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Differential Dynamical Effects of Macromolecular Crowding on an Intrinsically Disordered Protein and a Globular Protein: Implications for In-Cell NMR Spectroscopy

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In-cell NMR[1–3] provides information about how the crowded environment in cells, where the concentration of macromolecules reaches hundreds of grams per liter,[4] affects protein structure and dynamics. Several successes, including target protein overexpression in *Escherichia coli* [1,5–9] and injection of isotope-enriched protein into *Xenopus laevis* oocytes,[10,11] have been reported, but in-cell NMR remains in its infancy, and several potential problems need to be addressed. One problem is protein leakage from the cell during the experiment.[12–14] When this occurs, sharp signals from the protein molecules in the less viscous media mask the broader signals from the protein molecules in the more viscous cytosol. Here we examine two proteins. The intrinsically disordered protein, α-synuclein (αSN, ~14 kDa), does not leak and is observed by in-cell NMR. The globular protein, chymotrypsin inhibitor 2 (CI2, ~7 kDa),[15] leaks, and the remaining intracellular CI2 is not detectable. We show that the difference in detectability between αSN and CI2 is consistent with a differential dynamical response to macromolecular crowding.

Figure 1A shows the ^{15}N–^{1}H HSQC spectrum of an in-cell NMR experiment on αSN. The spectrum is consistent with that from previous studies.[9,16] Figure 1B shows the spectrum from the supernatant collected immediately after sample preparation. Only metabolite signals[17] are observed. Figure 1C shows the spectrum from the supernatant recovered after the in-cell NMR experiment. Again, only metabolites are observed. The data demonstrate that the αSN spectrum in panel A comes from αSN in the cell. We have obtained similar results with the intrinsically disordered protein FlgM.[8] We performed the same experiments with CI2 expressing cells. In contrast to αSN, all three spectra are nearly identical (Figure 1E–G) (and typical of a CI2 spectrum[18] in dilute solution). These data suggest that CI2 leaks from the cells. SDS-PAGE confirms that ~20% of the CI2 is lost from cells.

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Supporting Information Available: Material and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

Encapsulation in alginate microcapsules[19] stabilizes cells[20] and may prevent leakage. To test if encapsulation might be useful for in-cell NMR, we first tried αSN-expressing cells. The encapsulated cells yield a typical αSN spectrum (Figure 1D), proving that encapsulated cells can provide useful in-cell spectra.

We repeated the experiment with CI2-expressing cells. No CI2 signal was observed even though we increased the sensitivity by accumulating the data for a longer time compared to the other samples (Figure 1H). However, a typical CI2 spectrum was recovered after dissolving the encapsulates with EDTA (data not shown). These observations suggest that the signal from the intracellular CI2, which we know is present in detectable amounts, is too broad to observe. We reasoned that the broadening arises from an alteration in the dynamics of CI2, either from binding a larger species in cells or from the higher viscosity of *E. coli* cytoplasm, which can be 10–11 times that of water.[21,22]

Why would the intrinsically disordered proteins αSN and FlgM react differently compared with a globular protein CI2 to the increased viscosity in cells such that we detect αSN and FlgM, but not CI2? The ability to detect a protein by high-resolution NMR depends on its dynamics, which are affected by viscosity. In terms of NMR, dynamics are reflected in the relaxation rates, R_1 and R_2 of the observed nuclei.[23] If R_1 is too small, the nuclei do not relax between pulses, lowering the sensitivity of the experiment. If R_2 is too large, the resonances are too broad to detect. In general, smaller proteins, flexible proteins, and proteins in less viscous solutions exhibit larger R_1 values and smaller R_2 values than do larger proteins, ordered proteins, and proteins in viscous solutions.

To test the idea that R_1 and R_2 for αSN and CI2 react differently, we studied the response of the proteins to viscosity increases induced by the macromolecular crowding agent poly (vinylpyrrolidone) (PVP, 300 g/L, 40 kDa average molecular weight). We used PVP because it is soluble, has protein-like properties, and does not interact strongly with proteins.[24] Figure 2 shows the relaxation rates of backbone ^{15}N nuclei of αSN and CI2 in dilute buffer and in PVP solution.

