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Dear Dr. Li:

Thank you for submitting the above-named article to BBA - Biomembranes.

I am sorry to inform you that your paper is not acceptable for publication. We have completed the review of your manuscript and a summary of the comments received from two expert reviewers is appended below. Significant shortcomings were identified in the work, for example, one reviewer points out that simulations with the glycoprotein form of the protein need to be done, to provide data that are physiologically more meaningful. Addressing this issue alone would take more time than what is normally allowed for a major corrections decision in our journal. The second reviewer also has a series of concerns that would need to be addressed. Your manuscript in its present form will therefore not be considered further for publication at this stage. However, the topic is within scope and if you can address at a later date the concerns of both reviewers in an appropriate manner, you may, in the future, resubmit it as a completely new submission (in that case please include a cover letter describing the history of the manuscript).

Yours sincerely,

Hans J. Vogel, Ph.D.
Executive Editor
BBA – Biomembranes

Please see Reviewers' comments:

Reviewer #1: A manuscript by Li et al. describes simulations study of HIV protein gp120, which plays an essential role in virus entry into a host cell. Authors used a few computational methods and analysis tools, which provide a nice framework for studying protein dynamics. Thus, the manuscript is interesting from a methodological point of view. The manuscript is also well written and nicely illustrated.

There is, however, an essential problem with the biological relevance of the results. Gp120 is essentially glycoprotein; carbohydrates constitute half of the protein mass. Therefore, it is difficult to believe that glycan does not affect protein behavior. My suggestion is that authors extend simulation on glycosylated protein, including at least core glycan (5 first sugars of conserved sequence), which may represent about 30% of carbohydrates. My recommendation to reject the manuscript is based on time limit given for revision which is too short to perform such calculations.

Response:
We thank the reviewer for careful reading and constructive comments on our manuscript.

It is expected that the glycan layer could influence gp120 behavior, but this issue is beyond our research purpose. In our study, two comparative MD simulation systems of open-state gp120 under the CD4-stripped and CD4-exist conditions were performed to investigate CD4 binding effects on the molecular dynamics, thermodynamics, and kinetics of gp120. We think that controlling a single variable parameter (CD4 exist or not) in the same experimental environment (open-state gp120 without carbohydrates constitute) can be considered as a reasonable design for our study. Moreover, previous MD simulation studies on gp120 with glycosylated and non-glycosylated variable loops showed no significant differences in molecular fluctuations between these two forms of gp120 (*Yokoyama et al.*, *2012*, *PLoS One*).

Fig. 1 A fully glycosylated gp120 (PDB ID: 6MEO, cyan) and open-state gp120 (PDB ID: 3J70, yellow). The glycans (sticks representation) stripped from 6MEO were introduced into our simulations.

However, MD simulations of the glycosylated gp120 suggested by the reviewer are interesting and worth doing. To preliminarily study how glycan affects the behavior of gp120, the glycans stripped

from a fully glycosylated gp120 (PDB ID: 6MEO, Fig. 1, cyan) (*Shaik et al.*, *2019*, *Nature*) were introduced into our simulations (PDB ID: 3J70, Fig. 1, yellow). Except for using GLYCAM06 force field (*Kirschner et al.*, *2008*, *J. Comput. Chem.*) to handle glycans, three 100-ns MD replicas for ligand-free and CD4-bound simulation systems were carried out under the same protocol in our manuscript.

According to time evolutions of the backbone root mean square deviation (RMSD, Fig. 2) values in MD simulations of the glycosylated gp120, ligand-free system experienced larger structural deviations and more dramatic conformational changes than the CD4-bound system, indicating that the former has a high structural flexibility and a stronger capability to alter conformation than the latter. This RMSD evaluation also implies that glycan does not influence our comparative results of the intrinsic dynamics of gp120 inhibited by the binding of CD4.

