

TECHNICAL COMMENT

DISORDERED PROTEINS

Comment on “Innovative scattering analysis shows that hydrophobic disordered proteins are expanded in water”

Robert B. Best^{1*}, Wenwei Zheng², Alessandro Borgia³, Karin Buholzer³, Madeleine B. Borgia³, Hagen Hofmann⁴, Andrea Soranno⁵, Daniel Nettels³, Klaus Gast⁶, Alexander Grishaev⁷, Benjamin Schuler^{3,8*}

Riback *et al.* (Reports, 13 October 2017, p. 238) used small-angle x-ray scattering (SAXS) experiments to infer a degree of compaction for unfolded proteins in water versus chemical denaturant that is highly consistent with the results from Förster resonance energy transfer (FRET) experiments. There is thus no “contradiction” between the two methods, nor evidence to support their claim that commonly used FRET fluorophores cause protein compaction.

Riback *et al.* (1) recently presented a “molecular form factor” (MFF) method addressing the well-known challenges (2) of analyzing small-angle x-ray scattering (SAXS) data for unfolded or intrinsically disordered proteins (IDPs) (3, 4). Combined with the precision of SAXS measurements coupled to size exclusion chromatography, their method yielded the following results: (i) Unfolded proteins in water have a polymer scaling exponent $\nu \approx 1/2$, near the theta-solvent condition where protein-protein and protein-solvent interactions are balanced; in denaturant, this increases to $\nu \approx 3/5$, the limit where the protein-solvent interactions dominate. (ii) This change of scaling exponent is accompanied by an increase in radius of gyration (R_g) of 10% to 20%, depending on the sequence. We are pleased that these findings are in overall agreement with SAXS and Förster resonance energy transfer (FRET) studies from our laboratories (3, 5, 6) and others (4).

The chain expansion observed by Riback *et al.* helps to resolve a long-standing controversy between SAXS and FRET experiments (7): With increasing denaturant concentration, FRET ex-

periments had generally shown chain expansion, while until recently (3, 4) most SAXS studies observed no statistically significant change of R_g [(8) and references therein]. Their results are consistent with our recent collaborative study in which we compared SAXS and FRET estimates of R_g for each of two proteins (necessary because chain dimensions can be sequence-dependent). We found that the results are mutually consistent if both data types are analyzed with state-of-the-art methods (3, 6) (Fig. 1). A second study of a large group of IDPs reached a similar conclusion (4).

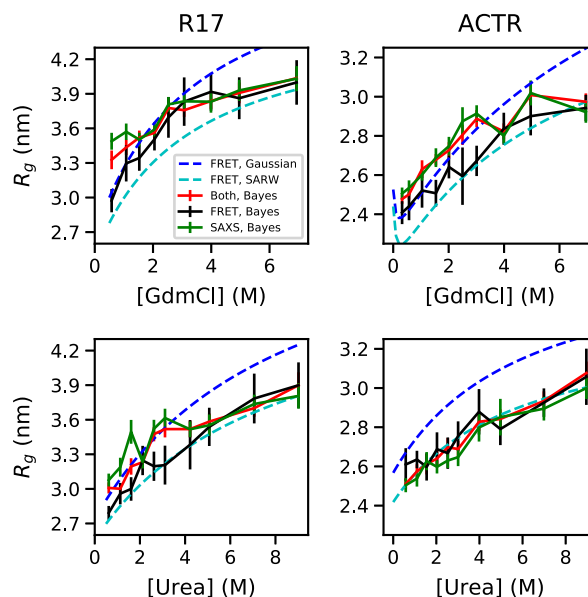
The main reasons for the discrepancy were deficiencies in the analysis of both SAXS and

FRET data. Earlier SAXS experiments underestimated expansion because the unfolded state of the foldable sequences studied could only be accessed above a certain denaturant concentration (3), as also pointed out by Riback *et al.*, and because obtaining precise and accurate R_g values from SAXS data of IDPs using the Guinier approximation is challenging (1, 3, 4). The former difficulty has been overcome by studying destabilized or intrinsically disordered proteins (1, 3, 4), the latter by improved analysis such as Bayesian ensemble refinement (3, 4, 6, 9) or the closely related MFF method (1). On the FRET side, the use of polymer models, such as a Gaussian chain or self-avoiding random walk (SARW), to interpret experimental results can overestimate the change in R_g (3, 10, 11) (Fig. 1), largely because the relative change of R_g upon chain expansion is intrinsically less than that of the end-to-end distance most commonly measured by FRET (3, 4, 10, 11). With ensemble refinement applied to either SAXS or FRET, or both combined, the data from both experiments yield consistent distributions of conformations, considering statistical error (3, 4, 6), as shown in Fig. 1 (3).

We therefore dispute the authors’ claim that their results are “in apparent contradiction to a variety of FRET measurements,” given that FRET experiments have not been reported on the sequences they studied. Their results are consistent with the magnitude of the change in both R_g and ν with denaturant inferred from recent FRET studies (3, 4), including the larger change in R_g and ν below 2 M GdmCl (Fig. 2A). Their ν values of 0.48 to 0.54 in water or low denaturant concentration are within the range (reflecting sequence-dependent variation of ν) obtained on the basis of ensemble refinement of data from previous FRET studies (3–6) (Fig. 2B).

