Published in final edited form as:
Curr Opin Struct Biol. 2009 February ; 19(1): 23–30. doi:10.1016/j.sbi.2008.12.004.

Biophysical characterization of intrinsically disordered proteins

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Summary

The challenges associated with the structural characterization of disordered proteins have resulted in the application of a host of biophysical methods to such systems. NMR spectroscopy is perhaps the most readily suited technique for providing high-resolution structural information on disordered protein states in solution. Optical methods, solid state NMR, ESR and x-ray scattering can also provide valuable information regarding the ensemble of conformations sampled by disordered states. Finally, computational studies have begun to assume an increasingly important role in interpreting and extending the impact of experimental data obtained for such systems. This article discusses recent advances in the applications of these methods to intrinsically disordered proteins.

Introduction

A disordered protein is commonly defined as one that does not adopt a well-defined native structure when isolated in solution under near-physiological conditions. A disordered protein region can be defined as a region that both meets the above definition when excised from the full-length protein to which it belongs and retains its disordered character in the context of the complete protein. Interest in disordered proteins has swelled as a result of the realization that such proteins, instead of the rare exceptions they were once envisioned to be, are unexpectedly and perhaps even astonishingly common in human and other genomes [1–3]. Furthermore, disordered proteins do not appear to simply occur as ‘filler material’ amongst functional well-structured proteins, and instead are associated with a variety of biological functions, many of them intimately related to human disease [4–6]. Therefore, characterization of the structural properties of such proteins must be considered an important complement to more typical structural studies of well-ordered proteins.

The absence of a well-defined structure in disordered proteins complicates the approach that must be taken when considering structural studies, since the most common goal, the determination of a unique high-resolution structure, is not attainable for the isolated protein. Instead, the goal of such studies is usually to obtain experimental constraints on the ensemble of states that is sampled by the polypeptide in question, including the detection of residual secondary structure, transient long-range contacts, and regions of restricted or enhanced mobility, with the hope that such information may prove informative regarding the associated biological function [7–9]. It should be noted that many disordered proteins do adopt more highly ordered conformations upon interactions with other cellular components [10]. In some cases, these bound states are subject to the same methods used for solving the structures of well-structured protein complexes, permitting a high-resolution view of the bound protein in question. However, in at least some cases, the bound states of disordered proteins remain highly non-compact and retain substantial mobility, and a brief discussion of structural studies of such bound states is therefore included.

Conflict of Interest
The author has no conflicts to declare.

In many ways intrinsically disordered proteins resemble denatured states of well-structured proteins. The latter have been subjected to greatly increased scrutiny in the past few decades, largely in the context of attempts to better understand the process of protein folding. While the focus herein is primarily on studies of intrinsically disordered proteins rather than denatured states, many of the techniques that are applied to the former have been adapted directly from those used in studies of the latter, and a number of reviews of such studies are therefore directly relevant [11–14].

Residual Secondary Structure

Secondary structure in disordered proteins is typically transient and confined to short individual helical or extended segments with ensemble-averaged structured populations ranging from a few nearly 100 percent. Because these segments often comprise a small fraction of the total protein sequence, they are typically difficult to detect using techniques for measuring overall secondary structure content such as circular dichroism or infrared spectroscopies. Solution state NMR is probably the technique best suited for the detection and delineation of such segments. Chemical shifts, with their exquisite sensitivity to environment and structure, remain the most powerful tool for achieving this goal. Deviations of observed shifts from those estimated for an idealized unstructured state (so called random coil shifts) can be used to assess both the location and population of transient (or full formed) secondary structure [7] (Figure 1). In cases of very low populations (10% or lower) uncertainties in the random coil shifts can lead to ambiguities in this type of analysis, which can be lessened to some extent by looking at shifts from several different nuclei, linear combinations of shifts, or more sophisticated moving averages [15]. Short range NOEs can be used to corroborate secondary structure propensities detected through chemical shifts [16,17], although the effects of local dynamics on such an analysis must be considered [18]. NMR coupling constants, which can be directly related to bond torsion angles, can also be used to pick up local secondary structure preferences [19,20], although effects behind local variability in such measurements are not as well characterized as in the case of chemical shifts [21].

