Comments to the Author

1. If the authors have adequately addressed your comments raised in a previous round of review and you feel that this manuscript is now acceptable for publication, you may indicate that here to bypass the “Comments to the Author” section, enter your conflict of interest statement in the “Confidential to Editor” section, and submit your “Accept” recommendation.

Reviewer #1: All comments have been addressed
Reviewer #2: (No Response)

We addressed the first reviewer’s comments, and the following is our response to the second reviewer’s concerns.

Reviewer #2: The manuscript by Chen et al. described the structure changes of addition mutant of E. coli Glutamine binding protein (GlnBP) upon ligand (glutamine) binding. The experimental methods of ITC, NMR, SAXS etc. were applied. The conclusion was the change of binding affinity was associated with quaternary structure change (inter-domain) caused by the lengthened linker mutant. While the inter-domain reorganization may contribute to the binding affinity changes, the contribution is one of many factors that could affect binding affinity. Potentially, electrostatic and hydrophobic interactions between protein side chains and Gln should dominate the interaction, not discussed in the manuscript. The authors don’t seem to have a clear experimental design or conclusion. In general, for a study like this, the hypotheses should be raised at the beginning, followed by experimental demonstration, result interpretation and final conclusion. Now, the manuscript lacks coherent reasoning or conclusion. In the end, there is no take home message from the study. The manuscript is highly immature and should not be published in the current format. Major issues are listed below.

We thank reviewer for the valuable comments. We did make a hypothesis that domain closure is critical for ligand binding at the beginning, which is the rationale for us to design the mutants and characterize the relationship between linker changes and ligand binding affinity. The reviewer is absolutely correct that electrostatic and hydrophobic interactions between side chain and Gln influence ligand binding. However, this has been demonstrated in many ligand binding proteins. Domain closure or how linker length affects ligand binding, has never been investigated to our knowledge. We maintain the overall protein sequence except the introduction of several alanines in the linker region to exclude the possibility that electrostatic or hydrophobic interaction determines ligand binding.

We add a few sentences in the discussion to highlight the significance of this study. We use NMR, SAXS, and ITC to characterize the ligand binding of GlnBP and corresponding structural changes. SAXS did show the structural changes for different GlnBP mutants. The complementary nature of these techniques provide biophysical insights of molecular binding. We generate GlnBP mutants with different domain linker length to investigate the perturbation of domain closure capability on protein ligand binding. We find that mutants of GlnBP with different domain closure capability show different ligand binding property. Longer linker allows the free domain closure that has the highest ligand binding affinity. Shorter domain linker blocks domain closure that has the lowest binding affinity. Breakage of disulfide bond releases the restriction on domain closure and restore ligand binding affinity. We discover a reversible way to control domain closure and ligand binding. The study shows the correlation between ligand binding affinity and domain closure, which is the major conformational change upon ligand binding and determinant for ligand
1. The result did not provide any quantitative correlation between ITC results and any others like SAXS or NMR. Therefore, the science advance on this binding topic is minimal.

ITC, NMR, and SAXS study different properties of protein complex. ITC studies the ligand binding affinity that shows different linker length perturbs the ligand binding affinity. SAXS shows that ligand binding gradually triggers domain closure and protein conformation changes. The results from three techniques are complementary to characterize the ligand binding. Nobody ever reports the quantitative relationship between SAXS, NMR, or ITC. It is not necessary to integrate the data from orthogonal techniques. Even if there is a quantitative relationship, it is going to be nonlinear and complex since the parameters for NMR is chemical shift changes per atom and SAXS shows the overall conformational changes. SAXS and NMR both provide aqueous structural information and we have demonstrated the ligand binding induced conformation changes from these two techniques. Since crystal structures of GlnBP in ligand free and bound state are available, we skip the generation of protein shape by SAXS and NMR structure determination.

2. The scattering of “Results” part was not acceptable, where sections were missing. The authors appeared to be random in narratively describing the results. Normally, sections of mutation selection, structure changes, correlation between xxx and yyy etc, should be in “Results”. For a biophysics study like this, a table of GlnBP protein of WT, mutant 1, mutant 2 … should be presented. This should be followed by structural characterization (NMR, SAXS, SV-AUC) at free and binding states of each protein. The authors should then infer from analytical results on the structural change of GlnBP at secondary, tertiary, quaternary structure levels. For example, NMR chemical shift could be sensitive to secondary and tertiary structure, relaxation could be sensitive to quaternary and oligomerization, SAXS could be sensitive to quaternary or domain structure, SV-AUC is for oligomerization. All of these standard reasonings are missing in the manuscript.

