nature research

Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: Cold sensitivity of the SARS-CoV-2 spike ectodomain
Corresponding author name(s): Dr. Priyamvada Acharya

Reviewer Comments & Decisions:

Decision Letter, initial version:

17th Aug 2020

Dear Priyamvada,

Thank you again for submitting your manuscript "Cold sensitivity of the SARS-CoV-2 spike ectodomain". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper, both experts in CoV spike biophysics/structure. 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.

You will see that the reviewers are positive about the interest of the findings, but they point to some missing data points and controls; inconsistencies between structural and biophysical analyses; potential overinterpretation of the negative-staining EM; lack of clarity in experimental conditions and statistics/reproducibility of the findings. Reviewer 2 finds (and we agree) that the use of the term "degradation" is confusing, and suggests "denaturation" (alternatively you could use "misfolding" or something along those lines).

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.

The text and figures will also require changes to conform to our editorial requirements. If you could send me the word document, I can do some checks now and give you some general guidelines; the figures will also require some streamlining, as they are overly busy right now, but I might not be able to give you specific instructions now, as there will be new data in the revision but I will send you at least some general guidelines.

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 3 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.

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-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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.

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We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

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

Sincerely,

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

The manuscript entitled "Cold sensitivity of the SARS-CoV-2 spike ectodomain" by Edwards et al. described an intriguing cold-destabilization effect of the SARS-CoV-2 spike protein, which has been under intensive structural and functional studies in light of the COVID-19 pandemic. The spike protein of SARS-CoV-2 is known to be thermal labile, and efforts have been made to increase its thermal stability by means of protein engineering, which are cited in this manuscript. While similar phenomena have been reported that prolonged incubation of the spike protein at 4 ºC can lead to deteriorated trimeric assembly of the spike protein, the key finding of this study demonstrated a very unusual feature that a brief incubation at 37 ºC (body temperature) for 3 hours can effectively restore the native-like quaternary structure of the spike protein as well as the antigenicity associated with the S2 domain (2G12 antibody). In contrast, prolonged incubation at 4 ºC partially unfold the spike protein to make the receptor binding domain (RBD) more accessible to host receptor, ACE2, and an RBD-driven antibody (CR3022 antibody). While the increased antigenicity and receptor binding affinity are a welcome feature for diagnostics purposes, this study underscored a potential concern that the structural information derived from these experimental conditions, namely, using spike proteins that have been incubated at a cold temperature for an extended period of time, could be misleading. According to the authors, the remedy for such undesired experimental artefacts is in fact a simple heat activation step that resembles to the host environment, i.e., body temperature. Considering the importance of maintaining a physiologically relevant conformation of the spike protein for basic and translational researches in COVID-19, the information provided by this study is indeed very timely and of general interest. I would recommend the publication of this manuscript after considering the following comments:

1. There appears to be some inconsistency in the structural and biophysical evaluations of the restoration of the native structure of the spike protein. In Line 62, it is said that the incubation of the spike protein at 37 ºC for one week can maintain 83% of the sample in native trimeric conformation while incubating at 4 ºC for one week causes severe destabilization of the trimer assembly (only 5% of the sample remains in the native conformation). A three-hour heat activation (4 -> 37 ºC) could restore the trimeric assemble to a similar level as that of the fresh sample (Figure 1E), but the DSC analyses showed the presence of a significant unfolding peak at ca. 48 ºC for the 4 -> 37 ºC recovered sample. If the low-melting temperature peak corresponded to a partially unfolded trimer, which was reflected in the same changes in the elution peaks of the size exclusion chromatography (Extended Figure 2E), why was it not reflected in the negative stain EM (NSEM) image analysis?
2. Along the same line, if continuous incubation of the sample at 37 ºC did not affect the trimeric assembly (even improved somewhat), why was such a condition not used for the following biophysical and biochemical analyses as a control?
3. How were the error bars shown in Figure 1E defined? Did they come from different batches of NSEM samples? If so, how many samples were analyzed?
4. Figure 1G, caption: A standard deviation cannot be derived from just two data points (N=2). More data points are needed.
5. Figure 1H: What are the experimental errors of the SPR-derived data?
6. Figure 2F: Can the authors provide the same statistical analysis of experimental errors for the percentages of native-like spikes as illustrated in Figure 1E?

Reviewer #2:
Remarks to the Author:
The manuscript by Edwards et al characterized the stability of a purified SARS-CoV-2 spike protein ectodomain variant at different temperatures. This "stabilized" variant of spike protein ectodomain

was originally reported by the Jason McLellan Lab and it was generated by introducing 2 prolines (PP) in the S2 part of the protein to prevent S protein transition into the postfusion state. The authors used a variety of biochemical/biophysical methods and negative stain EM to characterize this purified spike protein variant stored at different temperatures and the results indicated that this spike protein ectodomain variant is unstable when stored at 4 °C for prolonged period of time. For this conclusion the results presented in the manuscript appear to be solid providing useful information for production and storage of this widely used SARS-CoV-2 spike protein ectodomain construct.

Beside this conclusion, the authors compared negative stain EM (NSEM) images of freshly purified spike protein, spike protein stored at 4 °C for 7 days and 3 hour incubation of spike protein which had been previously stored at 4 °C for 7 days. Based on this comparison, the authors concluded that spike protein that had become "degraded" when stored at 4 degrees could be recovered back to "well-formed spikes" by incubation at 37 °C for 3 hours. The recovered protein had morphologies similar to the fresh protein by NSEM. While this finding is novel and interesting I found this comparison is confusing and misleading for several reasons:

1. Use of "degradation" (line 70 and 78) is confusing, it normally means protein being converted into smaller species by proteolysis, in this case I believe the authors are trying to describe the loss of "well-formed spike" structures after storage at 4 °C which can be subsequently recovered somewhat by 37 °C incubation. I speculate loss of the "well-formed spike" reflected a change in quaternary structure which should be more appropriately described as "denaturation" instead of "degradation" (it would also be nice if an SDS-PAGE gel can be shown to confirm that there is no degradation i.e. no loss of intact band after 7 days of storage at 4 °C)

2. The "well-formed" trimer description appears to be solely based on morphological similarity by NSEM images. Are there any differences by image analyses of NSEM images of fresh protein and 37 °C recovered protein? Although I believe NSEM does not have enough resolution to reveal differences such as RBD open and close (which the fresh protein images can be served as a good control as that protein should have both close and open conformations from previous studies.)

3. Subsequent size-exclusion chromatography, DSC, antibody and ACE2 binding data (Extended Data Figure 2, Figure 1 FGH, Extended Data Figure 4 and 5) show that the 37 °C recovered spike behaves most similar to 22 °C stored protein, especially in ACE2 and CR3022 binding. In ELISA assays, the 22 °C stored protein behaved almost the same in most cases as the 4 °C stored protein which is not "well-formed" by NSEM. More confusingly, the authors did not provide NSEM image of 22 °C stored spike and size-exclusion chromatography, DSC, antibody and ACE2 binding data for fresh protein were not presented. Also in the ELISA the authors did not include any data on 37 °C recovered spike or fresh protein. I feel that the data presented are conflicting to support the notion that the 37 °C recovered spike is "well-formed" similar to fresh protein. In addition, as mentioned above, there are significant amount of missing data in NSEM, size-exclusion chromatography, DSC, antibody and ACE2 binding assays, and ELISA, preventing a proper comparison between different conditions.