Figure 2A shows the R_1 values in buffer and in PVP. For most positions, the values for αSN changed little in buffer compared to 300 g/L PVP, even though the viscosity of the PVP solution is >50 times that of the dilute solution. Values from CI2, however, decrease 3–4 fold in PVP compared to dilute solution. The differential viscosity-induced decrease in R_1 values for CI2 compared to αSN would make it more difficult to detect CI2 in cells, consistent with our observations (Figure 1).

Figure 2B shows the R_2 values. For αSN, R_2 increased between 1.5- and 6-fold in PVP compared to buffer, while the values for CI2 increases between 3- and 40-fold. The increases for CI2 compared to αSN under crowded conditions would also make it more difficult to detect CI2 in cells, again consistent with our observations (Figure 1). These changes in R_1 and R_2 for CI2 are not caused by aggregation of the protein in PVP because NMR-detected diffusion experiments are consistent with a monomeric protein (Supporting Information). In summary, our data show that the ordered globular protein CI2 is more sensitive to viscosity than the intrinsically disordered protein αSN and that this increased sensitivity is expected to degrade spectra for ordered proteins in cells.

The atomic-level explanation of these differential effects lies in differences in global and local motions for ordered and disordered proteins. Because of their rigidity, the relaxation rates for globular proteins are most sensitive to global motion, which is described by a single rotational correlation time.[23] Disordered proteins, on the other hand, are flexible. Their motions are best described by considering an ensemble of interconverting conformers where every residue has a different effective correlation time.[25] In essence, the flexibility of disordered proteins lessens the deleterious effect of viscosity on their spectra.

To the best of our knowledge, this is the first report of a differential dynamical response of disordered and ordered proteins to macromolecular crowding. Our data suggest that it will be easier to detect in-cell signals from disordered proteins compared to ordered proteins and that a focus on flexible side chains will be advantageous for in-cell NMR of ordered proteins.[26] Because the cytoplasm of eukaryotic cells is less viscous than that of *E. coli* cells,[27] our observations imply that high resolution in-cell protein NMR data may be easier to acquire in eukaryotic cells.

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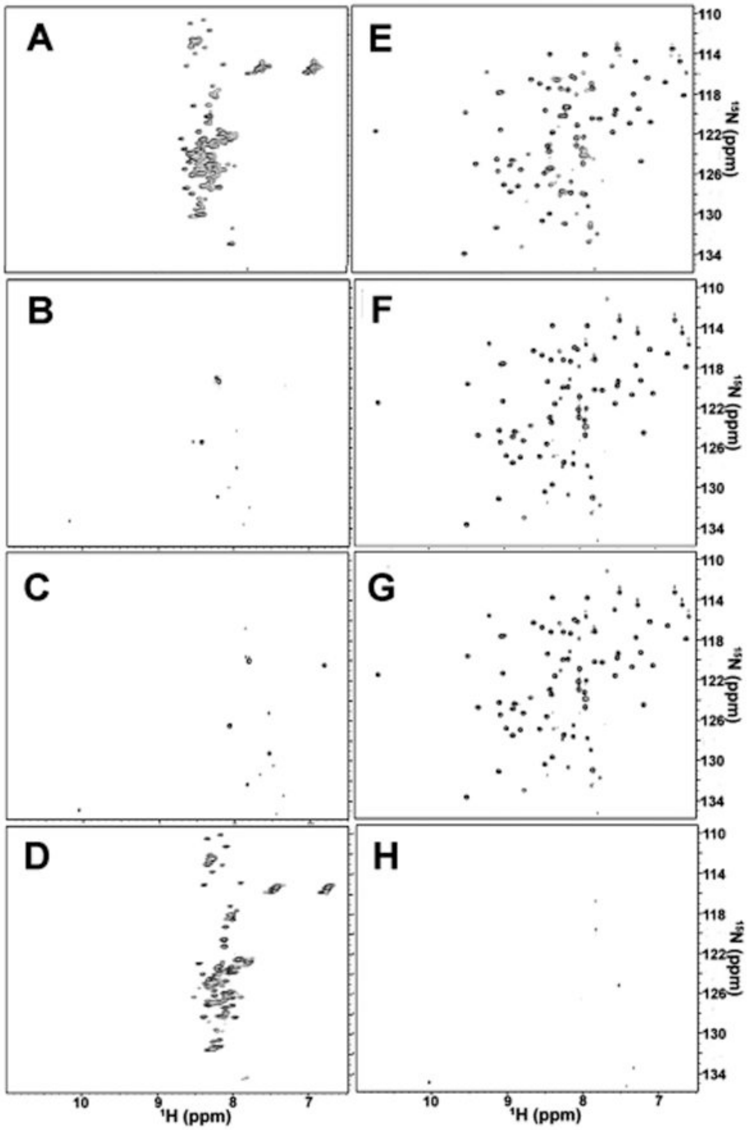