Most recently, residual dipolar couplings (RDCs) have been shown to be sensitive to local secondary structure in disordered or unfolded proteins. In anisotropic media, polypeptide chain segments can align weakly along a preferred direction, leading to a weak recoupling of the spin-spin dipolar interaction and resulting in measurable NMR line splittings. Because bond vectors (and in particular NH bond vectors) can be orthogonally aligned in helical or extended structures, different local structure leads to opposite effects on the observed RDCs, leading to a characteristic signature for each type of secondary structure [22] (Figure 1). However, RDCs do not disappear for proteins even under strongly denaturing conditions expected to disrupt all secondary structure, possibly because they are dominated by contributions from highly extended conformations within the ensemble [23]. As a result, while robust helical structure is evidenced as RDCs of opposite sign [22,24], more transient helical structure may lead to only a decrease in RDC amplitude without a sign reversal [25,26] (Figure 1). A potential way to mitigate this difficulty may be to analyze RDC differences from those observed under strongly denaturing conditions [22,25]. At present, however, quantitative interpretation of disordered protein RDCs in terms of residual secondary structure awaits further work.

Many parameters that can be measured using solution state NMR can in principle be determined using solid state NMR as well. One advantage of solid state methods is that they may eliminate rapid interconversion of different populations within a single sample, facilitating the direct measurement of structural parameters such as chemical shifts for each sub-ensemble. A recent report demonstrates the use of solid state NMR to examine secondary structure preferences based on chemical shifts in a denatured villin headpiece subdomain [27], suggesting that this technique may also be applicable to the characterization of disordered proteins.

Long-range Contacts

Long-range contacts in disordered proteins are inherently transient and therefore difficult to detect. Furthermore, a clear definition for such contacts is important, since it is to be expected that different regions within a highly flexible disordered polypeptide chain will occasionally approach or contact each other in the absence of any preferred interactions. In general, this expectation has to be made explicit based on statistical or computational models of the distance distribution between two residues or locations within a polypeptide chain sampling an idealized random coil ensemble. Subsequently, such a model can be used to predict the corresponding experimental data in the absence of preferred contacts, which can then be compared with data obtained from actual measurements.

The overall dimensions of fully unfolded protein ensembles are well-predicted by statistical models [28] but are expected to decrease in the presence of long-range interactions. Measurements of parameters such as the hydrodynamic radius (R_h) or radius of gyration (R_g) of a protein can be used to detect such a decrease [29,30]. Techniques such as size-exclusion chromatography or analytical ultracentrifugation, which can easily differentiate between particles with dramatically different R_h have not typically been used for studies of disordered proteins, possibly because of relatively small anticipated changes. Instead, pulse-field gradient (PFG) NMR, small-angle x-ray scattering (SAXS) and fluorescence correlation spectroscopy (FCS) measurements have been more commonly used. PFG experiments enable measurements of R_h for a disordered protein [31–33], which can indicate the presence of long-range contacts within an ensemble, but they are not able to pinpoint specific regions or residues involved in such contacts. FCS measurements of fluorescently tagged disordered proteins can also provide diffusion coefficients [34,35]. In addition, however, FCS is sensitive to fluorophore quenching reactions that occur as a function of internal dynamics [12]. If specific quenching groups can be identified in the protein, some information regarding specific contacts can be obtained [36]. SAXS measurements principally provide a measurement of the radius of gyration (R_g) for a disordered protein ensemble [37]. However, for systems containing both fully ordered and disordered regions [24,38,39], or even less ordered and more ordered domains [40] pair distribution functions from SAXS data can provide powerful constraints on the relative topology of the different domains.

Experimental methods that report on distances are best suited for detecting specific long-range contacts. Fluorescence energy transfer, a sensitive measurement of specific distances, has proven useful in this context. Although various donor-acceptor pairs can be used, tryptophan is often convenient for disordered proteins as it rarely occurs within their native sequences. Acceptors used in recent reports include modified tyrosine residues (3-nitrotyrosine) [41] and 1,5-IAEDANS-labeled cysteine residues [42]. Dyes from the Alexa family (Invitrogen) have also been used effectively [36]. Measurements provide detailed information on the distance distribution between specific sites in a disordered protein, which can be used to constrain conformational ensembles. A shortcoming, however, is that multiple constructs with different donor-acceptor locations are necessary for a comprehensive characterization.