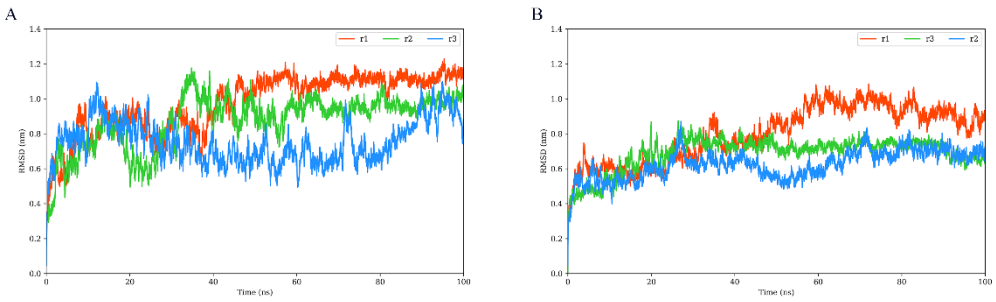

Fig. 2 Time evolution of backbone root-mean-square deviation (RMSD) values of glycosylated gp120 with respect to the starting structure calculated from three replicas (r1-3) in ligand-free (A) and CD4-bound (B) systems.

After clustering two vectors from the center of mass (COM) of the bridging sheet to the COM of the V1/V2 region and the COM of the V3 tip, a similar tendency of conformational transitions (Fig. 3) was observed in MD simulations of the glycosylated gp120. From the starting conformational state (Fig. 3A, green in ligand-free system with glycans, Fig. 3D, blue in CD4-bound system with glycans), glycosylated gp120 can sample intermediate conformation (Fig. 3C, green) only under ligand-free condition. This indicates that compared to the binding of CD4, glycans can only exert a limited effect on the conformational transitions of gp120. Our results of CD4-barrier on the conformational transitions are also applicable after adding glycans.

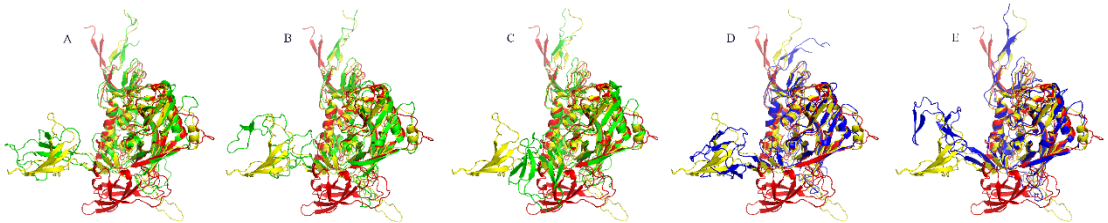

Fig. 3 Representative structures of the glycosylated gp120 in the ligand-free (A-C, blue) and CD4-bound (D-E, green) systems were superimposed to the closed-state (PDB ID: 5FYJ, red) and open-state (PDB ID: 3J70, yellow) gp120.

For the following two reasons, we think it is appropriate to add discussion about potential effects of carbohydrates constitute in this manuscript. Detailed MD simulations of glycosylated gp120 will be expanded as our future work.

1. This study we reported here focuses on the effects of CD4 binding to the molecular dynamics, thermodynamics, and kinetics of gp120. Comparative MD simulations of the ligand-free and CD4-bound gp120, although containing no glycans, could still reflect the molecular differences gp120 upon CD4 binding.
2. The MD simulations of the glycosylated gp120 we performed here are not sufficient to construct the Markov state model and can be further optimized. We look forward the reviewer to provide more professional advice for our future work.

Reviewer #2: The manuscript "CD4-binding obstacles in the conformational transitions and allosteric communications of HIV gp120" by Li et al applies molecular simulations, Markov modeling and network-based analysis to describe conformational states during the HIV-1 Env transitions from Env State 1 (closed conformation) to State 3 (open or CD4-bound conformation). The authors used computational tools and available CD4-bond Env structure as a starting point and analyzed the possible pathways by which gp120 or gp120-CD4 can transition to different states. The in silico methods/analysis provides some theoretical information but the study still needs to show consistency with concepts and experimental data available in numerous publications. Specifically, the mechanism of CD4 binding has been well investigated leading to current understanding that CD4 may bind State 1 or capture and stabilize State 3. There are also experimental data that describe intermediate states.