4. The CR3022 antibody binds much stronger to the 37 °C recovered protein than to protein stored at 37 °C (Figure 1H and Extended Data Figure 5B). Recent report (Huo et al, Cell Host & Microbe, https://doi.org/10.1016/j.chom.2020.06.010) showed that the epitope of CR3022 is highly cryptic in prefusion trimers and a rotation of the "up"/"open" RBD is needed to expose the cryptic epitope. In addition, in the same report it was shown that prolonged incubation with CR3022 is needed for CR3022 to bind the cryptic epitope resulting in trimer disintegration. The data presented in this paper

show differential binding of CR3022 antibody to 37 °C recovered protein and 37 °C stored protein suggesting there are likely substantial structural differences between these two proteins, and the 37 °C recovered spikes may not be "well-formed" similar to fresh protein if they expose the cryptic epitope.

Summarized by points above, I think the manuscript is written in a narrative which suggests the 37 °C recovered protein is a "well formed" spike similar to fresh protein or 37 °C stored protein. However, the presented biochemical/biophysical data suggest it is more like 22 °C stored protein. The biochemical/biophysical data also imply 37 °C stored protein, 22 °C stored protein, 4 °C stored protein and 37 °C recovered protein all have distinct structures with different capacity to bind antibodies/ACE2. Therefore, there is a discrepancy between the data and the narrative. I would advise the authors to carefully resolve these discrepancies or providing further experiment evidence, for example high-resolution EM structure of the 37 °C recovered protein to support their current narrative.

There are several minor issues:

1. In the method section, the author did not describe which temperature they use to produce the spike protein, based on their results this parameter can significantly change the state of the produced "fresh protein" - initial material of the study, can the authors clarify how purifications had been done? Also based on their result should it be recommended to purify this protein under room temperature or 37 °C?

2. For the rS2d-HexaPro produced, the authors tried to use NSEM and classification to ascertain the spike proteins are all closed, again I think NSEM does not have enough resolution to tell the difference between open and close conformations especially with potential artefact associated with heavy metal staining. A proper control should be done. For example, image analyses of NSEM images of the PP construct which is known to have open/close conformations should be done. However, I do believe the rS2d-HexaPro is mostly closed as three other reports (Rory et al, NSMB, Xiong et al, NSMB 2020, McCallum et al NSMB, 2020) all have confirmed this disulfide pair can disulfide-linked the spike protein monomers in the closed conformation, although the authors failed to cite the latter two refs. In addition, the ACE binding data provided by the authors supported their notion.

3. The authors failed to cite Xiong et al, NSMB 2020 regarding to their conclusion that closing the trimer solve the cold sensitivity issue. In that report, it has been demonstrated 2 ways of disulfide links (one of them is the same as the rS2d) allowed production of fully closed spikes which can be stored at 4 °C for prolonged period of time.

Author Rebuttal to Initial comments

Point-By-Point Response to Editor and Reviewers

Editor:

Summary

Thank you again for submitting your manuscript "Cold sensitivity of the SARS-CoV-2 spike ectodomain". … we now have comments (below) from the 2 reviewers who evaluated your paper, both experts in CoV spike biophysics/structure. 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.

You will see that the reviewers are positive about the interest of the findings, but they point to some missing data points and controls; inconsistencies between structural and biophysical analyses; potential overinterpretation of the negative-staining EM; lack of clarity in experimental conditions and statistics/reproducibility of the findings. Reviewer 2 finds (and we agree) that the use of the term "degradation" is confusing, and suggests "denaturation" (alternatively you could use "misfolding" or something along those lines).

Response:

We are delighted to hear these positive comments from the editor and the reviewers. We thank the editor and the reviewers for their critiques, comments and suggestions that we address below in our point-by-point response. We have replaced “degradation” by “denaturation” as suggested by the editor and Reviewer 2.

Other changes made to the manuscript based on editor’s feedback and comments are as follows:

1. We have briefly described the mutations in the “2P” construct early in the text (Lines 4750) and refer to this construct as “2P” after this first mention.
2. We have moved the tables containing the DSF and DSC Tm numbers in Figure 1f and 1g to Supplementary Tables 1 and 2, respectively,.
3. We have included a brief description of the DSF assay (Lines 95-98).
4. We have added details to the SPR and ELISA methods, including the temperatures at which the assays were run.
5. We have included characterization data for the two patient-derived antibodies (Supplementary Figures 4 and 5). Epitoppe mapping by ELISA are shown in

   Supplementary Figure 4 and Neutralization data are showing in Supplementary Figure 2. We have added methods for the neutralization assays. Ethics statement that reads as follows has been included in methods section: “Peripheral blood was collected following informed consent on a Duke University Medical Center approved Institutional Review Board protocol.” (Lines 252-253).

Reviewer 1:

Summary

The manuscript entitled "Cold sensitivity of the SARS-CoV-2 spike ectodomain" by Edwards et al. described an intriguing cold-destabilization effect of the SARS-CoV-2 spike protein, which has been under intensive structural and functional studies in light of the COVID-19 pandemic. The spike protein of SARS-CoV-2 is known to be thermal labile, and efforts have been made to increase its thermal stability by means of protein engineering, which are cited in this manuscript. While similar phenomena have been reported that prolonged incubation of the spike protein at 4 ºC can lead to deteriorated trimeric assembly of the spike protein, the key finding of this study demonstrated a very unusual feature that a brief incubation at 37 ºC (body temperature) for 3 hours can effectively restore the native-like quaternary structure of the spike protein as well as the antigenicity associated with the S2 domain (2G12 antibody). In contrast, prolonged incubation at 4 ºC partially unfold the spike protein to make the receptor binding domain (RBD) more accessible to host receptor, ACE2, and an RBD-driven antibody (CR3022 antibody). While the increased antigenicity and receptor binding affinity are a welcome feature for diagnostics purposes, this study underscored a potential concern that the structural information derived from these experimental conditions, namely, using spike proteins that have been incubated at a cold temperature for an extended period of time, could be misleading. According to the authors, the remedy for such undesired experimental artefacts is in fact a simple heat activation step that resembles to the host environment, i.e., body temperature. Considering the importance of maintaining a physiologically relevant conformation of the spike protein for basic and translational researches in COVID-19, the information provided by this study is indeed very timely and of general interest. I would recommend the publication of this manuscript after considering the following comments:

Response:
We appreciate the nice summary of the manuscript by this reviewer and the positive comments on the timeliness of the work. We have now addressed all of the concerns expressed by the reviewer in the form of changes to the manuscript as detailed in our response below.

