J|A|C|S COMMUNICATIONS

Published on Web 05/11/2009

Use of Protonless NMR Spectroscopy To Alleviate the Loss of Information Resulting from Exchange-Broadening

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Solution NMR spectroscopy plays a pivotal role in chemical biology to provide unique structural and dynamical information about biomolecules at atomic resolution.[1] Spurred by recent technical and methodological developments, NMR studies have begun to provide detailed structural characterization of protein complexes with molecular weights approaching 10^6 Da.[2] These achievements rely on the ability to assign resonances of individual backbone amide groups whose hydrogen atoms are exchangeable with bulk solvent. In cases of flexible or disordered loop regions, such exchange reactions may be fast enough to cause severe line-broadening of the amide ^{1}H resonances, resulting in a loss of correlations to the attached ^{15}N nuclei and hence of the corresponding structural and chemical information. The issue is particularly acute in the case of intrinsically disordered proteins (IDPs)[3] for which NMR spectroscopy is uniquely valuable as a means of characterization. Moreover, in addition to effects associated with solvent exchange, abundant conformational fluctuations can also give rise to severe exchange broadening, notably for partially folded states of proteins whose characteristic is of great importance in the definition of folding or misfolding phenomena.

One of the IDPs studied in great detail by solution NMR techniques is human α-synuclein (αSyn), the fibrilar aggregates of which are the main constituent of Lewy bodies, the cellular hallmark of Parkinson's disease.[4,5] Structural information about αSyn has largely come from measurements of NMR chemical shifts, ^{15}N spin-relaxations, residual dipolar couplings (RDCs), and paramagnetic relaxation enhancements (PREs),[6] all of which are dependent on the availability of resolved amide ^{1}H−^{15}N correlations. Under physiological conditions, neutral pH and body temperature, however, αSyn exhibits a drastic loss of observable backbone amide resonances that renders inaccessible spectral information about the first 100 of its 140 residues (Figure 1; left panel), which has been attributed to rapid hydrogen exchange.[7] As a result, most NMR studies on IDPs and αSyn, in particular, have been carried out at low temperatures and, in some cases, acidic conditions, in order to reduce the exchange rates sufficiently to obtain high-resolution spectral information.[8] Temperature, however, is a key parameter for governing the folding processes associated with a given protein that in part determine the aggregation propensity, oligomerization, and further fibrilization. Indeed, αSyn exhibits temperature-sensitive aggregation propensity rendering no observable amyloid formation at 288 K over a prolonged period of incubation (Figure 2), suggesting that aggregation-prone species may not be sufficiently populated at low temperatures, rendering structure-based rationalizations of the experimentally observed aggregation properties inadequate. Consequently, there is an urgent call for experiments to probe the physicochemical properties of IDPs in vivo and to rationalize in vitro data with in vivo findings.[9]

We describe here the application of protonless NMR spectroscopy to αSyn to demonstrate its power under biological-relevant conditions when proton-based NMR spectroscopy is limited by

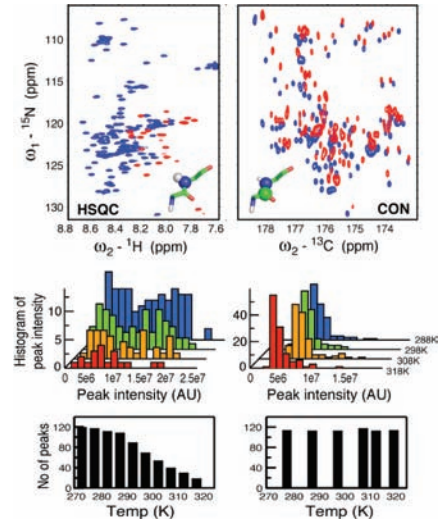

Figure 1. Comparison of overlaid ^{1}H−^{15}N HSQC (left panel) and CON spectra (right panel) of αSyn recorded at 288 K (blue) and 318 K (red) (top). Histograms of peak intensities (middle) and the numbers of observed crosspeaks (bottom) are shown as a function of temperature, illustrating the gain of information by using ^{13}C detection.

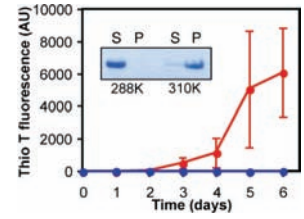

Figure 2. Aggregation kinetics of αSyn at 310 K (red) and 288 K (blue) monitored by the fluorescence of thioflavin T over six days (in triplicates). Inset: SDS-PAGE analysis showing that, while αSyn remains in the soluble fraction (S) at 288 K after six days, it is mostly found in the pellet (P) as fibriliar aggregates at 310 K.

exchange effects. Protonless NMR spectroscopy was initially developed for proteins that contain paramagnetic centers where direct detection of ^{13}C, which has a low gyromagnetic ratio compared to ^{1}H nuclei, helps to reduce undesirable relaxation losses.[10] The ^{13}C detection method has recently been applied to IDPs where well-resolved ^{13}C−^{15}N correlation CON spectra could be obtained,[11] thanks to the large chemical shift dispersion of backbone carbonyl carbons; indeed, complete backbone assignments of αSyn were determined by using a suite of protonless NMR experiments,[10] but at a low temperature.