Response:
We thank the reviewer for careful reading and constructive comments on our manuscript.

In this revision, more descriptions and discussions consistent with the available experimental data listed by the review have been added.

Specific comments follow.

1. The term "unliganded" and "liganded" are outdated as HIV-1 gp120 is almost always crystalized with antibodies and not unliganded, and "liganded" can be ambiguous as it may represent a complex with CD4 or antibodies. The authors should use the new terms in the HIV-1 Env field that were coined in 2016 (Herschhorn et al. mBIO 2016) after the identification of a new Env intermediate state throughout the manuscript. State 1 (for the closed conformation), State 2 (intermediate conformation), and State 3 (open or CD4-bound conformation).

Response:
We now use “closed” and “open” instead of “unliganded” and “liganded”, respectively, to describe the conformational states of gp120. The multiple conformational states detected in our simulations have been corresponded to the closed conformation (State 1), intermediate conformation (State 2), and open conformation (State 3) (*Herschhorn et al., 2016, MBio*). In order to avoid the confusion caused by the conformational states labeled by number, we use "open", " intermediate" and "closed" directly in this revision.

2. The CD4 receptor can bind to either Env conformational state. Binding to State 1 induces the transition to State 3 and binding to State 3 stabilizes this conformational state. There is not any "different role". Please delete or rephrase the sentence: "However, significant structural rearrangements between these two states and recent biophysical observations suggest that CD4 may play a different role" from the abstract. Also remove any of these concepts from the discussion section. If the authors believe that capturing State 3 is a dominant mechanism, they should provide strong experimental evidence.

Response:
We rephrase related concepts in abstract and discussion in this revision. In our study, we want to

declare capturing the open state is one of molecular mechanism. Comparative MD simulations of the ligand-free and CD4-bound systems demonstrate different conformational transitions of gp120 from open state to closed state (seeing graphical abstract, Fig. 2, and Fig. 3 in our manuscript), suggesting that gp120 is intrinsically dynamic from open state to closed state, whereas the binding of CD4 blocks these conformational transitions.

3. The authors should include the relevant references for identified Env intermediate conformations. They should at least add:

a. Herschhorn A. et al mBIO 2016 for the intermediate state;

b. Alsahafi N. et al Cell Host & Microbe 2019 for a new conformational state related to ADCC activity; and

c. Lu M. et al Nature 2019 for discussion on BG505SOSIP state.

The authors should further discuss how these experimental-defined intermediates are related to their in silico Env states.

Response:

The references listed by the review have been discussed and cited in this revision.

4. The authors should validate their finding/results using available experimental data in the literature. Changes of residue 193 lead to substantial conformational changes that are consisted with the ability of L193 to form a hydrophobic core maintaining State 1. Changes to more hydrophilic residues correlate with transitions to downstream conformations. The authors should introduce in silico all amino acids to position 193, calculate the most stable conformation for each and show the correlation between opening of the trimer and hydrophobic changes. Similarly, the I423A change should stabilize State 3-like Env conformation.

Response:

Residual mutation plays a role in conformational changes of gp120, but this issue is out our research aim. In our study, two comparative MD simulation systems of open-state gp120 under the ligand-free and CD4-bound conditions were performed to investigate CD4 binding effects on the molecular dynamics, thermodynamics, and kinetics of gp120.

In fact, our group is preparing a study about molecular dynamics of gp120 from different HIV-1 isolates, which taken from an experimental report (*Seaman et al., 2010, J. Virol.*). In that study involving multiple HIV isolates will investigate the relationship between sequence variations and structural dynamics.

We have added discussion of residual mutation in this revision. We hope that the reviewers and editors will support our request to separate this issue into an independent work.

5. Delete the sentence: "It is doubtful whether such so significant structural change of gp120 between these two states should be attributed to the binding of CD4." Structural rearrangements as the result of CD4 binding are documented in numerous publications using functional, biochemical and biophysical assays.

Response:
We deleted the above sentence.