Figure 1.

^{1}H–^{15}N HSQC spectra of αSN (left panels) and CI2 (right panels): (A, E) in-cell spectra; (B, F) spectra of supernatants collected immediately after preparing the cells; (C, G) spectra of supernatants collected immediately after completing the spectra; (D, H) spectra from encapsulated cells.

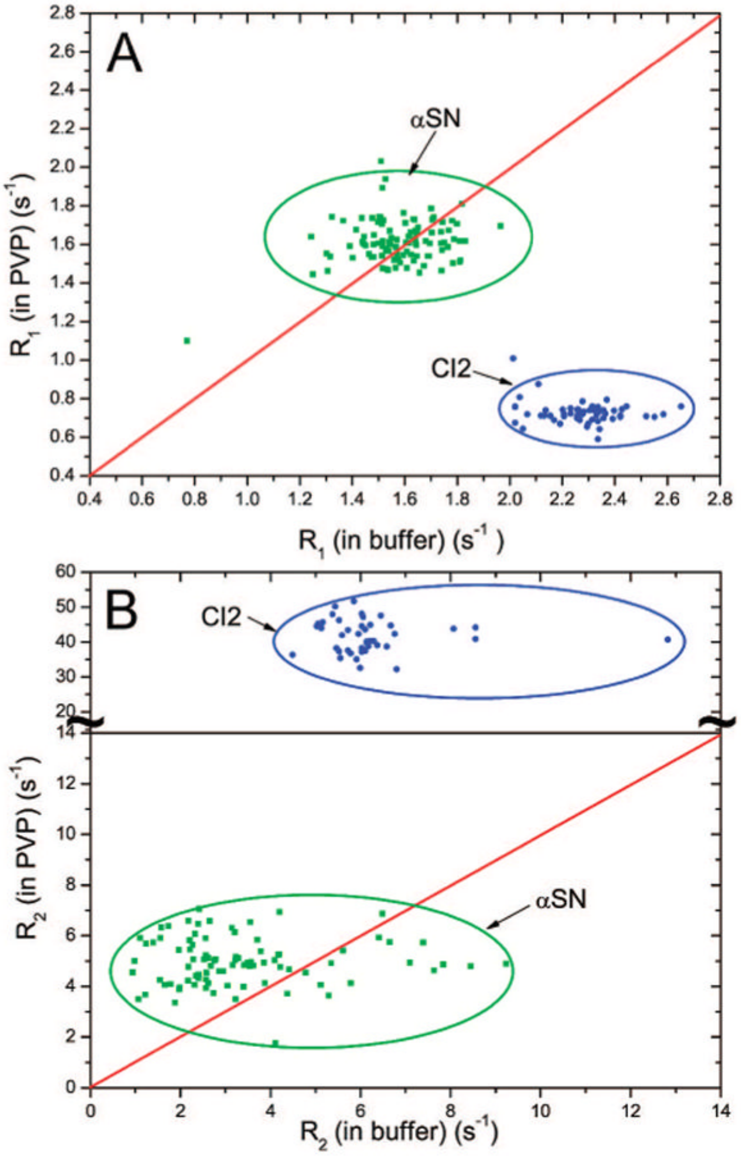

Figure 2.

R_1 (A) and R_2 values (B) of αSN and CI2 in 300 g/L PVP and in dilute solution (25 °C). The red lines indicate a unitary slope.