Protonation-dependent conformational variability of intrinsically disordered proteins

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Received 21 March 2013; Revised 20 June 2013; Accepted 21 June 2013
DOI: 10.1002/pro.2304
Published online 2 July 2013 proteinscience.org

Abstract: Intrinsically disordered proteins (IDPs) are characterized by substantial conformational plasticity and undergo rearrangements of the time-averaged conformational ensemble on changes of environmental conditions (e.g., in ionic strength, pH, molecular crowding). In contrast to stably folded proteins, IDPs often form compact conformations at acidic pH. The biological relevance of this process was, for example, demonstrated by nuclear magnetic resonance studies of the aggregation prone (low pH) state of α-synuclein. In this study, we report a large-scale analysis of the pH dependence of disordered proteins using the recently developed meta-structure approach. The meta-structure analysis of a large set of IDPs revealed a significant tendency of IDPs to form α-helical secondary structure elements and to preferentially fold into more compact structures under acidic conditions. The predictive validity of this novel approach was demonstrated with applications to the tumor-suppressor BASP1 and the transcription factor Tcf4.

Keywords: intrinsically disordered proteins; protein meta-structure; pH dependence; structural biology; biomolecular NMR; EPR spectroscopy

Intrinsically disordered proteins (IDPs) display conformational plasticity and undergo structural rearrangements upon changes of pH. Here we provide experimental evidence for α-helix formation and structural compaction of IDPs under acidic conditions. Sequence analysis of a large set of IDPs revealed a general tendency to form α-helical secondary structure elements and to preferentially fold into more compact structures under acidic conditions suggesting that elements sensitive to protonation-induced stabilization might be crucial in folding-upon-binding events.

Introduction

Intrinsically disordered proteins (IDPs) display remarkable physicochemical properties that allow
facilitates the hydrophobic collapse.9

This folding transition is initiated by the formation into a partially folded conformation at lower pH.

tively folds from a denatured state at neutral pH

disorder.12 In addition to modulating the ionization lead to oncological processes and lysosomal storage

tions11 and pH alterations in the acidic organelles

Protonation and deprotonation are fundamental chemical mechanisms to modulate ionization states and physicochemical properties of biomolecules and therefore of great relevance for biological systems. In eukaryotic cells, the pH in different organelles is highly variable ranging from 7.2 to 7.4 in the cytosol and nucleus to 4.0–5.5 in the endosomes and lysosomes.10 Thus, pH dysregulation frequently leads to cellular dysfunction and ultimately to disease phenotypes. For example, tumor cells have been shown to behave abnormally under acidic cellular pH conditions11 and pH alterations in the acidic organelles lead to oncological processes and lysosomal storage disorder.12 In addition to modulating the ionization states of molecules and their interaction patterns with authentic binding partners, alteration of pH can also induce structural changes in proteins. Typically, proteins are only marginally stable and small changes in environmental conditions can induce substantial structural rearrangements. Given the small stability differences between the folded and unfolded state(s), slight changes of structure-disrupting factors (concentration of chemical denaturants, pH, or temperature) may lead to substantial and even cooperative changes in the population of folded and unfolded states, respectively. It is important to note that partial proton transfer is often sufficient for catalytic acceleration of chemical reactions and complete proton transfer might not be necessary. This is described in the concept of general versus specific acid catalysis in physical organic chemistry and enzymology. Protonation of a given substrate modifies its electronic state and may yield, for example, a strong electrophilic center that may be prone to attacks of nucleophiles. In return, this means that a reduction of electrostatic repulsion among deprotonated, acidic residues of a protein does not necessarily require complete protonation of acidic side chains.