Specific comments:

1) There appears to be some inconsistency in the structural and biophysical evaluations of the restoration of the native structure of the spike protein. In Line 62, it is said that the incubation of the spike protein at 37 ºC for one week can maintain 83% of the sample in native trimeric conformation while incubating at 4 ºC for one week causes severe destabilization of the trimer assembly (only 5% of the sample remains in the native conformation). A three-hour heat activation (4 -> 37 ºC) could restore the trimeric assemble to

a similar level as that of the fresh sample (Figure 1E), but the DSC analyses showed the presence of a significant unfolding peak at ca. 48 ºC for the 4 -> 37 ºC recovered sample. If the low-melting temperature peak corresponded to a partially unfolded trimer, which was reflected in the same changes in the elution peaks of the size exclusion chromatography (Extended Figure 2E), why was it not reflected in the negative stain EM (NSEM) image analysis?

Response:

We appreciate the reviewer bringing this up. One reason for the apparent discrepancy is likely due to how the spike samples are treated before and during measurements using the different techniques employed in this study. NSEM is the only technique where the spike could be captured directly from the condition it was incubating at and fixed on the grid, with minimal lag time and/or exposure to other temperatures.

The DSC measurements, on the other hand, were performed after each sample was purified by SEC. The 4 ºC incubated sample was purified by SEC at 4 ºC, whereas the 22 ºC and 37 ºC samples were purified by SEC at room temperature. We have now explicitly clarified this in the methods: "Samples that had been incubated at 22 ºC or 37 ºC were purified by SEC at room temperature and the sample incubated at 4 ºC was purified by SEC at 4 ºC (Extended Data Figure 2), diluted to 0.2–0.3 mg/mL in HBS, and degassed for 15 min at room temperature prior to analysis. DSC measurements were performed immediately after SEC purification of the samples." Therefore, the 4 -> 37 ºC recovered sample was exposed to room temperature (~25 ºC) for a few hours prior to DSC measurements. Given that the low Tm peak is present in the DSC profile of the 4 -> 37 ºC recovered sample, we have added the following sentence to the DSC results (Lines 109-112): "Thus, the DSC results confirm that storage at 4 ºC destabilizes the spike compared to samples stored at 22 ºC or 37 ºC, and that returning the destabilized spike to 37 ºC for 3 hours substantially restores its stability, although the presence of the low-Tm peak suggests that the recovery is partial.'

2. *Along the same line, if continuous incubation of the sample at 37 ºC did not affect the trimeric assembly (even improved somewhat), why was such a condition not used for the following biophysical and biochemical analyses as a control?*

Response:

We have included 37 ºC 1-week incubation datapoints in the DSF, DSC, SPR and ELISA measurements. This condition was missing was in Figure 2f (% spike from NSEM data on different stabilized spike mutants). We have now added 37 ºC 1-week data to the bar graph.

3. *How were the error bars shown in Figure 1E defined? Did they come from different batches of NSEM samples? If so, how many samples were analyzed?*

Response:
The error bars shown in Figure 1e came from independent experiments performed with different protein lots. The number of samples analyzed in each case are noted at the bottom of each column. We have clarified this in the Figure legend: "Solid bars indicate averages, with number of samples indicated at the bottom of each column. The error bars indicate standard error of the mean, except when N=2, where the error bars indicate range."

4. *Figure 1G, caption: A standard deviation cannot be derived from just two data points (N=2). More data points are needed.*

Response:
The DSC profile presentred in Figure 1g is representative of 2 technical replicates. We now show the two experimental replicates in Extended Data Figure 3. We have moved the Table listing the melting temperatures (Tm) to Supplementary Table 2. The melting temperatures are now listed as mean±range.

5. *Figure 1H: What are the experimental errors of the SPR-derived data?*

Response:
We have now included error bars for the SPR data in Figure 1h. These are derived from technical replicates (N=3), and the data shown is representative of at least 5 independent experiments, two of these are shown in Supplementary Figure 2.

6. *Figure 2F: Can the authors provide the same statistical analysis of experimental errors for the percentages of native-like spikes as illustrated in Figure 1E?*

Response:
We have now added error bars to Figure 2f.

Reviewer 2:

The manuscript by Edwards et al characterized the stability of a purified SARS-CoV-2 spike protein ectodomain variant at different temperatures. This "stabilized" variant of spike protein ectodomain was originally reported by the Jason McLellan Lab and it was generated by introducing 2 prolines (PP) in the S2 part of the protein to prevent S protein transition into the postfusion state. The authors used a variety

of biochemical/biophysical methods and negative stain EM to characterize this purified spike protein variant stored at different temperatures and the results indicated that this spike protein ectodomain variant is unstable when stored at 4 °C for prolonged period of time. For this conclusion the results presented in the manuscript appear to be solid providing useful information for production and storage of this widely used SARSCoV-2 spike protein ectodomain construct.

Response:
We thank the reviewer for this succinct summary of our study and for the positive comments

Beside this conclusion, the authors compared negative stain EM (NSEM) images of freshly purified spike protein, spike protein stored at 4 °C for 7 days and 3 hour incubation of spike protein which had been previously stored at 4 °C for 7 days. Based on this comparison, the authors concluded that spike protein that had become "degraded" when stored at 4 degrees could be recovered back to "well-formed spikes" by incubation at 37 °C for 3 hours. The recovered protein had morphologies similar to the fresh protein by NSEM. While this finding is novel and interesting I found this comparison is confusing and misleading for several reasons:

1. Use of "degradation" (line 70 and 78) is confusing, it normally means protein being converted into smaller species by proteolysis, in this case I believe the authors are trying to describe the loss of "well-formed spike" structures after storage at 4 °C which can be subsequently recovered somewhat by 37 °C incubation. I speculate loss of the "well-formed spike" reflected a change in quaternary structure which should be more appropriately described as "denaturation" instead of "degradation" (it would also be nice if an SDS-PAGE gel can be shown to confirm that there is no degradation i.e. no loss of intact band after 7 days of storage at 4 °C)

Response:
We agree with the reviewer that use of the term "degradation" is confusing. We have now replaced this with "denaturation" in the text. We have also now included SDS-PAGE gel images (Supplementary Figure 1) confirming that the spikes are not degraded upon prolonged storage at 4 °C.

2. The "well-formed" trimer description appears to be solely based on morphological similarity by NSEM images. Are there any differences by image analyses of NSEM images of fresh protein and 37 °C recovered protein? Although I believe NSEM does not have enough resolution to reveal differences such as RBD open and close (which the fresh protein images can be served as a good control as that protein should have both close and open conformations from previous studies.)

Response:
In a previously published study (Henderson et al., NSMB Nat Struct Mol Biol. 2020 Jul 22. doi: 10.1038/s41594-020-0479-4 (Extended Data Figure 4) we have shown that 3D reconstructions obtained using NSEM can distinguish between "up" and "down" RBD states, and the ratios are in agreement with

those reported in published cryo-EM studies. For this study, we have developed a metrics based on 2D classifications to report on the percentage of spike in a purified sample. This allows rapid assessment of spike preparations with smaller datasets than what we would collect for good quality 3D reconstructions. Based on analyses of these smaller datasets, we have not found differences between NSEM reconstructions of fresh protein versus 37 °C recovered protein, either in the morphologies or in the ratios of up/down popualations.