NMR long-range NOEs, traditionally used for obtaining topological distance constraints in well-structured proteins, have proven difficult to observe in disordered proteins. This likely results from two causes: first, the contacts in disordered proteins may be too short lived to allow for efficient buildup of NOE signals, and second, the very high degeneracy of side chain chemical shifts makes it very difficult to reliably assign long-range NOEs involving side chain nuclei. Nevertheless, successful detection of medium- and long-range NOEs in an unfolded state of a well-structured protein was reported in a study employing selective labeling strategies to greatly reduce the number of proton signals [43]. Thus, further advances may increase the applicability of NOE methods to intrinsically disordered systems.

Paramagnetic relaxation enhancement (PRE) has perhaps been most successful at detecting long-range contacts in disordered protein ensembles [44–47]. The method, which was again used first in studies of unfolded or partially folded proteins [48–50], relies on the dipolar interaction between nuclear spins and the spin of an unpaired electron, typically introduced in the form of a nitroxide compound, the so called spin label, conjugated to a cysteine residue. Alternately, chelating groups can also be used to introduce a paramagnetic metal [51,52]. Spins in the physical proximity of the spin label relax more efficiently, leading to broadened signals, an effect that is reliably detectable to approximately 25 ångströms [49]. Positions near the site of spin-label attachment in the polypeptide sequence are covalently constrained to be in the spatial vicinity of the spin label and always exhibit resonance broadening. For ideal random coils, however, residues increasingly distant in sequence from the labeled position spend relatively less time in the proximity of the label and should exhibit monotonically decreasing degrees of broadening. Deviations from this expected pattern are indicative of preferential long-range stabilizing interactions. Importantly, most reports to date measure PRE effects by comparing signal intensities in a single pair of spectra acquired in the presence and absence of a paramagnetic spin label. This approach is subject to systematic errors, and more accurate measurements are recommended to enable quantitative interpretation of PRE data [53,54].

In addition to measurements reflecting inter-spin distances, other NMR parameters have the potential to report on long-range contacts in disordered polypeptides. Chemical shifts are exquisitely sensitive to environmental perturbations, and might be expected to reflect any alteration of long-range interactions. Surprising, however, transient contacts that are observable using PRE methods do not appear to be robustly reflected in chemical shifts [45, 55], possibly because of their highly fluctional nature. RDCs have also been proposed to be sensitive to long-range contacts, with reports attributing differences between predicted and observed RDCs to such interactions [56,57]. More recently, however, this interpretation has been brought into question by data suggesting that local transient secondary structure is responsible for the disagreement between the modeled and observed RDC data [26] (Figure 1 and Figure 2).

Dynamics

Disordered proteins are highly, but not uniformly, flexible. Different mobilities in different regions may be linked to function, making the characterization of dynamics in such proteins highly desirable. A number of techniques can provide dynamics information for specifically labeled sites. For example, FCS can report on the timescale of conformational dynamics associated with the quenching of specific intrinsic or conjugated fluorophores [36,58]. ESR can also be used to assess the flexibility of the polypeptide backbone at the location of spin-label attachment [42,59,60]. In contrast to such site-specific techniques, solution state NMR is capable of providing highly detailed dynamics information simultaneously at different sites throughout a protein, and has thus been the preferred tool for characterizing dynamics in disordered systems.

NMR measurements indicate that disordered proteins often exhibit rapid (on the NMR timescale) backbone motions throughout the polypeptide chain [16,61], in contrast to well structured systems where such motions are typically restricted to hinge or loop regions. When exceptions to this observation occur they are typically associated with secondary structure formation [17,62,63] or also local hydrophobic clusters [47,64], and are usually of interest from a functional perspective. Slower motions (on the NMR timescale), which have been attributed to conformational exchange arising from long-range contacts in unfolded proteins [65,66], have not thus far been commonly observed in disordered systems [67], although potential evidence for their presence exists [63]. However, such conformational exchange has been documented in folding-upon-binding events involving disordered proteins [68,69]. An

accompanying article in this issue discusses recent developments in the study of such disorder-to-order transitions.

