be helpful.

Thank you for this suggestion. As noted in our response to reviewer 1, we have now added this to the text.

An analysis of the raw HDX-MS data sets from Raghuvamsi et al and Huang et al to test the hypothesis that they simply missed the bimodal distributions of key peptides as described in the supplement would be great if those datasets are available. Obviously, if they are not available then such an analysis is not possible.

The raw MS data needed for this analysis has not been provided. However, since time of submission, we have had several discussions with authors from both of those papers about the differences in their experiments. Both groups agree with our assessment in our supplemental information about why the bimodal peptides were not obvious in their analysis. D’Arcy, who carried out the HDX from Huang et. al, has since revised her text to include analysis of distribution widths (that are consistent with overlapping bimodals that result in wider than expected isotope distributions in the regions where we observe bimodals). Raghuvamsi et al. have also confirmed that the increased temperature of their studies (37C) dramatically affects the ability to detect these two conformations. We have updated and expanded this discussion in the supplemental text and clearly stated in the main manuscript that there are no inconsistencies between the different data sets.

Minor comments
- “UK variant” should be “Alpha variant” or “B.1.1.7 variant.”

We agree and have changed all uses of “UK” to either “B.1.1.7 (alpha)” or “alpha” in the text.

- The claim that Alpha might have higher infectivity because of faster conversion to B state is speculative. This possibility is still worth mentioning, but should be more clearly framed as speculation.

We have rewritten these statements to clarify the speculation.

- What do the authors posit triggers the transition from A to B in the context of real viral infection? Clearly it isn't temperature there.

As noted by the reviewer, we have no reason to posit that these temperature effects are driving things in the context of viral infection. We used temperature as a means to control the relative population of the two conformers and learn something about the energetics. Instead, we might speculate that binding to ACE2 traps the RBD in the up state, a step that appears to also trigger the conversion to the open trimer. Thus trapping one or more RBDs in the up state may increase the likelihood of the three-up conformer, which releases a major source of interprotamer contacts, allowing transition to the B state. Furthermore, while we have no data for this, this may be greatly accelerated in the virus, in the absence of the two stabilizing prolines and and in the presence of proteolytic cleavage. In sum, while this transition may be more favorable on a real virus, it may be less - we do not have evidence for what occurs on the virus and hope we made that clear in the text.

Decision Letter, first revision:

23rd Nov 2021

Dear Susan,

Thank you for submitting your revised manuscript "The SARS-CoV-2 spike reversibly samples an open-trimer conformation exposing novel epitopes" (NSMB-A45292A). It has now been seen by a subset of the original referees and their comments are copied below. The reviewer finds that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

****To facilitate our work at this stage, we would appreciate if you could send us the main text as a Word file. Please make sure to copy the NSMB account (cc'ed above).*****

Please don't hesitate to contact me if you have any questions.

With kind regards,

Beth

Beth Moorefield, Ph.D.
Senior Editor
Nature Structural & Molecular Biology

Reviewer #1 (Remarks to the Author):

I appreciate the revisions and clarifications the authors have made to their revised manuscript. The figures also more clearly convey the results of the experiments and analysis than in the original submission. The study brings to light an important aspect of SARS-CoV-2 spike constructs that people in the field should be aware of.

Final Decision Letter:

21st Jan 2022

Dear Dr. Marqusee,

We are now happy to accept your revised paper "The SARS-CoV-2 spike reversibly samples an open-trimer conformation exposing novel epitopes" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

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