3. Subsequent size-exclusion chromatography, DSC, antibody and ACE2 binding data (Extended Data Figure 2, Figure 1 FGH, Extended Data Figure 4 and 5) show that the 37 °C recovered spike behaves most similar to 22 °C stored protein, especially in ACE2 and CR3022 binding. In ELISA assays, the 22 °C stored protein behaved almost the same in most cases as the 4 °C stored protein which is not "well-formed" by NSEM. More confusingly, the authors did not provide NSEM image of 22 °C stored spike and size-exclusion chromatography, DSC, antibody and ACE2 binding data for fresh protein were not presented. Also in the ELISA the authors did not include any data on 37 °C recovered spike or fresh protein. I feel that the data presented are conflicting to support the notion that the 37 °C recovered spike is "well-formed" similar to fresh protein. In addition, as mentioned above, there are significant amount of missing data in NSEM, size-exclusion chromatography, DSC, antibody and ACE2 binding assays, and ELISA, preventing a proper comparison between different conditions.

Response:

For the purpose of this study, we have defined "fresh" spike sample to be one that was purified the same day the cells were harvested. The purification was completed within 8 hours and the samples were not frozen. We have now clarified this in the text when we first mention "fresh" spike and have included details of the purification in the methods. In the text, we state: "The 2P spike was produced in 293F cells at 37 °C and the purification was performed at room temperature and completed within 6-8 hours (Supplementary Figure 1a-c)". (Lines 62-64). In the methods section, we have added the following: "All protein purification steps including Strep tag purification and SEC were performed at room temperature. The spikes were purified the same day that the cells are harvested and the purification was completed within 6-8 hours." (Lines 209-211).

Given the definition for a "fresh" sample as described above, it is challenging to include a "fresh" sample in all of the studies described in the paper. We were able to capture NSEM data with the fresh sample since that involved making NSEM grids with the freshly purified sample at the end of the working day. These grids were then stored to be imaged later. We were also able to include SEC profiles since these were part of the purification. We have presented SEC profiles for "fresh" spike in Extended Data Figure 1 and Supplementary Figure 1a. ELISA and DSC measurements take much longer to perform so including a "fresh" spike sample for these measurements was not practical. We have now, however, included SPR data on CR3022 and 2G12 binding on "fresh" spike as well as the same spike that was flash frozen in liquid N2 for long-term storage, then thawed for testing (Supplementary Figure 2). We found that

freeze-thaw does affect the antigenicity of the 2P spike, which is not surprising for a molecule that is so sensitive to temperature effects. We also found that incubating the spike sample briefly at 37 °C following freeze-thaw can restore "fresh"-like antigenicity.

For ELISAs, given that they are widely-used in antibody discovery and serology assays, our goal was to demonstrate the importance of taking into account the conditions the spike was stored at for achieving consistent results. We believe the data we have presented does that. Based on the new data we have added in Supplementary Figure 2, we now include a recommendation for storing and using the spike. We have added the following to the text: "Based on these data, in order to obtain consistent results in binding studies, we recommend flash-freezing spike samples in single use aliquots, thawing at 37 °C followed by a brief (~20 min) incubation at 37 °C before using the 2P spike in binding studies." (Lines 131-134).

Depending on the analysis method, the handling of the spike samples have differed in this study. In ELISA, for example, all the samples, irrespective of the temperatures they were incuibated at, are exposed to room temperature for the duration of the assays that spans a few hours and includes incubations and washes. Despite this, clear differences are observed based on how the spike was stored prior to the assay. SPR, on the other hand, allows us to set the sample compartment temperature to match the temperature the spike was incubated at, thus allowing tighter control of the temperature the spike is exposed to during the experiment. Indeed we find differences in the measured 4 -> 37 ºC recoveryof the 2P spike C, depending on whether the spike continued to be stored at 37 °C until right before the assay (Figure 1h and Supplementary Figure 2) or whether the spike was allowed to equilibrate at 22 °C/RT (Extended Data Figure 4). Overall, these observations point to the susceptibility of the 2P spike to temperature induced changes and thus the importance of adopting consistent protocols to ensure reproducibility of critical assays.

We have now included a representative image of a NSEM micrograph for 22 °C stored spike (Supplementary Figure 1d).

4. The CR3022 antibody binds much stronger to the 37 °C recovered protein than to protein stored at 37 °C (Figure 1H and Extended Data Figure 5B). Recent report (Huo et al, Cell Host & Microbe, https://doi.org/10.1016/j.chom.2020.06.010) showed that the epitope of CR3022 is highly cryptic in prefusion trimers and a rotation of the "up"/"open" RBD is needed to expose the cryptic epitope. In addition, in the same report it was shown that prolonged incubation with CR3022 is needed for CR3022 to bind the cryptic epitope resulting in trimer disintegration. The data presented in this paper show differential binding of CR3022 antibody to 37 °C recovered protein and 37 °C stored protein suggesting

there are likely substantial structural differences between these two proteins, and the 37 °C recovered spikes may not be "well-formed" similar to fresh protein if they expose the cryptic epitope.

Response:
We agree with the reviewier that the 37 °C recovered spike are not equivalent to the fresh spike. That the spike is able to recover from cold-induced denaturation upon storage at 37 °C simply shows that some of the denaturation is reversible. Based on the data presented in this paper, we do not recommend storing the spike at 4 °C. As mentioned in the response to the comment above, we have now added a few sentences of how we recommend the spike be stored and used.

Summarized by points above, I think the manuscript is written in a narrative which suggests the 37 °C recovered protein is a "well formed" spike similar to fresh protein or 37 °C stored protein. However, the presented biochemical/biophysical data suggest it is more like 22 °C stored protein. The biochemical/biophysical data also imply 37 °C stored protein, 22 °C stored protein, 4 °C stored protein and 37 °C recovered protein all have distinct structures with different capacity to bind antibodies/ACE2. Therefore, there is a discrepancy between the data and the narrative. I would advise the authors to carefully resolve these discrepancies or providing further experiment evidence, for example high-resolution EM structure of the 37 °C recovered protein to support their current narrative.

Response:
The purpose of this study was to:

1. Report on the cold sensitivity of the widely used SARS-CoV-2 S ectodomain 2P construct.
2. Study this phenomenon using several complementary methods.
3. Explore the nature of the cold-denaturation and show that it is reversible to some extent although, as the reviewer correctly stated, not completely.
4. Demonstrate that an engineered spike with an interprotomer disulfide that links the RBD and the S2 domain is stabilized against this cold-induced denaturation.

Given these goals, we believe a high-resolution EM structure of the 37 °C recovered protein is out of the scope of our aims for this study. We see now how the narrative may have been confusing, and we thank the reviewer for pointing this out. We have now inserted statements that specifically say that the recovery at 37 °C is not complete.