Computation

Despite the detailed information that can be obtained on residual secondary structure, transient long-range contacts, and dynamics in disordered proteins, describing the ensemble of conformations sampled by such systems remains a considerable challenge. Computational methods increasingly play an important role in helping to visualize the implications of experimentally observed constraints, as well as in generating de novo predictions and simulations. An early contribution was the development of the program AGADIR, which accurately predicts regions of significant helix propensity [70]. To date, no similarly successful predictor of strand propensity has been reported, possibly due to the more highly cooperative nature of helical structure. The reliability of residual secondary structure analysis based on NMR chemical shifts has been evaluated using molecular dynamics simulations [71] and improved statistical algorithms have been proposed [15].

Interpretation of RDCs has been facilitated by several approaches developed to better understand unfolded states of well-structured proteins. Calculations based on global alignments of large ensembles of conformations generated solely based on volume exclusion and backbone torsion angle parameters from loop regions of well-structured proteins [23,72] were found to reproduce experimental data for several systems. An approach using local alignment of chain segments in ensembles generated based on volume exclusion biased by experimentally determined secondary structure propensities gave similarly good agreement using considerably smaller ensembles [73]. Interestingly, a correlation between amino acid bulkiness and RDCs in disordered proteins has been observed and also interpreted to reflect locally extended conformations due to steric constraints [57]. The interpretation of SAXS data from disordered proteins has also been extended by computational generation and selection of consistent ensembles of polypeptide conformations [74,75].

Similarly, visualizing the implications of long-range contacts detected through PRE measurements has been facilitated by the development of computational analyses. PRE measurements have been used to constrain molecular dynamics simulations in order to generate an ensemble that is consistent with the experimental data. Comparison with an ensemble generated to resemble an excluded-volume random coil model allowed identification of regions involved in preferentially formed long-range contacts, as well as analysis of representative conformers [44]. An alternative approach uses PRE data to prune large ensembles of generated structures in order to enrich for conformations that are consistent with the data [52]. Either of these methods can also incorporate additional experimental constraints. More recently, models based on interactions of clusters of high AABUF (average area buried upon folding) value were found to successfully reproduce detailed features of PRE profiles [76].

Finally, de novo molecular dynamics simulations are also proving useful in providing general insights into the behavior of disordered proteins, especially for cases such as homopolymers that are particularly challenging experimentally [77–79].

Ordered States

Upon interacting with cellular components such as other proteins, membranes, or the cytoskeleton components, intrinsically disordered protein can fold into conformations that are more highly ordered. Folding can be restricted to short elements of secondary structure [80–82], or can encompass lengthy regions of the polypeptide chain [83]. If the resulting complex can be isolated in a well-structured form, standard methods can be applied to solve the structure of the ordered conformation. In some cases, however, it seems that the bound state retains a

significant degree of mobility and in addition remains highly non-compact, precluding a typical structure determination. In such cases, the techniques described above can prove as useful in describing the bound state as they are in characterizing the free state, but additional become applicable as well. Solution state NMR relies largely on short inter-proton distances to constrain molecular structure at high resolution. For non-compact conformations, such constraints are rare or absent. In the absence of such constraints, a recent approach, molecular fragment replacement, has been developed which relies predominantly on RDCs, combined with the database of known structures, to assemble local trajectories of polypeptide chain segments into a complete structure [84]. This approach was combined with long-range PRE restraints to calculate a structure of the protein alpha-synuclein in its detergent micelle-bound state [51]. Longer-range distance constraints can also be obtained using pulsed ESR spectroscopy, as illustrated recently in studies of micelle-, bicelle- and vesicle-bound alpha-synuclein [85,86].

Conclusions

Detailed biophysical studies are likely to prove crucial in clarifying the relationship between biological function and underlying structure for intrinsically disordered proteins, much as they have for well-structured ones. Over the past several years, the application of increasingly sophisticated methods has revealed that disordered proteins are far from homogeneous statistical random coil polymers and instead exhibit a rich diversity of local and even long-range structural preferences, as well as dynamics, that are likely to be of functional import. The transient nature of such preferences makes them more challenging to detect and characterize than the stable structures of well-ordered proteins. Nevertheless, continuing technical improvements as well as a further increase in the amount and quality of available data, combined with improvements in computational methods, should improve our fundamental understanding of these systems and their increasingly recognized roles in biology.

