The University of Michigan

Department of Chemistry
Biophysics Program
2006 Chemistry
930 N. University Avenue
Ann Arbor, Michigan 48109-1055

Charles L. Brooks III, Ph.D.
Cyrus Levinthal Distinguished
Professor of Chemistry and Biophysics
Warner-Lambert/Parke-Davis
Professor of Chemistry
Professor of Biophysics

Phone	734/647-6682
Fax	734/6471604
E-mail	brookscl@umich.edu

August 6, 2018

Professor Ron Levy
Department of Chemistry
Temple University
Philadelphia, PA

Dear Ron,

Please find our revised manuscript entitled "Approaching Protein Design with Multisite λ Dynamics: Accurate and Scalable Mutational Folding Free Energies in T4 Lysozyme" (ID PRO-18-0114), co-authored by RL Hayes, JZ Vilseck and CL Brooks III.

We have revised the manuscript per the reviewer comments and provide a detailed response to the reviewers below. We hope that out paper is now acceptable for publication in Protein Science.

Sincerely,

Charles L. Brooks III
Cyrus Levinthal Distinguished University Professor
of Chemistry and Biophysics
Warner-Lambert/Parke-Davis Professor
of Chemistry
Professor of Biophysics
Professor of Chemistry
Chair of Biophysics

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Charles L. Brooks III
Cyrus Levinthal Distinguished University Professor
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Warner-Lambert/Parke-Davis Professor of Chemistry
Professor of Biophysics

Referee(s)' Comments to Author:

Reviewer 1

Comments to the Author
This is a high quality paper that is easy to referee. Lambda-dynamics is extended to the protein design problem of computing stabilities for a large library of lysozyme sequences, which differ at a few positions. Lambda-dynamics has improved over the years in important ways and it provides here accurate folding free energy differences for 240 lysozyme sequences using a single MD simulation. This is at the lower limit of bona fide protein design problems but the method has the obvious potential to scale further. The method opens new possibilities for protein design, providing high-accuracy, medium-throughput simulations that can be applied to a problem eg after a first, very high-throughput pass has been done with a less accurate design tool. Overall, the methodology is very original and important. The quality of the data and the discussion are high. The presentation is clear and complete. The paper is very well-suited to Protein Science.

Thanks for a favorable review. Your explanation of potential use cases is quite insightful.

Reviewer 2

Comments to the Author
This manuscript reports on the performance of a computational method for computing free energy differences known as multisite λ dynamics. Application is to predicting changes in protein stability upon mutation of side chains in T4 lysozyme, for which there is a wealth of data. A major advantage of MSλD is the need for only simulating the ends states and that the free energy can be computed for multiple sequences simultaneously. This allows the search of a much larger sequence space than possible with FEP, which is restricted to one site at a time and several intermediate simulations between the end states are needed. The multisite results are encouraging and a nice general discussion of the value of MSλD for protein design is presented. On the other hand, the Methods and Supplementary sections are highly technical and directed to someone already intimately familiar with MSλD. Nevertheless, because the methodology is directed toward protein design, and the results indicate accurate free energy differences can be obtained, the paper should be of interest to the general audience of Protein Science if the following points can be addressed in a revised ms.
A. The multiple site calculations are of most interest but some additional explanation is needed for how the calculation is done and what is the outcome. The results in Fig 2 are a major part of the work being reported, so that clarifying the points below would help the reader understand these results.

1. The sentence on p. 8 "The 3 site, 4 site and 5 site systems comprised 8, 24, and 240 sequences with 6, 14, and 9 experimental measurements at the same pH" needs to be explained. The 8, 24 and 240 is some combination of the mutants listed in table 3 but it's not clear how the number of sequences is determined. And, how is this very large number of sequences (8+24+240) connected to the much smaller number of experimental measurements (6+14+9)? The basis for these numbers needs to be spelled out a bit more.

We added more details to the introduction of these numbers on page 8, and note that we only compare the experimentally measured sequences with experiment.

2. What are the experimental data plotted in Figure 2 in the case of multisite mutations? The table in ref 49 with experimental data shows single mutation free energy changes. Double mutants are typically not the sum of single mutants, so what is compared for multisite FE

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of Chemistry and Biophysics

Warner-Lambert/Parke-Davis Professor of Chemistry
Professor of Biophysics

differences? Are the MSλD results somehow using FE values for only single sites relative to WT? Further, the figure is labeled “All multisite mutants” yet the description quoted above in #1 gives 8+24+240 sequences, which clearly is not the number of values plotted in figure 2. So what is meant by “all multisite mutants”?