We thank reviewer for this valuable comment. We add section titles in the results part. We add one more section to introduce the design logic behind the mutants with different length of domain linker as the following:

**Generation of GlnBP mutants with different domain linker length**

We create N170C (Asn 170 was mutated to Cys) mutant and introduce extra cysteine at C terminal that will form disulfide as an artificial linker, which controls the magnitude of domain closure based on our structural analysis. The N170 and C terminal residues are located at the interface between two domains. Our hypothesis is that the linkage of these two residues will perturb the domain closure that will affect ligand binding affinity consequently. We introduce domain linker with 0 to 5 alanine that show different magnitude of restriction on domain closure. The linker composed of disulfide bond can be reversibly broken by reducing reagents.

We add a few sentences to each section of results to give detail explanation about the physical and biological meaning of the data.

We agree with the reviewer that correlation between xxx and yyy etc, should be in “Results”. We show the results in a reversible way. The mutants we generate can introduce a reversible linker. The ligand binding affinity is perturbed and restored after domain linker formation or breakage. Reversibility is a stronger evidence than correlation analysis. The biophysical measurements of diverse GlnBP mutants are not
complete between the structural changes are minimal and ligand binding is the most important property we are interested.

3. The 1st paragraph in "Results" belongs to method description. 
The first paragraph illustrates how we prepare and characterize ligand free GlnBP since GlnBP from cell lysate contain both ligand free and bound GlnBP. We treat the purified sample with detergent and remove the ligand. If the sample is a mixture of ligand free and bound format, the following binding assay will have measurement errors.

4. Page 2 Paragraph 1, the statement “two domain linked by a flexible “hinge” region” was not correct. We changed to “The 2 domains were linked by 2 rigid β-strands”

5. The method section missed the description of SV-AUC method.
We thank reviewer for pointing this out. We add one paragraph to describe the SV-AUC method as the following:

**Analytical ultracentrifugation**

Sedimentation-velocity experiments were performed at 20 °C in a Beckman XL-I analytical ultracentrifuge using an An50Ti rotor. Aliquots of protein and reference buffer were loaded into a sedimentation-velocity cell equipped with a dual-sector charcoal-Epon centerpiece. The reference buffer used for studies of the ligand-free protein was an eluted fraction lacking protein, as determined by A280, obtained from size-exclusion chromatography of the protein sample. The reference buffer for centrifugation of GlnBP in the presence of ligands was the buffer from dialysis. Following a 2-h temperature equilibration, the sample was centrifuged at 35,000 rpm. The radial distribution of the sample was monitored with Rayleigh interference optics. Data were acquired at 2-min intervals for 300 radial scans. The data set was analyzed globally to obtain the sedimentation coefficient (c(s)) and molecular mass (c(M)) distributions using Sedfit.

The manuscript by Chen et al. remained immature after the revision. Though a series of artificial linker mutants were generated, the mutant protein was only subject to ITC study, not NMR, SAXS or AUC. The NMR study on the same WT protein and ligand has been extensively studied by others before. The authors’ current studies were isolated on different proteins, and lacked quantitative results or correlation. This type of scattering in study was not reflected in “title”, which implied NMR and SAXS were applied on mutants, actually not. A table listing protein variants, methods and results were suggested to the authors, but authors did not take the suggestion. The main conclusion of longer linker introducing tighter binding between protein and ligand was commonly assumed in protein biochemistry because of less steric restriction. The study using ITC only verifies the common knowledge. A scientific approach should drive from the avenue of free energy landscape to interpret results and give more quantitative insight to molecular interaction. Due to the less quantitation and correlation, the manuscript did not deliver any robust new findings that merit the publication.

We really thank reviewer for this point, in our abstract, we want to approve hypothesis that the freedom of closure is vital for ligand binding, and our experiments approved this hypothesis. We change the manuscript title to “Reversible domain closure modulates GlnBP ligand binding affinity” that summarize the chief finding more appropriately. The mutants are variants of GlnBP with different linker length from 0-
5 alanine residues. Thus, we think it is not necessary to list these simple mutants with one more table. The reviewer comments that “The study using ITC only verifies the common knowledge”. Our manuscript is the first study to investigate the correlation between domain closure and ligand binding. ITC for ligand binding is a common knowledge. The usage of ITC in characterization of domain closure is novel. We thank review for the suggestion about energy landscape. Our focus is the relationship between domain closure and ligand binding affinity. Gibbs energy changes is a concrete demonstration or explanation of our conclusion. ITC and SAXS are direct measurements to approve the relationship between domain opening and ligand binding.