LETTER

The importance of being quantitative

A recent PNAS article (1) presents single-molecule fluorescence (SMF) data on the microsecond folding protein BBL, showing a bimodal FRET efficiency distribution near the denaturation midpoint, which is interpreted as direct evidence of two populations of molecules (folded and unfolded) separated by a free-energy barrier. This contrasts with our previous conclusion that BBL folds downhill (a single population that shifts during unfolding) based on quantitative analysis of a wealth of thermodynamic and kinetic data (see, for example, ref. 2). More surprisingly, we have independently performed SMF experiments on BBL and see a sharp unimodal distribution that shifts from high to low FRET efficiencies at increasing chemical denaturant (ref. 29 in ref. 1).

The only apparent differences between SMF experiments are that we use less bulky dyes, attach them through short flexible linkers, and employ a new photo-protection cocktail that we purposely developed to achieve high photon fluxes with minimal photobleaching. Huang et al. (1) argue that our SMF experiments do not resolve the two peaks because the thresholds are lower and the flexible linkers decrease the dynamic range in FRET efficiency. However, this argument is incorrect. Our photon fluxes and thresholds are higher (we quoted means, not peak values) and with much less photodamage. The dynamic range in FRET efficiency is equivalent with $\approx$0.45 change between water and high denaturant. Furthermore, at the denaturation midpoint, our FRET efficiency distribution is a single peak with near shot-noise limited width even at thresholds $>$100; and there is good quantitative agreement with the ensemble experiments on the labeled and unlabeled proteins

At first glance, the SMF data presented by Huang et al. (1) seem to agree with barrier-limited folding, but quantitative analysis reveals serious inconsistencies. Their experiments use highly saturating illumination intensities for their FRET acceptor (A647) (3) and are dominated by photobleaching. There is severe broadening relative to shot-noise, which only seems to affect the "unfolded" peak. Thus, the actual denaturation midpoint obtained by peak integration greatly differs from visual estimates, and is off by $\approx$1 M denaturant relative to the bulk data. Another important issue has to do with threshold and binning-time effects. In these experiments, the sub-millisecond folding/unfolding of BBL should result in some dynamic averaging, the same phenomenon known as chemical exchange in nuclear magnetic resonance. When this effect is properly quantified by using the Gopich-Szabo theory (4) and all the relevant numbers (thresholds, binning times, apparent diffusion time, and BBL folding rate), it turns out that the folded and unfolded peaks should merge into a single broad peak as threshold and binning time increase in Huang et al.'s Fig. 4 (1). However, the two peaks follow the opposite trend, showing no signs of being in conformational exchange at any rate faster than $\approx$1/(3 milliseconds).

These observations strongly suggest that the two populations represent undesired heterogeneity in their SMF experiments rather than barrier-limited folding. Therefore, no conclusion can be extracted from their data before all of these issues are properly sorted out, proving once more that distinguishing between downhill and barrier-limited folding requires thorough quantitative analysis rather than a rule of thumb.

***Luis Alberto Campos*[a], *Jianwei Liu*[b], *and Victor Muñoz*[a,1]**
[a]*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Ramiro de Maeztu 9, Madrid 28040, Spain; and* [b]*Department of Chemistry, Stanford University, Stanford, CA 94305-5080*

1. Huang F, Ying L, Fersht AR (2009) Direct observation of barrier-limited folding of BBL by single-molecule fluorescence resonance energy transfer. *Proc Natl Acad Sci USA* 106:16239–16244.
2. Li P, Oliva FY, Naganathan AN, Muñoz V (2009) Dynamics of one-state downhill protein folding. *Proc Natl Acad Sci USA* 106:103–108.
3. Kong X, Nir E, Hamadani K, Weiss S (2007) Photobleaching pathways in single-molecule FRET experiments. *J Am Chem Soc* 129:4643–4654.
4. Gopich IV, Szabo A (2007) Single-molecule FRET with diffusion and conformational dynamics. *J Phys Chem B* 111:12925–12932.

Author contributions: V.M. designed research; L.A.C. and J.L. performed research; L.A.C., J.L., and V.M. analyzed data; and V.M. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: vmunoz@cib.csic.es.