While most of the mutations in ref 49 are point mutants, several dozen are multisite mutants. We have modified the text to mention we only compare with the experimentally measured sequences. We also changed the titles of the figures from “all multisite mutants” to “all multisite systems”.

3. How are the sites treated in a multisite MSλD run? It is my understanding that all targeted sites are scaled in a multidimensional λ space in a single run. If one residue has two or more substitutions (e.g. V111 in Table 3), are all of the amino acid types mutated in one run? How are the interactions handled between residues of different sites? For example, do mutants at residue 99 interact with mutants at residue 102 in the 4 site system?

We modified the single site section to mention all mutations at a site are present in the same simulation, and added the sentence “Interactions between side chains at different sites are scaled by the product of their λ variables, so mutating side chains at two sites only interact when they are both on, which allows MSλD to explicitly account for coupling between sites,” to the multisite section to address this confusion. So the answer to your question is yes, whichever side chain is on (with nonzero λ) at residue 99 will interact with whichever side chain is on at site 102.

4. What is set to zero in Figure 1 and 2? Presumably it is WT but it is not specified in the caption.

We added the phrase “free energies are plotted relative to wild type with C54T/C97A” to both captions.

5. How is convergence assessed for the MSλD results? Is there some measure of in terms of the evolution of λ?

Convergence is typically assessed through the statistical uncertainty in the 5 duplicate runs. Table S2 in the Supporting Information shows the lack of convergence that can occur if optimal biasing potentials are not used. Convergence is also touched on in Figure 3c since SSλD simulations should be more converged than MSλD simulations because fewer sequences need to be sampled. Temporal convergence is beyond the scope of this article, but we ran 20 to 40 ns because this is in the ballpark of what is conventional with MSλD (10 to 30 ns in recent publications).

B. The point that MSλD is more efficient than FEP is made repeatedly and argued in a descriptive manner with statements about the number of simulations for FEP vs MSλD, etc. The efficiency of MSλD makes sense, but could a more quantitative comparison be given, such as a rough estimate of the relative CPU time required for a large scale study using FEP compared to using MSλD?

No, a more quantitative comparison cannot be given without expending a lot of computer time. In another study of drug binding, we ran FEP on a small subset, roughly 7%, of the ligands studied with MSλD, but the FEP simulations took more time than the MSλD simulations. In that study a difference of a factor of 20-30 was estimated. The central issue is that one has to obtain free energy estimates of comparable precision on the same system, as some systems (e.g. small to large vs. large to small mutations in proteins) converge at different rates. We ran our simulations for roughly the same amount of time as

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standard FEP simulations, but obtained far better precision and accuracy. Running long enough FEP simulations to obtain that level of precision would be intractable. We added a long discussion to the SI and mention it in the main text.

C. The authors should make some kind of comparison between the mutant structures from MSλD with the crystal structures. In particular, there are structural changes noted in ref 49 associated with the L99A cavity. Such a comparison would be useful, particularly given that the authors speculate that relaxation of the structure influences their results.

This was the most difficult and most fruitful suggestion. We have added 3 pages to the SI and two paragraphs and a figure to the main text discussing structural relaxation in the context of solved structures. Ironically, the structural relaxations for L99 are quite rapid, but this is not the case for several of the other sites.

Some minor points on the written presentation are the following:
1. P. 5: “C54T/C97A background” should be defined

Done

2. Footnote to Table 2 is a squared quantity but the values are labeled root mean squared

Thanks, we have corrected the equation in tables 2 and 4

3. P. 10 last paragraph: “5/2 sequences” and “23/13 sequences.” What is the slash?

The slash denotes either FSWITCH or PME simulations. We have reworded the sentence to clarify.

4. P. 15 The first paragraph of section Prospects of MSλD for Protein Design is not clear. Suggest rewriting.

We have rewritten this section to clarify the difference between state-of-the-art protein design methods which are generative, and the FEP approach which is currently only accurate enough to perturb existing sequences.

5. P. 21-22 bottom. One sentence states only side chain dihedrals are scaled, yet there is discussion about phi and psi angles also being scaled. Please clarify.

Some dihedrals including C_β involve the same rotatable bond as the ϕ and ψ angles. We have clarified this point in the text.

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