In this study, we have used this approach to follow well-resolved backbone ^{13}C−^{15}N correlations of αSyn in CON spectra, recorded at temperatures from 278 to 320 K (Figure 1, right panel). Monitoring the chemical shift changes reveals a general nonlinear response with the sample temperature, giving rise to marked discontinuity at about 298 K for amide nitrogen flanking the

10.1021/ja902307q CCC: $40.75

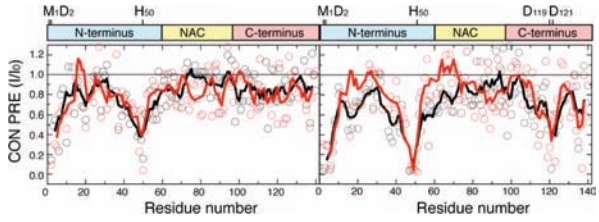

Figure 3. CON-based PRE of αSyn in the presence of 40 (left) and 400 μM (right) Cu(II). Data obtained at 283 and 310 K are shown in black and red, respectively. The PREs for individual residues are shown in open circles and running averages with a window size of five data points in thick lines. Regions that correspond to the N-terminus, NAC, and C-terminus are indicated at the top of this figure, with additional labels that indicate locations of the primary (M_1 and D_2), secondary (H_{50}), and very low affinity (D_{119} and D_{121}) binding sites.

hydrophobic NAC region (residues 40−70) and carbonyl carbon in the first helical region (residues 15−30) of αSyn (Figure S2); from this point (298 K) and above, the majority of the amide ^{1}H−^{15}N correlations become unobservable using conventional proton detection methods (Figure 1; left panel). These data, and those derived from resolved Cα and Cβ resonances in CBCACO experiments[10] (Figures S3 and S4), indicate that, at higher temperatures, αSyn undergoes significant conformational rearrangements, particularly in the C-terminal region, which is likely to result in a more elongated, that is, more expanded, conformation as has been shown previously[6i] leading to the exposure of the NAC region that is at the origin of αSyn aggregation (Figure S4). This is in line with the previous finding that polyamine binding at the C-terminus of αSyn accelerates the aggregation process.[6b] Importantly, our results underline the notion that αSyn does not aggregate at lower temperatures most likely because of the structural compaction which impedes the intermolecular contacts associated with the exposure of the NAC region (Figure 2).

We next set out to obtain PRE data that is most amenable to relate the global structural properties at a residue-specific level by monitoring relaxation effects induced by the binding of paramagnetic Cu(II) ions to αSyn at 283 and 310 K (Figure S5). By recording CON spectra of 100 μM αSyn in the presence of a substoichiometric (40 μM) and a saturating amount (400 μM) of Cu(II), we were able to observe the expected PREs at both temperatures (Figures 3 and S6). In line with previous studies (at 283[6j] and 288 K[6k]), PREs were observed in the vicinity of a high affinity binding site at the N-terminus ($K_{d1} \sim 0.5$ μM) and of a second binding site at H_{50} ($K_{d2} \sim 50$ μM) in the presence of 40 μM Cu(II) at both temperatures, indicating that Cu(II) binding to this region is specific at such substoichiometric concentrations, and that the binding is not so sensitive to temperature (Figure 3a).

Because of the lower gyromagnetic ratio of ^{13}C compared to that of ^{1}H, direct ^{13}C detection enables Cu(II) PREs to be observed at concentrations that saturate a second binding site (Figure 3). Moreover, with most crosspeaks remain detectable in the CON spectra, chemical shift changes upon Cu(II) binding can also be extracted, which show similar trends as do the PREs (Figure S6). In the presence of 400 μM Cu(II), the regions that flank the second binding site exhibit significantly reduced PREs at 310 K compared to those observed at 283 K, while the PRE of the binding residues, D_2, H_{50}, D_{119}, and D_{121}, remains essentially unaffected (Figure 3), indicating that at body temperature (310 K) the structural ensemble undergoes a significant reduction in the degree of compaction of the structure in the vicinity of the residues that initiated the aggregation process, namely the NAC region. Detailed structural elucidation under current experimental setups should therefore provide further insight into the molecular basis of the Cu ion-induced aggregation process.

Protonless NMR spectroscopy is in general well-suited for fast relaxing systems, and it is therefore ideal for PRE measurements that provide unique structural information for defining the global conformations populated by IDPs[6b,d] under physiological conditions. Additionally, the ^{13}C−^{15}N correlations of IDPs in CON spectra are generally far more dispersed, that is, better resolution, in comparison with proton-based detection methods, making identification of minute chemical shift perturbations more tractable. In summary, we have used αSyn as an example to illustrate the importance of characterizing the structural ensembles of IDPs under physiological conditions and the advantage of using protonless NMR spectroscopy to extract residue-specific information thereof, which is crucial for relating these structural features to the biological functions of this important class of proteins.

Acknowledgment. We thank the staff and acknowledge the use of the Biomolecular NMR Facility, Department of Chemistry, University of Cambridge. S.-T.D.H. is a HFSP Long-Term Fellow (LT00798/2005) and in part supported by NSC of ROC, Taiwan (NSC97-2917-1-564-102). C.W.B. is an EMBO Long-term Fellow. C.M.D. acknowledges with gratitude supports from the Wellcome Trust and the Leverhulme Trust.

Supporting Information Available: Detailed description of experimental procedures and additional NMR spectra and analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA902307Q