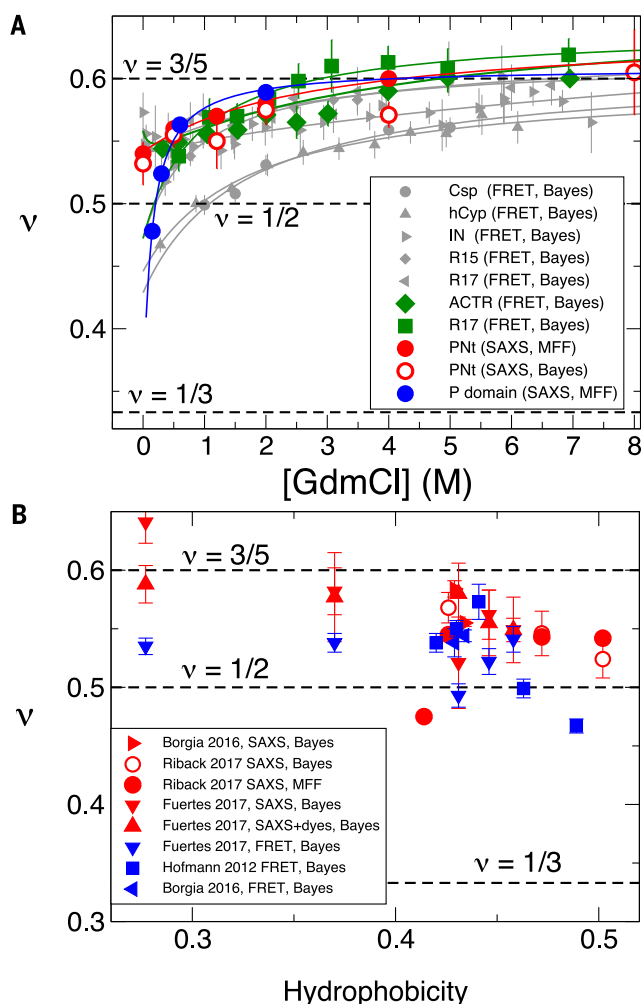
Despite this consistency, the authors suggest that “addition of fluorophores with hydrophobic character may lead to chain compaction and may

Fig. 1. R_g from Bayesian ensemble refinement against experimental data for unfolded proteins in denaturant using FRET, SAXS, or both experiments. Results are shown for two proteins (ACTR, R17) in urea and guanidinium chloride (GdmCl) (3). R_g values from FRET using Gaussian chain or SARW models (3) are shown for reference.



¹Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892, USA. ²College of Integrative Sciences and Arts, Arizona State University, Mesa, AZ 85212, USA. ³Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland. ⁴Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel. ⁵Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA. ⁶Physical Biochemistry, University of Potsdam, 14476 Potsdam, Germany. ⁷National Institute of Standards and Technology and Institute for Bioscience and Biotechnology Research, Rockville, MD 20850, USA. ⁸Department of Physics, University of Zurich, 8057 Zurich, Switzerland. *Corresponding author. Email: robert.best2@nih.gov (R.B.B.); schuler@bioc.uzh.ch (B.S.)

Fig. 2. Polymer scaling exponents, ν , for unfolded or disordered proteins. (A) Denaturant-dependent ν from SAXS data of Riback *et al.* for PNT (red) and P domain (blue) (1) compared with those from FRET data for a variety of unfolded and disordered proteins (gray) (5) and for the IDP ACTR and a destabilized spectrin R17 domain (green) (3). Exponents were obtained from Bayesian ensemble refinement (3) of primary FRET or SAXS data ("Bayes") or MFF analysis where indicated. Curves are fits to a binding model (5) or to a polyelectrolyte model for IN and ACTR (3). (B) Scaling exponents versus Kyte-Doolittle hydrophobicity (15) (rescaled between 0 and 1) for the same proteins in water or low denaturant concentration, as well as additional data for a set of IDPs in water from Fuertes *et al.* (4). Results for MFF (1) and Bayesian ensemble refinement (3) are highly consistent. Error bars indicate statistical error.



contribute to FRET signal changes" (1). Although some extremely hydrophobic FRET fluorophores can indeed cause additional compaction under native conditions, ensemble refinement identified the inconsistency between the resulting FRET and SAXS data (3). However, results for the more hydrophilic fluorophores most commonly used were in good agreement with SAXS data (3) (Fig. 1). Furthermore, a recent tour-de-force SAXS study of proteins with and with-

out fluorophores showed only small perturbations and no systematic changes of R_g and ν upon labeling (4) (Fig. 2B). The evidence presented by Riback *et al.* to support their claim comes not from a protein but from earlier small-angle neutron scattering (SANS) and FRET measurements on polyethylene glycol (PEG) (12). PEG lacks complications from a folded state, such as those that previously (13) caused these authors to overlook ubiquitin expansion (1). The PEG study,

however, used old protocols to analyze the data. Applying such earlier methods to a protein lacking a folded state, the authors had determined that "fully reduced ribonuclease A does not expand at high denaturant concentration" (14), but they now find an expansion for the same protein [figure 3C in (1)].

Riback *et al.* thus do not provide a convincing basis for their assertion that the conclusions of FRET and SAXS experiments are contradictory. Rather, their results add to the increasingly consistent picture of unfolded and intrinsically disordered proteins that has been emerging in recent years.

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