In this study, we use the recently developed meta-structure concept, a computational approach,13 to analyze structural changes in IDPs under acidic conditions. The meta-structure analysis provides quantitative (on a per residue basis) information about residue structural complexity (compactness) and local secondary structure. Compactness is inversely related to local residue solvent-exposure, whereas local secondary structure values are defined analogously to the NMR secondary Co chemical shift (α-helices: positive; β-sheets: negative). Numerous applications have already demonstrated the applicability of this approach.14–17 In this study, we computationally consider the effect of lowering the pH by altering the primary sequence through replacing the negatively charged carboxylate groups of Asp/Glu residues with neutral (hydrogen-bond donor) amide groups of Asn/Gln residues. The predictive validity of our meta-structure approach is illustrated by its application to the IDPs BASP1, a tumor-suppressor down regulated by the oncogenic transcription factor Myc,18 and Tcf4, a transcription factor involved in the Wnt signaling pathway.19

Results

The meta-structure derived compactness and local secondary structure parameters were used to assess pH-induced changes in protein structural features. Residue-specific compactness values quantify the spatial embedding of individual residues within the 3D protein structures. Residues in the interior of a structure exhibit large compactness values, whereas residues located on the surface and exposed to the solvent display small (even negative in case of conformationally highly flexible segments) values. The meta-structure derived secondary structure parameter is defined in analogy to the well-established NMR 15N/13C chemical shift index, with positive values for α-helices and negative values indicating the presence of an extended conformation. First, we evaluated the performance of the approach by comparing calculated compactness values of IDPs (taken from the DisProt database) with well-folded proteins deposited in the PDB database. As can be seen from the histograms shown in Figure 1(A), IDPs are characterized by significantly smaller compactness values (~320) compared to their well-folded counterparts (~330), indicating that compactness
values are valuable quantitative probes for structural compaction of proteins. Then, we analyzed whether the calculated local secondary structure parameters are indicative of α-helices and β-strands. To facilitate the analysis, protein backbone dihedral angles φ and ψ were extracted for a set of 857 proteins taken from the PDB database (protein structure database designed by Kontaxis et al.20). Dihedral angle combinations were transformed into a single geometrical parameter using the well-established relationship: 3cosΩ = 1 − 4cosφ(ψ + ψ)/2.21 Figure 1(B) shows that meta-structure derived secondary structure values are consistently positive for residues located in α-helical segments, whereas residues populating extended structural elements (β-strands or polyproline II helices) display negative values. It has to be noted that, although α-helices can be directly inferred from the meta-structure analysis, negative secondary structure values are found for both β-strands and other extended segments (polyproline II helices). As a summary, we conclude that changes in local structure preferences (e.g., α-helix to β-strand transition) can be correctly identified by this approach.

We have applied our meta-structure approach to two well-studied examples of acid-induced structural changes of IDPs. A recent NMR analysis of the pH-dependence of α-synuclein revealed significant structural adaptions in the C-terminal (Asp/Glu-rich) region. Although at near neutral pH residues in the C-terminal part populate extended conformations, propensities for α-helical elements are increased on lowering pH.8 Surprisingly, independent FTIR data suggested an increase in populations of β-sheet conformations.22 The secondary structure change was accompanied by an overall compaction of the polypeptide chain at low pH as probed by pulsed-field gradient (PFG) measurements of the hydrodynamic radius, R_H, and long-range paramagnetic relaxation enhancement data.8 Guinier analysis of SAXS data also showed that at neutral pH the radius of gyration of α-synuclein amounts to R_g = 40 Å, whereas it drops to ~30 Å at low pH.22 The meta-structure analysis shown in Figure 2(A) convincingly supports these findings: (i) increase in α-helical propensities in the C-terminal (Asp- and Glu-rich) region; (ii) increased population of β-sheet conformations in the N-terminal region that houses the aggregation relevant NAC region of α-synuclein; and (iii) significant local compaction of the C-terminal region.