6. Rephrase "There are about 75% unliganded state and 25% liganded state at 4°C, whereas an inverted distribution of 25% unliganded state and 75% liganded state was observed at 37°C, suggesting from a thermodynamic perspective that gp120 is intrinsically able to sample a variety of conformational states." to reflect that these data is based on one method/structure. smFRET experiments show the primary HIV-1 Env are dominantly in State 1.

Response:
We deleted the description about conformational distributions at different temperatures.

7. V3 is not emanating from the bridging sheet but from under the stem of the bridging sheet.

Response:
We fixed this mistake in this reversion.

8. Please add that hydrogen-deuterium exchange analysis, which is referenced, was performed with soluble BGSOSIP trimer.

Response:
More description of hydrogen-deuterium exchange (*Guttman et al., 2014, Structure*) was added.

9. The authors used Clade G X1193.c1 SOSIP.664. They should explain why they used this structure and discuss how representative this structure is with regard to different Envs from different HIV-1 strains.

Response:
We choose the model of gp120 based on the available structure. The cryo-EM structure of HIV gp120 complexed with CD4 (PDB ID: 3J70) (*Rasheed et al., 2015, Structure*) is the only one full-length, open-state model when we carried out MD simulations. We illustrated this issue more clearly in this reversion. Other HIV strains are not considered in our study.

10. Delete or rephrase "the liganded state can be considered as a high free energy state, which can intrinsically transfer into the ground state (i.e. the unliganded state) of gp120 due to a lower free-energy level." Based on abundance of scientific data and reports, State 1 (closed conformation) occupies a HIGH energy well and CD4 binding facilitate the transition to LOWER energy state.

Response:
Evident from the single-molecule fluorescence resonance energy transfer (smFRET) experiment about conformational dynamics of gp120 (*Munro et al., 2014, Science*) shows that gp120 exhibits different conformational distributions and dominant conformations at different conditions. In the condition without any ligands (Fig. 1, unliganded), in the presence of soluble CD4 (Fig. 1, $sCD4_{D1D2}$),

and in the presence of $sCD4_{D1D2}$ and 17b (Fig. 1, $sCD4_{D1D2}$/17b), the dominant conformation is presented by low (~0.10), high (~0.60), and intermediate (~0.30) FRET, respectively.

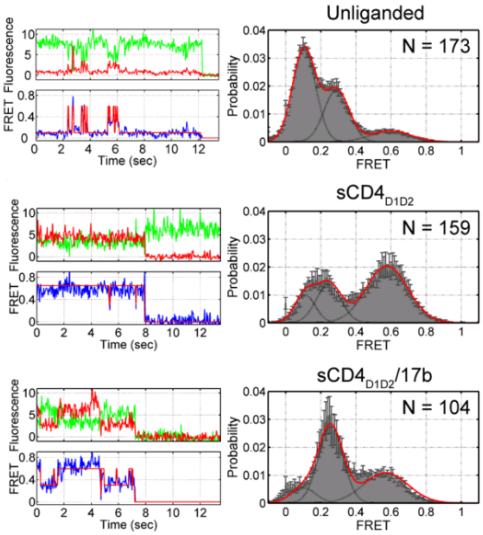

Fig. 1 Figure from single-molecule fluorescence resonance energy transfer (smFRET) experiment (*Munro et al., 2014, Science*). (left) Representative fluorescence trajectories. (Right) FRET trajectories were compiled into a population FRET histogram and fit to the sum of three Gaussian distributions (red).

Based on smFRET (Fig. 1, top right), in absence any ligands, or under the "unliganded" condition, open state (high FRET) locates at a high energy level and could intrinsically transfer into the dominant, lower-energy closed state (low FRET). This supports our ligand-free simulations, which suggesting that gp120 is intrinsically dynamic from open state to closed state.

However, we rephrase the above sentence, and clarify the relationship between our simulations and the available scientific data and reports in this reversion.

11. The HR1 of gp41 is exposed on the Env surface when CD4 binds. The authors should add this information and discuss the potential changes to this region in their in silico modelling.

Response:
The possible effects of gp120 dynamics on the HR1 of gp41 have been added and discussed in this reversion.