For the NSEM data we state: "In summary, these data showed that the 2P spike denatured upon storage for at 4 °C for a week, and this denaturation could be at least **partially** reversed by incubating the sample at 37 °C following cold-storage." (Lines 80-82)

For the DSC results we state: “Thus, the DSC results confirm that storage at 4 °C destabilizes the spike compared to samples stored at 22 °C or 37 °C, and that returning the destabilized spike to 37 °C for 3 hours substantially restores its stability, although the presence of the low-Tm peak suggests that the recovery is partial.” (Lines 109-112)

Finally, based on all of these data, we recommend the following for obtaining consistent results when using the 2P spike ectodomain: “Based on these data, in order to obtain consistent results in binding studies, we recommend flash-freezing spike samples in single use aliquots, thawing at 37 °C followed by a brief (~20 min) incubation at 37 °C before using the 2P spike in binding studies.” (Lines 131-134)

Minor issues:

1. In the method section, the author did not describe which temperature they use to produce the spike protein, based on their results this parameter can significantly change the state of the produced “fresh protein” - initial material of the study, can the authors clarify how purifications had been done? Also based on their result should it be recommended to purify this protein under room temperature or 37 °C?

Response:

The spike proteins were produced in 293F cells at 37 °C. We have now added this and other details to the methods section. We purify the spike proteins at room temperature. Since discovering its cold sensitivity we have strictly avoided any exposure to 4 °C for these spike proteins during purification. We also purify the protein the same day that the cells are harvested and the purification is completed within the day. The purified protein is flash frozen and stored in -80 °C in single-use aliquots. Each aliquot is thawed and briefly inbuated (~20 min) at 37 °C before use. We have now added all these details to the purification methods.

2. For the rS2d-HexaPro produced, the authors tried to use NSEM and classification to ascertain the spike proteins are all closed, again I think NSEM does not have enough resolution to tell the difference between open and close conformations especially with potential artefact associated with heavy metal staining. A proper control should be done. For example, image analyses of NSEM images of the PP construct which is known to have open/close conformations should be done. However, I do believe the rS2d-HexaPro is mostly closed as three other reports (Rory et al, NSMB, Xiong et al, NSMB 2020, McCallum et al NSMB, 2020) all have confirmed this disulfide pair can disulfide-linked the spike protein monomers in the closed conformation, although the authors failed to cite the latter two refs. In addition, the ACE binding data provided by the authors supported their notion.

Response:

While NSEM reconstructions lack the resolution of cryo-EM, the up/down RBD transitions can be resolved by NSEM. Indeed, we have performed the control that the reviewer has suggested here. We have performed similar NSEM analysis on the 2P construct and our results are published here: Henderson et al., NSMB Nat Struct Mol Biol. 2020 Jul 22. doi: 10.1038/s41594020-0479-4 (Extended Data Figure 4). We have shown in this paper that not only can we resolve up/down RBD conformation in NSEM reconstructions, the ratios of "up" vs "down" agree with ratios obtained from cryo-EM reconstructions and NSEM analysis can distinguish between up/down variations in differently stabilized constructs. We have now added the following sentence to the text: "Similar to the rS2d construct9, rS2d-Hexapro was shown by NSEM to be

100 % in a 3-RBD-down conformation (Figure 2D-E, Extended Data Figure 7 and

Supplementary Movie 1)." (Line 140-142)

3. The authors failed to cite Xiong et al, NSMB 2020 regarding to their conclusion that closing the trimer solve the cold sensitivity issue. In that report, it has been demonstrated 2 ways of disulfide links (one of them is the same as the rS2d) allowed production of fully closed spikes which can be stored at 4 °C for prolonged period of time.

Response:
We thank the reviewer for pointing out this omission. We have now included a reference to the

Xiong et al, NSMB 2020 paper to the text: "An independent study also reported that the 985C383C disulfide bond, as well as an introduced disulfide between residues 987 and 413, prevented denaturation of the spike when stored at 4 °C[15]." (Lines 147-149)

Decision Letter, first revision:

25th Sep 2020

Dear Priyamvada,

Thank you again for submitting your manuscript "Cold sensitivity of the SARS-CoV-2 spike ectodomain". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports during these busy times. Nevertheless, we now have comments (below) from the 2 reviewers who originally 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.

You will see that the reviewers acknowledge the revisions, but there are still a few pending issues including missing data points and figure labeling issues (reviewer 1); in addition, reviewer 2 finds that additional analyses of the conformational state of the well folded spike should be feasible and provide important information. With regard to statistics (reviewer 1 point 5), I suggest plotting individual data points. Finally, while reviewer 1 asks for further discussion, we acknowledge this might not be possible given the restrictions of our Brief Communication format.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response. I can again offer guidelines for the revision, if you send me a word file for main manuscript. 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.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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.

[FOR STRUCTURAL MS] If there are additional or modified structures presented in the final revision,

please submit the corresponding PDB validation reports.

[FOR MS WITH CROPPED GELS] Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

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

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

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.

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Note: 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.

Sincerely,

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Referee expertise:

Referee #1:

Referee #2:

Referee #3:

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors made good efforts in addressing both reviewers' comments in the revised manuscript. Given the differences in sample preparation, the observed temperature-dependent structural and functional integrities varied quite significantly, as pointed out by Reviewer #2. Accordingly, the authors elaborated on the differences observed by different techniques, stressed that the restored structural and functional integrities were partial in most cases, and made a recommendation to store and reactivate the spike protein after long-term storage (lines 131-134), which is very useful for the readership. While the revision made significant improvements after addressing both reviewers' comments, there quite a few errors that require correction and/or clarification. I also felt that the discussion on the highly stabilized spike variants should be elaborated further in the context of their use as vaccine candidates and basic research on the structure-function relationships. Specific comments are as follows:

1. Regarding the analyses of the highly stable HexPro and rS2d-Hexpro, their resistance to heat and cold treatments is an attractive feature that also leads to very high production yield in a CHO cell expression system (Figure 2c). However, the engineered disulfide bonds also restricted the folding

dynamics of the spike protein, leading to a constantly all-RBD-down conformation (Figure 2e and Extended Data Figure 7). Considering that the receptor ACE2 binding to the spike protein requires the RBD in an up conformation, and that most effective neutralizing antibodies that have been reported in the literature bind to the RBD in an up conformation as nicely demonstrated here in (Figure 1h and Extended Data Figure 4), will the engineered spike proteins still be useful for eliciting neutralizing antibodies? A very comprehensive review on the structures of antibodies in complex with the spike protein is published by Bjorkman and colleagues (https://www.biorxiv.org/content/10.1101/2020.08.30.273920v1), which could be cited in this manuscript when discussing the importance of the conformational changes of the RBD in antigenicity.