A second example is given by prothymosin α. It was shown that prothymosin α exhibits an increase in α-helical content from nearly 0% at high pH to about 15% at low pH, coincident with a considerable reduction of the radius of gyration (high pH: 37.8 Å; low pH: 27.6 Å).9 Inspection of Figure 2(B) shows that the significant increase in compactness and α-helical content compared to neutral conditions is correctly identified by the meta-structure analysis. Summing up, these two applications clearly demonstrated that the novel meta-structure approach provides reliable residue-specific information about the impact of protonation on local second structure and structural compaction.

In a second step, we applied our approach to two IDPs: BASP1, a tumor-suppressor, and Tcf4, a transcription factor involved in Wnt signaling. BASP1 is an N-myristoylated protein that is involved in neurite outgrowth and plasma membrane organization23 and has been shown to be involved in transcription regulation by acting as a co-suppressor of WT1 function (Wilms’ tumor suppressor protein 1).24,25 Figure 3(A) shows the results of meta-structure analysis of BASP1. From the
residue plot it can be deduced that under acidic conditions (pH 2) BASP1 displays larger fractions of α-helical regions as compared to pH 6. Furthermore, it samples more compact conformations, as indicated by the significantly larger compactness values. The meta-structure-predicted overall compaction of BASP1 was experimentally verified using PFG NMR diffusion measurements (PFG-DOSY), although significant scatter in the extracted diffusion constants impaired a detailed quantitative analysis. Additional support for the structural compaction of BASP1 was obtained from well-established NMR saturation experiments. It was shown that the SOFAST-HMQC experiment can efficiently be used to probe 1H-1H spin diffusion or NOE effects, when a selective inversion pulse (H\text{sat}) is applied on aliphatic protons before the start of the pulse sequence. Two data sets are recorded with (I\text{sat}) and without (I\text{ref}) the inversion pulse H\text{sat}. The intensity ratio (\lambda\text{NOE} = I\text{sat}/I\text{ref}) measured in the two SOFAST-1H-15N-HMQC spectra depends on spin diffusion effects and thus probes the structure and dynamics of proton spin networks (density and mobility) in proteins. In well-structured, globular proteins spin diffusion is highly efficient leading to \lambda\text{NOE} << 1, whereas in loosely folded proteins (random coils, molten globules) \lambda\text{NOE} \approx 1. Thus, we have applied HET-SOFAST-HMQC to BASP1 to probe structural compaction under high and low pH conditions. The significant decrease of \lambda\text{NOE} on lowering pH (0.75–0.60) again indicates structural compaction of BASP1 under low pH conditions. The overall compaction of BASP1 at low pH was independently corroborated by electron paramagnetic resonance (EPR)-based double electron-electron resonance (DEER) measurements. Figure 3(B) shows DEER time traces obtained at different pH values (pH 1 and pH 6). Changes in solution pH clearly influence effective modulation depths, \Delta_{\text{eff}}, and the pair-wise distance distributions, P(r). At low pH, the effective modulation depths, \Delta_{\text{eff}}, is larger and P(r) is shifted toward shorter distances. More specific information about local structural dynamics changes was obtained using residue-specific NMR data. Inspection of Figure 3(C) displays a clear correlation between meta-structure derived (Fig. 3(C), top) and experimental NMR-derived differential secondary structural propensity (SSP) changes [SSP (pH2) - SSP (pH6); Fig. 3(C), middle]. Consistently, most pronounced secondary structural changes are found in the glutamic acid-rich region 30–100, whereas smaller secondary structural changes were observed for segments around residues 180 and 230. The regions of increased α-helical content at low pH coincide with regions displaying reduced local mobility under acidic conditions as indicated by differential 15N T2 values (15N T2 (pH6) - 15N T2 (pH2)). These regions appear as local maxima in the differential 15N T2 versus residue plot [Fig. 3(C), bottom]. Interestingly, the global compaction of BASP1 at low pH is further indicated by larger 15N T2 values (smaller apparent correlation time \tauC) [Fig. 3(C), bottom]. It should be noted that on structural compaction BASP1 retains significant internal mobility that is significantly different to the behavior of well-folded (ordered) proteins. These local changes in internal dynamics lead to residue-specific modulation of the experimental 15N T2 values, for example, local secondary structure formation.