Acknowledgements

This work was supported by NIH/NIA grants AG019391 and AG144051, the Irma T. Hirschl Foundation and a gift from Herbert and Ann Siegel.

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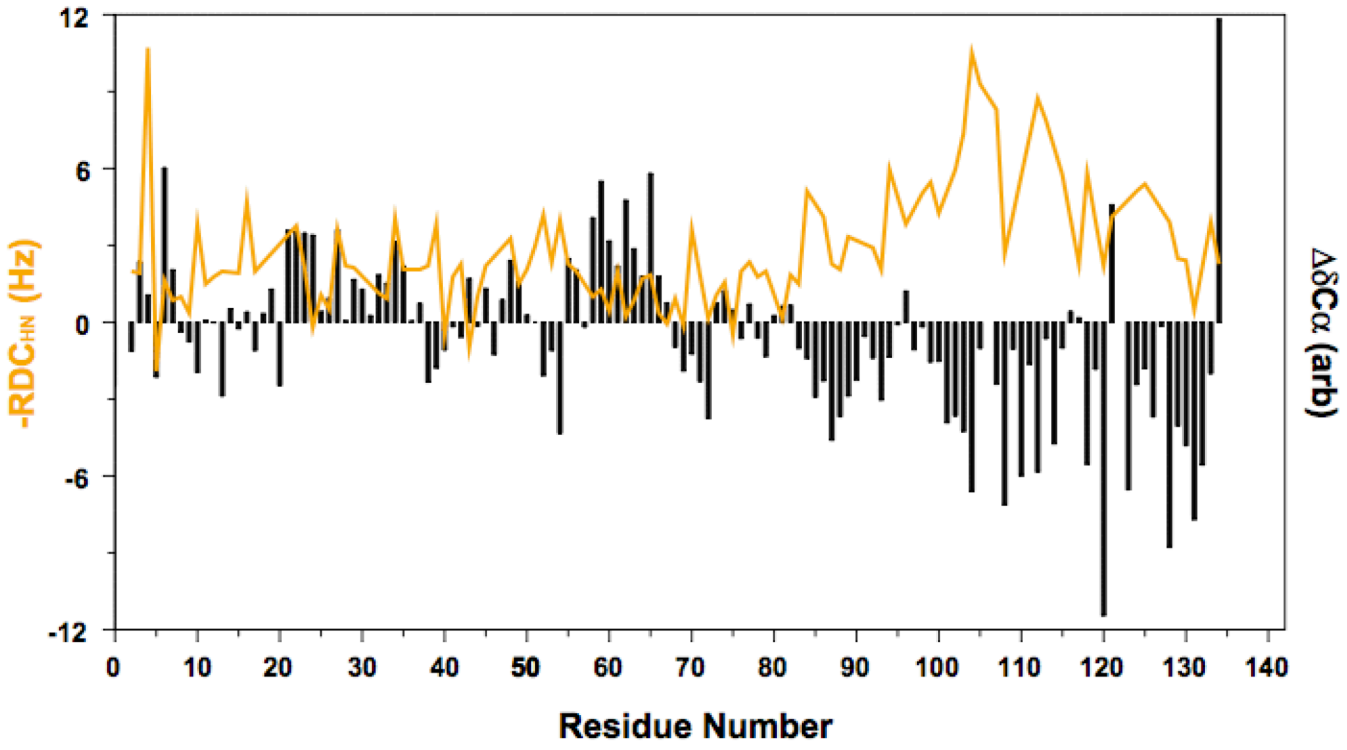

Figure 1. NMR α-carbon secondary chemical shifts and NH RDCs for β-synuclein

Chemical shift deviations from random coil values indicate a preference for extended structure (negative values) in the C-terminal region and helical structure (positive values) in the N-terminal region. Large amplitude RDCs in the C-terminal region correlate qualitatively with negative shift deviations and likely reflect locally extended chain segments. Smaller amplitude RDCs in the N-terminal region occur largely in regions of positive shift deviations, although the RDCs do not change sign as would be expected for more highly populated helical structure. Adapted from [26].

