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Dr. Susan Marqusee
Biophysics and Computational Biology Editor, *Proceedings of the National Academy of Sciences*

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Dear Dr. Marqusee,

Please find enclosed our manuscript titled "*Synonymous mutations can alter protein dimerization through localized interface misfolding involving self-entanglements*" that we are submitting to *PNAS* as a Research Report.

The ability for proteins to dimerize, as measured by K_{D}, has been shown to be influenced by synonymous mutations, which change the mRNA sequence but not the protein's primary structure. This is odd, because from the Anfinsen's Thermodynamic Hypothesis predicts that the same native structure should form regardless of the mRNA sequence – and hence the same binding affinities should occur. This indicates there are long-term changes in protein structure due to these synonymous mutations. For example, the protein FRQ's ability to post-translationally dimerize decreased by more than half when its mRNA sequence was "optimized" to translate more quickly (PMID: 23417067). The structural and kinetic origins of this phenomenon is unknown. In the enclosed manuscript we use multi-scale simulations of the synthesis and maturation of two *E. coli* homodimers to suggest the structural origins of this phenomenon, and verify it with new experimental data.

Our results indicate that synonymous mutations alter the probability a protein forms non-covalent lasso-entanglements that perturb the protein's dimer interface, leading to significant changes in its dimer affinity. These entangled states are near-native in structure, allowing them to bypass cellular quality control mechanisms, and are kinetic traps, as disentanglement of the misfolded state takes a long time – these characteristics indicate they can influence long-time-scale protein function.

We note that this manuscript was previously submitted to *PNAS* (Manuscript ID: 2021-17827), but was not reviewed. Since this prior submission, we have made significant updates to the manuscript. We now include explicit comparison of our coarse-grain simulation results to limited proteolysis mass spectrometry data generated in Stephen Fried's lab. In addition, we carried out additional simulations and computed potentials of mean force for key entangled protein conformations.

This manuscript represents a significant advance in our understanding of how proteins can retain "memory" of their translation kinetics long after synthesis is complete. In concert with our recent publications on this type of misfolding being common across the *E. coli* proteome (PMID: 35654797) and their influence on enzymatic activity (PMID: 36471044) of

entanglements, these results help explain widespread observations of the effects of synonymous mutations on protein structure and function from the past several decades of research in the fields of biochemistry, molecular biology, and biotechnology.

Reviewers with expertise in this area include:

(1) Kingshuk Ghosh (University of Denver) – Computationalist who has studied protein conformational space.

(2) William Jacobs (Princeton University) – Theorist with expertise in co-translational protein folding and protein misfolding.

(3) Robert Best (National Institute of Diabetes and Digestive and Kidney Diseases) – Theorist with expertise in coarse-grained simulations of proteins.

(4) Eugene Shaknovich (Harvard University) – Theorist who has studied co-translational protein folding.

(5) Yi Liu (University of Texas Southwestern Medical Center) – Experimentalist who demonstrated the FRQ protein can misfold to soluble but less-functional states that influence its ability to dimerize.

(6) Dave Thirumalai (University of Texas, Austin) – Theorist on protein folding.

Please do not hesitate to contact me if you require any additional information.

King regards,

Ed O’Brien