The second IDP analyzed was the DNA-binding T-cell factor/lymphoid enhancer factor (Tcf4/LEF), a prominent binding partner of β-catenin. β-Catenin is a central unit in the Wnt signaling pathway, and constitutive activation of the Wnt signaling pathway is involved in the development of various human malignancies, like colorectal carcinomas, melanomas, and ovarian carcinomas. Although detailed structural information of unbound Tcf4 is still lacking, it was shown by CD spectroscopy that Tcf4 in...
its apo-state is devoid of significant secondary structure elements and is reminiscent of a random coil. This lack of global structure is also reflected in small meta-structure derived compactness. The average residue compactness of Tcf4 was found to be about 54, which is considerably smaller than the average value for a stably structured, globular protein (about 300). Meta-structure calculations performed under acidic conditions revealed that at low pH Tcf4 is more compact and significantly more \( \alpha \)-helical [Fig. 4(A)]. To verify this prediction, NMR signal assignment was performed at neutral and low pH (2.0). The increase in \( \alpha \)-helical content at low pH was clearly observed in the SSP analysis [Fig. 4(B)]. Interestingly, the two \( \alpha \)-helical regions (\( \alpha_1 \) and \( \alpha_2 \)) displaying more \( \alpha \)-helical propensities under acidic than under neutral conditions constitute major parts of the Tcf4 interaction interface and provide most of the binding energy for the \( \beta \)-Catenin/Tcf4 complex [Fig. 4(C)]. It is particularly noteworthy, that the

**Figure 3.** Experimental verification of meta-structure derived structural changes of BASP1 on lowering pH. (A) Differences in meta-structure derived local secondary structure (black) and compactness values (gray) between acidic and neutral conditions. (B) Schematic illustration of coarse-grained structural changes of BASP1 on changes in solution pH, as detected by DEER spectroscopy. On lowering pH, the two labelling sites (C110 and C229) spatially approach, indicating compaction of the IDP (top). This is evident from time traces (lower left) at pH 6 (red) and at pH 1 (blue) and the corresponding distance distributions (lower right). (C) Comparison between meta-structure derived secondary structure changes (upper trace), NMR derived secondary structure propensities (SSP) changes (middle trace) and differential local conformational dynamics probed by \(^{15}\)N \( T_2 \) relaxation on lowering pH (lower trace).
region $\alpha_1$ exhibits the largest SSP changes [Fig. 4(B,D)] and thus points to a significantly larger conformational plasticity of this part of Tcf4.

Finally, to analyze whether the observed pH-dependent conformational changes might constitute a conserved feature within the IDP domain, we performed a large-scale analysis of human IDPs. Because the average mean compactness value for a protein in the PDB is about 300, we used a threshold of 200 for the selection of human IDPs. Only proteins with mean compactness values smaller than this threshold were considered (in total 1013 proteins were analyzed). We thus only consider globally disordered proteins and exclude globular proteins with (long) unstructured linker segments.

For this set of human IDPs, we calculated local secondary structure and compactness values for high and low pH forms. The histogram of pair-wise secondary structure and compactness differences is shown in Figure 5. Overall, we found a slight but significant preponderance of $\alpha$-helical content at low pH and increased compactness values on decreasing pH seem to be a conserved feature for IDPs. Moreover, considering only longer residue stretches (larger than 7 residues) with consistent local secondary structure changes, the preponderance of $\alpha$-helical structures at low pH becomes even more pronounced [Fig. 5(A), insert].