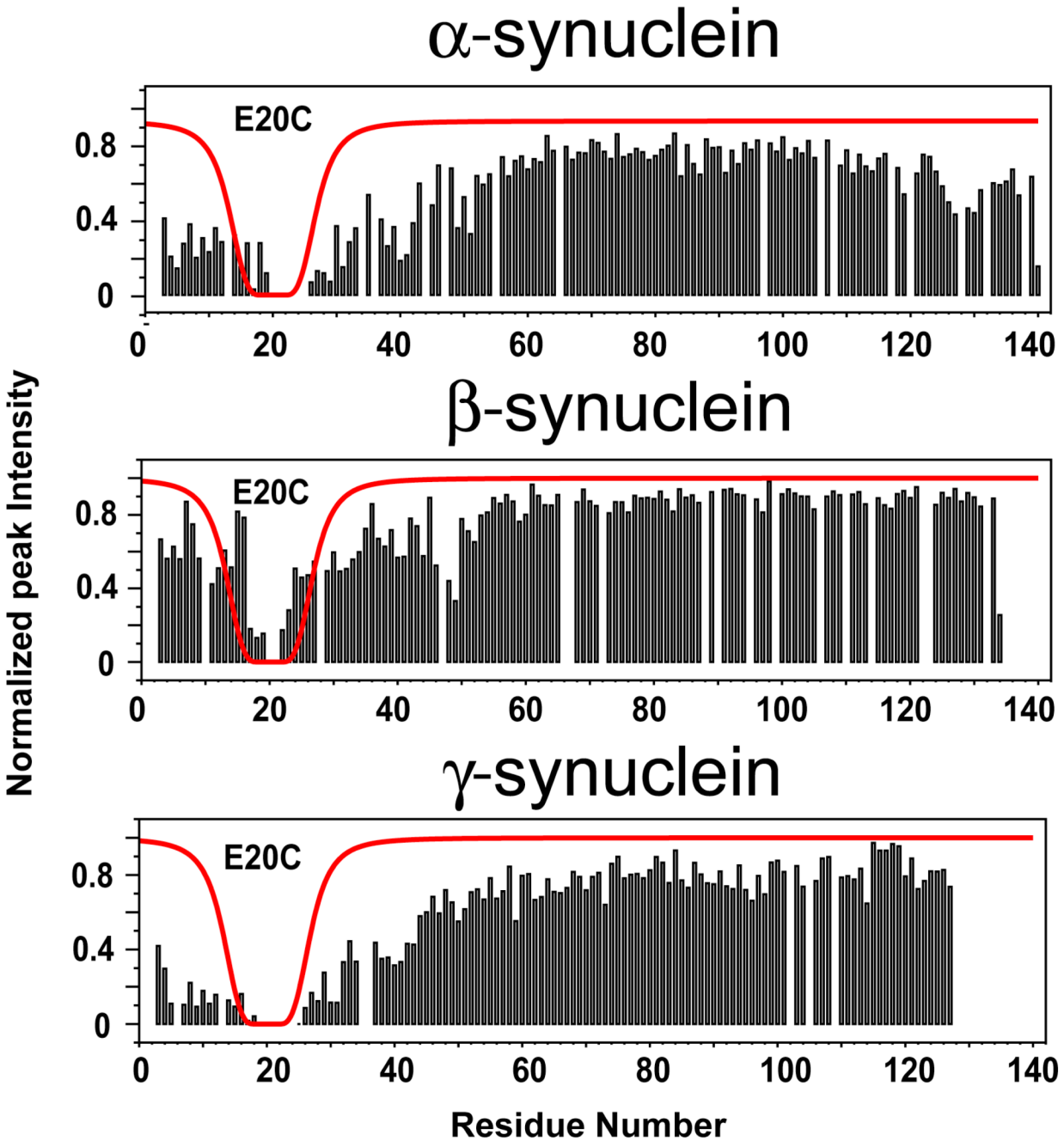

Figure 2. NMR PRE in α-, β- and γ-synuclein

A paramagnetic spin label attached to a cysteine introduced at position 20 leads to clear broadening in the C-terminal tail of α-synuclein, but not of β- or γ-synuclein, suggesting long-range N- to C-terminal contacts are present in the former but not in the latter. The red lines represent the predicted broadening for an idealized random coil. Adapted from [26].