2. The authors mentioned that prolonged incubation at 37 ºC following the cold incubation can lead to slight aggregation (Line 74) while continuous incubation of the spike protein at 37 ºC for one week also showed evidence of higher molecular weight species (Line 77). Here the authors referred to Supplemental Figure 1e in Line 75 for the reactivation and the continuous 37 ºC incubation to Supplemental Figure 1 in Line 78. First of all, the caption of the Supplemental Figure 1 (It's written as "Extended Data" Figure 1 in the corresponding figure legend, which should be corrected throughout the manuscript in line with NSMB's guideline to authors) did not define the experimental conditions and details of the SEC profiles, making it difficult to figure out where the "higher molecular weight species" was referred to. Second, there was not Supplemental Figure 1e as described in Line 75. Did the authors refer to Extended Data/Supplemental Figure 2? Furthermore, the SEC profiles shown in Extended Data Figures 1 and 2 were quite different. It is unclear what the "good" and "bad" samples shown in Extended Data Figure 1 correspond to in Extended Data Figure 2. A clear and consistent cross-reference of the experimental data and their descriptions should be made. The authors should also include the SEC profile of a "fresh" sample in Extended Data Figure 2e as a reference.

3. Likewise, the SPR profiles and their corresponding response unit bar charts in Extended Data Figure 4 appeared to be mistakenly labeled and paired. While the figure caption stated that panels a, b and c corresponded to ACE2, CR3022 and 2G12 binding, the labels above the SPR profiles were inconsistent. Importantly, the corresponding bar charts were not in the correct order. In the following ELISA profiles (panels d-g), there were only three datasets and the 4 ºC, 7d -> ºC was missing. Why were the data of the recovered sample omitted? The same issue occurred in Extended Data Figure 5 c-e.

4. In Figure 1h, the max response unit for CR3022 binding to the spike protein was 20-fold higher than that for 2G12. Considering that the SPR respond unit reports on the mass change upon complex formation and that the two antibodies have similar molecular weights, why did the two antibodies showed such a big difference in the SPR responses? The difference became even more obvious in Extended Data Figure 4a-c in which the ACE2 binding only showed single digit response units, in a binding process with a Kd value in low nM range. How can such a difference occur for these strong binders?

5. In Figures 1 & 2, it is stated that "The error bars indicated standard error of the mean, except when N=2, where the error bars indicate range." Standard errors can be significantly smaller than standard deviations, depending on the data size. The authors should explicitly state which data points have N=2 and how many technical replicates (or biological replicates) were made for the remaining ones where standard errors were reported.

6. For clarify, I would recommend to change the coloring scheme for the temperature treatments: the red (4ºC, 7d) and pink (4 ºC, 7d -> 37 ºC 3h) are very similar. Additionally, the descriptions should

be explicit and consistent: In Extended Data Figure 2, the pink line is defined as (4 ºC, 7d -> 37 ºC 3h) whereas in most of the other figures, the "3h" is omitted.

Reviewer #2:
Remarks to the Author:
After examining the authors' previous paper (Henderson et al., Nat Struct Mol Biol. 2020), I am now convinced that image analysis of negative-stain EM images following the authors procedure can distinguish open and closed spikes.

As previously stated the authors presented strong evidence of cold denaturation. The authors also presented a novel finding that 37 °C can revert the cold-denatured spike back to "well-formed" spike. But the previous interpretation of biochemical data was confusing.

After the authors' reversion, particularly when they interpret that "the recovery is partial", I can now understand from the data that 37 °C incubation can only convert a certain population of the denatured spike back to "well-formed" spike, the presence of unconverted, still denatured spikes in the "rebound" sample likely complicated the assay results and caused confusions when it was ignored in the previous interpretation.

I found it still unsatisfactory that the authors failed to comment on the conformational state of the 37 °C recovered "well-formed" spike. The new data supplied by the authors (Supplementary Figure 3) suggest 37 °C recovered spike is mainly in a closed state with reduced CR3022 binding. Since the authors have acquired negative-stain EM images of the recovered spike (Figure 1d), a simple image analysis should be able to find out the "closed"/"open" ratio of the recovered spike. I believe such analysis will strongly strengthen this novel finding, providing insight into the recovery process. Such info will also be valuable when other research groups choose to use authors' method of 37 °C incubation to renature spike protein.

In summary, I found the manuscript much easier to read and understand after the revision, and I found the narrative is now acceptable with the presented data. I would like to strongly support the publication of the article if the authors can perfect this interesting paper by including info regarding the conformational state of the 37 °C recovered "well-formed" spike.

Minor comment:

Line 123/124, "Extended Data Figure 5" should be referred at the end of the sentence "Both antibodies showed different binding profiles depending on the temperature at which the spike was stored," for easy reading.

Author Rebuttal, first revision:

Reviewer 1:

Summary

The authors made good efforts in addressing both reviewers' comments in the revised manuscript. Given the differences in sample preparation, the observed temperature-dependent structural and functional integrities varied quite significantly, as pointed out by Reviewer #2. Accordingly, the authors elaborated on the differences observed by different techniques, stressed that the restored structural and functional integrities were partial in most cases, and made a recommendation to store and reactivate the spike protein after long-term storage (lines 131134), which is very useful for the readership. While the revision made significant improvements after addressing both reviewers' comments, there quite a few errors that require correction and/or clarification. I also felt that the discussion on the highly stabilized spike variants should be elaborated further in the context of their use as vaccine candidates and basic research on the structure-function relationships.

Response:
We are glad that the reviewer is largely satisfied with the revisions we had made to the manuscript in response to the very helpful comments from both reviewers. We have addressed the remaining issues raised by the reviewer as detailed below.

Specific comments:

1) Regarding the analyses of the highly stable HexPro and rS2d-Hexpro, their resistance to heat and cold treatments is an attractive feature that also leads to very high production yield in a CHO cell expression system (Figure 2c). However, the engineered disulfide bonds also restricted the folding dynamics of the spike protein, leading to a constantly all-RBD-down conformation (Figure 2e and Extended Data Figure 7). Considering that the receptor ACE2 binding to the spike protein requires the RBD in an up conformation, and that most effective neutralizing antibodies that have been reported in the literature bind to the RBD in an up conformation as nicely demonstrated here in (Figure 1h and Extended Data Figure 4), will the engineered spike proteins still be useful for eliciting neutralizing antibodies? A very comprehensive review on the structures of antibodies in complex with the spike protein is published by Bjorkman and colleagues (https://www.biorxiv.org/content/10.1101/2020.08.30.273920v1), which could be cited in this manuscript when discussing the importance of the conformational changes of the RBD in antigenicity.

Response: We have described the all-RBD-down rS2d spike (without the HexaPro mutations) in a previous publication (Henderson et al, 2020, NSMB), where we have discussed in detail the

vaccine implications of a RBD-down state stabilized spike. Briefly, while the disulfide-linked down state locked double mutant (rS2d) would presumably not elicit antibodies that require an "up" RBD conformation, it would still be capable of eliciting potent RBD-directed antibodies such as S309 that are able to bind the down state RBD, as well as NTD and S2 directed responses.

Owing to the space limitations of the NSMB Brief Communications format, and because we have discussed the vaccine implications of the all-RBD-down spike in our previous publication, we are limiting our discussions in this paper to the cold sensitivity of the furincleavage deficient 2P spike ectodomain and the abrogation of this cold sensitivity in the stabilized rS2d-HexaPro construct.

