LETTER

Reply to Huang et al.: Slow proton exchange can duplicate the number of species observed in single-molecule experiments of protein folding

In contrast to the implications of the letter by Huang et al. (1), our previous work unambiguously showed that the protein BBL under denaturing conditions is a single, partly unfolded state (one-state folding) rather than a mix of folded and unfolded molecules (two-state folding) (2). This conclusion was based on ultrahigh-resolution single-molecule (sm) FRET experiments (>1 photon per microsecond) performed on a long BBL construct at pH 6 and using urea as denaturant. We also demonstrated analytically that the single peak in the FRET histograms of BBL could not be explained as two rapidly interconverting states (2).

Additionally, we reported supplementary bulk and smFRET data on the shorter BBL variant of Huang et al. (QNND-BBL), mentioning discrepancies with their guanidinium chloride (GdmCl) denaturation results (2). For these experiments, we used phosphate buffer instead of Mops. We have confirmed that the source of the discrepancy was the difference in buffers, as stated by Huang et al. (1). However, this issue does not affect in any way our original results and conclusions, which were based on different data. We also disagree with the assertions by Huang et al. about good and bad buffers for GdmCl denaturation and with the presumption that their reported bimodal smFRET histograms (1, 3) demonstrate that BBL folds in a two-state fashion.

The experiments that are ambiguous are, in fact, those of Huang et al. (1) because of the choice of GdmCl as denaturant near-neutral pH. Multimolar GdmCl solutions greatly alter the pK_a of all buffers. The effect goes opposite ways for acidic and amine buffers because ionic strength shifts ionization equilibria toward the charged species. They miscalculate these effects by ignoring that glass electrodes underestimate the true pH of concentrated GdmCl solutions (4). The corrected pH-meter scale (4) shows that, in concentrated GdmCl, 20 mM phosphate buffer initially set to pH 7 levels at pH ~6.2, whereas 50 mM Mops also set to pH 7 reaches values close to pH 8.5 (Fig. 1*A*). Therefore, Mops is actually inferior to phosphate as a buffer for protein denaturation by GdmCl.

The generic pH shifts induced by GdmCl are often ignored because most proteins do not titrate at neutral pH (4). However, BBL unfolding shows unusually strong pH dependence between pH 6.5 and 9 (figure 1b in ref. 1), which means its unfolding is tightly coupled to protonation of some group titrating in that range. BBL stabilization above neutral pH is indeed quite dramatic. Fig. 1*B* shows that the true stability at pH 7 is significantly lower than that measured with Mops buffer (which roughly corresponds to pH 8; Fig. 1*A*). Because the two histidines and all acidic residues of BBL titrate at lower pH (5), there must be a basic group with highly reduced pK_a in the native structure (large destabilization by protonation). In the conditions of Huang et al. (1), both pH and BBL protonation are moving targets, and proton transfer is slow (in contrast to pH 6). Consequently, the two peaks of their smFRET histograms (1, 3) might very well be two slowly interconverting protonation species of a partly unfolded protein, a scenario quite different from the authors' interpretation of conventional folded and unfolded states.

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The authors declare no conflict of interest.

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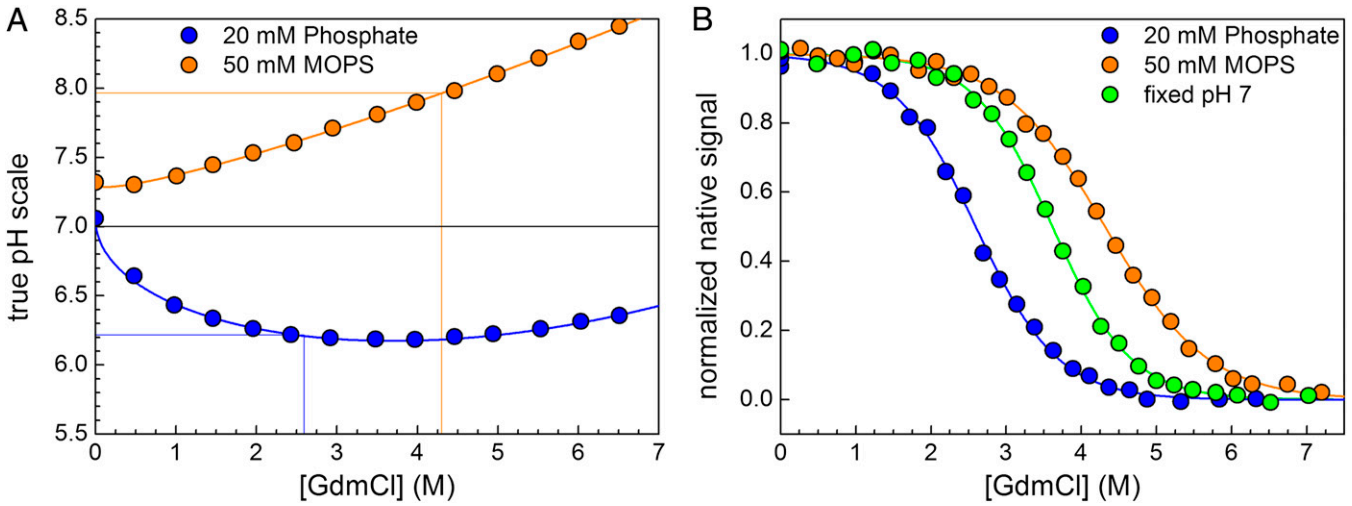

Fig. 1. Effects of GdmCl on the pH of buffered solutions and on the stability of QNND-BBL. (*A*) True change in pH [after proper correction of the pH-meter glass-electrode reading (4)] induced by GdmCl on 50 mM Mops buffer (orange circles) and 20 mM phosphate buffer (blue circles). Both buffers were prepared adding NaCl to an ionic strength of 250 mM and adjusting the pH to 7 (at 298 K) before mixing with GdmCl. The C_m of QNND-BBL on each buffer is indicated with a box of equivalent color. (*B*) GdmCl denaturation of QNND-BBL using 50 mM Mops buffer (orange), 20 mM phosphate buffer (blue), and 50 mM phosphate buffer in which the pH was adjusted to 7 at every concentration of GdmCl using the proper correction of the glass-electrode reading (green). In the three experiments NaCl was added to set the final ionic strength to 250 mM.