Theory, simulations, and experiments show that proteins fold by multiple pathways

William A. Eaton^{a,1} and Peter G. Wolynes^{b,c,1}

In PNAS (1), Englander and Mayne repeat an assertion, already made by them along with Bai and Sosnick in 1995 (2), that the modern energy landscape theory of protein folding, by emphasizing the parallelism of potential folding pathways, is incompatible with evidence that in some cases a single particular sequence of folding substructures dominates the set of folding trajectories. This assertion of the authors was false when made in 1995, and since then considerable collaborative work between the experimental and theoretical communities has made an irrefutable case against the notion that the only way proteins fold is by a single obligate pathway (ref. 3 and references therein), as implied in Englander and Mayne's article (1). Moreover, hydrogen–deuterium exchange experiments, of the type cited by Englander and Mayne (1) as being in conflict with theory, have in fact already been treated successfully using theories based on energy landscape ideas (4). Englander and Mayne (1) misrepresent or largely ignore thirty years of progress in protein-folding theory and appear almost dismissive of statistical mechanics and modern kinetics.

At an atomistic level, no two trajectories from the unfolded state to the folded state can possibly be identical, so there is an unimaginably large number of detailed pathways for folding a protein. Moreover, it is the rate enhancement from the combination of multiple microscopic paths and a bias toward native interactions intrinsic to a funneled landscape—not intermediates (5)—that resolve the Levinthal paradox (6). Englander and Mayne (1) acknowledge that multiple microscopic pathways are used to reach the

ensemble of transition-state structures, but then ignore this part of the transition paths, regarded by most as the critically important segment because reaching the transition state is rate-limiting. Even when describing pathways at a coarse-grained structural level, such as the order in which partial or complete substructures (or “foldons”) form, analyses of all-atom molecular dynamics simulations show that multiple pathways exist before and after the transition state and are in fact used for a large set of small proteins (see, for example, refs. 7 and 8).

Evaluating the number of different paths experimentally will require single-molecule measurements, which are still in their infancy. Nevertheless, the first experiments to address this issue already show multiple paths when intermediates are populated (9), and multiple paths even for two-state folding (10). As single-molecule experimental methods continue to improve in speed and structural resolution, doubtless multiple paths will be commonly observed.

There is no “clash” of theory and experiment, as suggested by Baldwin (5). Energy landscape theory includes the simple scenario of Englander and Mayne (1), while providing a much richer framework for analyzing protein folding (6) than does the purely verbal description set forth in the PNAS report (1), which by itself is incapable of making quantitative predictions for any protein. In sharp contrast, a large variety of folding mechanisms can be understood using the quantitative predictions of the landscape paradigm (6), which have been confirmed in the laboratory (3, 9, 10) and supported by all-atom simulations (7, 8, 11).

¹ Englander SW, Mayne L (2017) The case for defined protein folding pathways. *Proc Natl Acad Sci USA* 114:8253–8258.

² Bai Y, Sosnick TR, Mayne L, Englander SW (1995) Protein folding intermediates: Native-state hydrogen exchange. *Science* 269:192–197.

³ Wolynes PG, Eaton WA, Fersht AR (2012) Chemical physics of protein folding. *Proc Natl Acad Sci USA* 109:17770–17771.

⁴ Craig PO, et al. (2011) Prediction of native-state hydrogen exchange from perfectly funneled energy landscapes. *J Am Chem Soc* 133:17463–17472.

⁵ Baldwin RL (2017) Clash between energy landscape theory and foldon-dependent protein folding. *Proc Natl Acad Sci USA* 114:8442–8443.

^aLaboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; ^bDepartment of Chemistry, Rice University, Houston, TX 77005; and ^cCenter for Theoretical Biological Physics, Rice University, Houston, TX 77005

Author contributions: W.A.E. and P.G.W. wrote the paper.

The authors declare no conflict of interest.

Published under the PNAS license.

¹To whom correspondence may be addressed. Email: eaton@helix.nih.gov or pwolynes@rice.edu.

- 6 Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG (1995) Funnels, pathways, and the energy landscape of protein folding: a synthesis. *Proteins* 21:167–195.
- 7 Henry ER, Best RB, Eaton WA (2013) Comparing a simple theoretical model for protein folding with all-atom molecular dynamics simulations. *Proc Natl Acad Sci USA* 110:17880–17885.
- 8 Best RB, Hummer G (2016) Microscopic interpretation of folding ϕ -values using the transition path ensemble. *Proc Natl Acad Sci USA* 113:3263–3268.
- 9 Zoldák G, Rief M (2013) Force as a single molecule probe of multidimensional protein energy landscapes. *Curr Opin Struct Biol* 23:48–57.
- 10 Guinn EJ, Jagannathan B, Marqusee S (2015) Single-molecule chemo-mechanical unfolding reveals multiple transition state barriers in a small single-domain protein. *Nat Commun* 6:6861.
- 11 Best RB, Hummer G, Eaton WA (2013) Native contacts determine protein folding mechanisms in atomistic simulations. *Proc Natl Acad Sci USA* 110:17874–17879.