2. The authors mentioned that prolonged incubation at 37 ºC following the cold incubation can lead to slight aggregation (Line 74) while continuous incubation of the spike protein at 37 ºC for one week also showed evidence of higher molecular weight species (Line 77). Here the authors referred to Supplemental Figure 1e in Line 75 for the reactivation and the continuous 37 ºC incubation to Supplemental Figure 1 in Line 78. First of all, the caption of the Supplemental Figure 1 (It's written as "Extended Data" Figure 1 in the corresponding figure legend, which should be corrected throughout the manuscript in line with NSMB's guideline to authors) did not define the experimental conditions and details of the SEC profiles, making it difficult to figure out where the "higher molecular weight species" was referred to. Second, there was not Supplemental Figure 1e as described in Line 75. Did the authors refer to Extended Data/Supplemental Figure 2? Furthermore, the SEC profiles shown in Extended Data Figures 1 and 2 were quite different. It is unclear what the "good" and "bad" samples shown in Extended Data Figure 1 correspond to in Extended Data Figure 2. A clear and consistent cross-reference of the experimental data and their descriptions should be made. The authors should also include the SEC profile of a "fresh" sample in Extended Data Figure 2e as a reference.

Response: Following instructions from the editor we have re-organized the Extended Data Figures and Supplemental Figures.

The evidence for the slight aggregation of the spikes after prolonged incubation at 37 ºC following the cold incubation came from observations of spike clusters in the NSEM micrographs. This is now shown in Extended Data Figure 1e, with a circle drawn on the micrograph around a cluster of spikes visible in the micrograph for the 2P spike sample that was stored at 4 °C for one week followed by recovery at 37 °C for 6 days.

For samples that were incubated for 7 days at 37 ºC we observed increase in the higher molecular weight bands in SDS-PAGE gels (Supplementary Figure 3). We have edited the text for clarity: "SDS-PAGE analysis of 2P spike samples stored at different temperatures does not indicate any appreciable degradation of the spike, although we do observe an increase in higher

molecular weight bands for spike samples stored at 37 °C for a week (Supplementary Figure 3)." Extended Data Figures 1 and 2 are now Supplementary Figures 1 and 2, respectively. The SEC profiles look different in Supplementary Figures 1 and 2 because the SEC runs shown is Supplementary Figure 1 were performed with spike after purification using a Strep-Tactin affinity, whereas, for Supplementary Figure 2 a batch of Strep-Tactin + SEC purified spike was used to setup the incubations at different temperatures and SEC runs were performed with these samples post-incubation. This accounts for the absence of the higher molecular weight shoulders in the SEC plots in Supplementary Figure 2, compared to the ones in Supplementary Figure 1. In order to clarify this difference for readers, we have now added this sentence to the legend of Supplementary Figure 1: "The SEC and SDS-PAGE runs were performed with spike samples that were produced in 293F cells and purified from the cell culture supernatant using a StrepTactin column (see methods)." To the legend of Supplementary Figure 2 we have added the following sentence: "The spike preparations were purified by SEC before the start of the temperature incubations. The peak corresponding to the 2P spike was used discarding any shoulder and aggregates."

The "good" and "bad" spike preparations shown in Supplementary Figure 1 were examples taken from our early days of research on the SARS-CoV-2 2P spike, when we had not yet linked spike denaturation to cold storage. For all subsequent studies in this paper, the "good" spike preparation shown in Supplementary Figure 1a is representative of the 2P spike preparation used to start temperature incubations.

3. Likewise, the SPR profiles and their corresponding response unit bar charts in Extended Data Figure 4 appeared to be mistakenly labeled and paired. While the figure caption stated that panels a, b and c corresponded to ACE2, CR3022 and 2G12 binding, the labels above the SPR profiles were inconsistent. Importantly, the corresponding bar charts were not in the correct order. In the following ELISA profiles (panels d-g), there were only three datasets and the 4 ºC, 7d -> ºC was missing. Why were the data of the recovered sample omitted? The same issue occurred in Extended Data Figure 5 c-e.

Response: *The antigenicity data have been re-organized and consolidated to Extended Figures 2 and 3. We have added the data on the 4 ºC, 7d -> 37ºC to the figures. They are shown side-byside with the previous plot since the experiments were done on consecutive days with the same lot of protein.*

4. In Figure 1h, the max response unit for CR3022 binding to the spike protein was 20-fold higher than that for 2G12. Considering that the SPR respond unit reports on the mass change upon complex formation and that the two antibodies have similar molecular weights, why did the two antibodies showed such a big difference in the SPR responses? The difference became even more obvious in Extended Data Figure 4a-c in which the ACE2 binding only showed single digit

response units, in a binding process with a Kd value in low nM range. How can such a difference occur for these strong binders?

Response: We have consolidated the Extended Data Figures and the Supplementary Figures showing the antigenicity assays. These are now presented in Extended Data Figures 2 (binding to previously known ligands) and 3 (binding to new antibodies COVID-19 recovered patient). In Extended Data Figure 2 we show the raw SPR data corresponding the bar graphs shown in Figure 1d. To link the two figures we have now added the following sentence to the Figure 1 legend: "Error bars are derived from 3 technical repeats. The data shown are representative of at least 5 independent experiments. 2 independent repeats using separate protein lots are shown in Extended Data Figure 2." and we have added this sentence to the Extended Data Figure 2 legend: "The bar graphs in Figure 1d show data from Lot # 025MFK."

The SPR assays were performed by capturing the antibodies on an anti-Fc chip and flowing over a solution of the spike in running buffer to measure binding. Thus, the response levels are a function of the molecular weight of the spike rather than of the antibodies. The difference in the binding levels between 2G12 and CR3022 can arise from the following sources: (1) Small differences between the levels of the different antibodies captured on the anti-Fc chip, (2) The surfaces may not be equally active for both ligands, (3) The different epitopes to which these bind may not be presented equally optimally. The major contributor to the differences in binding levels for CR3022 and 2G12 is likely option #3. CR3022 binds an epitope on the RBD, whereas, 2G12 binds a quaternary glycan epitope in S2. We have shown that in a cryo-EM dataset of 2G12 bound 2P spike (https://www.biorxiv.org/content/10.1101/2020.06.30.178897v1) , a large population of the spike remains unbound (Figure S9 of biorxiv preprint referenced above). Indeed, that epitope presentation on the spike is a key determinant for binding level differences in the SPR experiments are underscored by this study where binding levels of the same spike samples to similarly prepared surfaces differ based on the temperature at which the spike was stored.

5. In Figures 1 & 2, it is stated that "The error bars indicated standard error of the mean, except when N=2, where the error bars indicate range." Standard errors can be significantly smaller than standard deviations, depending on the data size. The authors should explicitly state which data points have N=2 and how many technical replicates (or biological replicates) were made for the remaining ones where standard errors were reported.

Response: For the bar graph shown in Figure 1b, the number of replicates for each datapoint is now listed at the top of each bar. To the legend we have added: "Error bars shown are derived from 2-7 independent experiments with different protein lots, and represent standard error of mean where $N \geq 3$ and range where N=2, with the exact N indicated at the top of each bar. From left to right, bars show spike percentage in a fresh sample of spike; after the spike undergoes a

single freeze-thaw cycle; after it has been incubated for 5-7 days at 4 °C (red), 22 °C (green) or 37 °C (blue); and spike samples that were stored for 1 week at 4 °C, then incubated at 37 °C for 1 hour, 3 hours, or 4-6 days."

For the bar graph shown in Figure 2b, we have similarly listed the number of replicates for each datapoint at the base of each bar. To the legend we have added: "Error bars shown are derived from 2-4 independent experiments with different protein lots, and represent standard error of mean where N ≥ 3 and range where N=2, with the exact N indicated at the base of each bar."

6. For clarify, I would recommend to change the coloring scheme for the temperature treatments: the red (4ºC, 7d) and pink (4 ºC, 7d -> 37 ºC 3h) are very similar. Additionally, the descriptions should be explicit and consistent: In Extended Data Figure 2, the pink line is defined as (4 ºC, 7d -> 37 ºC 3h) whereas in most of the other figures, the "3h" is omitted.

Response: We have changed the color of the 4 ºC, 7d -> 37 ºC data points to a lighter pink and the 4ºC, 7d data points to a darker red, thus making it easier to distinguish between the two..

Reviewer 2:

After examining the authors' previous paper (Henderson et al., Nat Struct Mol Biol. 2020), I am now convinced that image analysis of negative-stain EM images following the authors procedure can distinguish open and closed spikes.

Response: We have now deposited the NSEM reconstruction of the rS2d-Hexapro spike that we have reported here (EMD-22934), the rS2d spike we had reported previously in the *Henderson et al., Nat Struct Mol Biol. 2020* paper (EMD-22809), as well as a number of our recent NSEM reconstructions of antibody-bound spikes (EMD-22923, EMD-22920, EMD-22921, among others) that all demonstrate that our NSEM reconstructions are resolved well enough to distinguish between the "up" and "down" RBD conformations.

As previously stated the authors presented strong evidence of cold denaturation. The authors also presented a novel finding that 37 °C can revert the cold-denatured spike back to "wellformed" spike. But the previous interpretation of biochemical data was confusing.

After the authors' reversion, particularly when they interpret that "the recovery is partial", I can now understand from the data that 37 °C incubation can only convert a certain population of the denatured spike back to "well-formed" spike, the presence of unconverted, still denatured spikes in the "rebound" sample likely complicated the assay results and caused confusions when it was ignored in the previous interpretation.

Response: We thank both reviewers for their helpful comments and critiques that helped us clarify the presentation of our results.

2. I found it still unsatisfactory that the authors failed to comment on the conformational state of the 37 °C recovered "well-formed" spike. The new data supplied by the authors (Supplementary Figure 3) suggest 37 °C recovered spike is mainly in a closed state with reduced CR3022 binding. Since the authors have acquired negative-stain EM images of the recovered spike (Figure 1d), a simple image analysis should be able to find out the "closed"/"open" ratio of the recovered spike. I believe such analysis will strongly strengthen this novel finding, providing insight into the recovery process. Such info will also be valuable when other research groups choose to use authors' method of 37 °C incubation to renature spike protein. In summary, I found the manuscript much easier to read and understand after the revision, and I found the narrative is now acceptable with the presented data. I would like to strongly support the publication of the article if the authors can perfect this interesting paper by including info regarding the conformational state of the 37 °C recovered "well-formed" spike.

Response: Following the reviewer's suggestion, we have now performed 3D classification of the particles picked from the NSEM data of 2P spike stored at 4 °C for a week followed by recovery at 37 °C for 3 hours. These data are presented in Supplementary Figure 6, and show that typical populations of 1-RBD-up and 3-RBD-down spike are observed in the sample following revovery. We have added this result to the text: "3D classification of spike particles from NSEM micrographs of 2P spike samples stored at 4 °C for 1 week followed by a 3-hour incubation at 37 °C, showed the typical populations of all-RBD-down and 1-RBD-up spike that we have reported previously from freshly prepared spike samples9 (Supplementary Figure 6)." NSEM reconstructions for the 2P spike after 1-week cold storage followed by 3-hour recovery at 37 °C are deposited with accession codes EMD-22967 and EMD-22968 for the 3-RBD-down and 1RBD-up states, respectively.

3.
Minor comment:

Line 123/124, "Extended Data Figure 5" should be referred at the end of the sentence "Both antibodies showed different binding profiles depending on the temperature at which the spike was stored," for easy reading.

Response: We have re-orgnaized the Extended Data Figures and Supplementary figures following the editor's instructions. The figure call-outs have also been edited. For this section, we now call the figures once in the beginning and not multiple times while describing the results:

"We also tested two antibodies isolated from a COVID-19 convalescent donor, with epitopes mapped to the ACE-2 binding site (AB712199) and to the S2 region of the spike (AB511584) (Extended Data Figure 3, Supplementary Figures 7-8). Both antibodies showed different binding profiles depending on the temperature at which the spike was stored, highlighting the importance of accounting for this cold-sensitive behavior of the S ectodomain for obtaining consistent results in serology assays when using the 2P spike or similar constructs." We agree with the editor and reviewer that this increases the readability of the text.

Decision Letter, second revision:

12th Nov 2020

Dear Priyamvada,

Thank you again for submitting your manuscript "Cold sensitivity of the SARS-CoV-2 spike ectodomain". After editorial assessment of the revision and response, we are happy to accept your paper, in principle, for publication as an Brief Communication in Nature Structural & Molecular Biology, on the condition that you revise your manuscript in response to our editorial requirements.

The text and figures may require revisions. Note that, within a week, we will send you detailed instructions for the final revision, along with information on editorial and formatting requirements. We recommend that you do not start revising the manuscript until you receive this additional information.

To facilitate our work at this stage, please send us the main text as a word file.

Data availability: this journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data 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:
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We hope that you will support this initiative and supply the required information. Should you have any query or comments, please do not hesitate to contact me.

In recognition of the time and expertise our reviewers provide to Nature Structural & Molecular Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Cold sensitivity of the SARS-CoV-2 spike ectodomain". For those reviewers who give their assent, we will be publishing their names alongside the published article.

If you have any questions, please do not hesitate to contact me directly.

Sincerely,
Ines

Ines Chen, Ph.D.
Chief Editor

Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

22nd Nov 2020

Dear Priyamvada,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "Cold sensitivity of the SARS-CoV-2 spike ectodomain" (NSMB-BC43832B). Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

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2. DATA DEPOSITION: We require deposition of coordinates (and structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). EM maps must be deposited in the EMDB. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. Proteomic datasets should be deposited in PRIDE. Accession codes must be provided in your final submission for acceptance, and entries must be accessible or HPUB at the galley proof stage.

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Best regards,
Ines

Ines Chen, Ph.D.

Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Final Decision Letter:

3rd Dec 2020

Dear Dr. Acharya,

We are now happy to accept your revised paper "Cold sensitivity of the SARS-CoV-2 spike ectodomain" for publication as a Brief Communication 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.

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