**Discussion**

IDPs are intriguingly sensitive to changing environmental conditions. Given that local changes of pH are of high relevance to *in vivo* functionalities of these protein species, local pH levels or proton concentrations are tightly controlled in living organisms. Although detailed studies of the effect of pH on IDP conformational space are rare, recent experimental findings indicate that a decreasing pH is likely to be accompanied by the formation of $\alpha$-helices in glutamic acid-rich regions and a subsequent folding transition to a non-molten globule state for which the hydrophobic collapse is facilitated by neutralization of negatively charged sidechains. In this study, we have introduced a novel
meta-structure approach that can be used to predict the pH dependence of IDPs using only primary sequence information. The pH-dependent differences in meta-structure derived local structure and compactness values were found to be in good agreement with experimental findings. Data obtained on a large set of intrinsically disordered human proteins show that IDPs fold under acidic conditions into more compact structures with higher α-helical content largely due to reduced electrostatic repulsion of negatively charged side chains. It was shown recently that the inherent context-independent α-helical propensities are significantly higher for Asn and Gln than for Asp and Glu, respectively. The meta-structure derived increases in α-helical propensities are not only related to individual (context-independent) amino acid specific properties but also take into account context-dependent (primary sequence) influences.

This observation is also particularly interesting in view of a recent finding showing that the evolution of IDP sequences is constrained and involves a significant bias in the amino acid composition. In contrast to globular, stably folded proteins structural compaction in IDPs is accompanied by the formation of α-helices and characterized by a low β-structure propensity. Therefore, avoiding aggregation despite preformed molecular recognition elements is an important driving force in protein sequence evolution of IDPs. We showed that similar mechanisms are responsible for maintaining soluble, functionally active and nonaggregating IDPs on protonation or under acidic conditions.

Furthermore, as illustrated with the β-catenin/Tcf4 protein complex, stabilization of preformed α-helices via (full or partial) protonation might also be of relevance for protein complex formation involving IDPs. Although globular proteins typically involve autonomously structured domains of about 60–300 amino acid lengths, IDPs mediate protein interaction using short sequence motifs. Depending on the context, they are called eukaryotic linear motifs, short linear motifs, molecular recognition features, or preformed structural elements (PSEs), although they are not entirely unrelated and share analogous features. Although protein recognition by IDPs often proceeds via folding-on-binding events (e.g., disorder-to-order transitions), there is growing evidence that even in the bound state IDPs (can) retain substantial conformational flexibilities, a conceptual view which is sometimes referred to as “fuzziness.” The Tcf4/β-catenin protein complex is an example for static disorder ("polymorphic") as Tcf4 can bind in several distinct conformations to β-catenin. Specifically, the Tcf4/β-Catenin binding interface comprises three epitopes centered at Tcf4 residues D16, E29 and D43 (PDB: 1JPW, 1JDH). The central epitope (E29), α1, shows considerable plasticity and docks to β-Catenin in several different conformations (extended or α-helical). Inspection of the Tcf4/β-Catenin structure (PDB: 1JPW, 1JDH) reveals that in this complex epitope α1 is surrounded by a basic patch comprising the positively charged β-Catenin residues K312, R342, K345, R376, and R386. The finding that protonation of negatively charged side-chains stabilizes α-helical structures provides an additional chemical mechanism for stabilizing recognition elements in IDP protein complexes. Favorable electrostatic interactions between the basic proton acceptor located at the surface of the IDP recognition element and the acidic proton donor of the interaction partner lead to stabilization.
of the partially PSE. However, it should be noted that to stabilize the basic proton acceptor complete proton transfer is not required, as even the realization of hydrogen bonds (e.g., partial proton transfer) can already lead to stabilization of the system. Based on our results obtained for the Tcf4/β-catenin protein complex we conclude that tuning of protonation efficiency might constitute a relevant chemical mechanism to modulate interaction affinities and associated recognition selectivity.

As a conclusion, the availability of a fast and reliable prediction tool for preformed α-helical elements sensitive to protonation-induced stabilization may be of great relevance for the (sequence-based) identification of folding-on-binding systems in different organisms and the analysis of recognition patterns with potential implications for large-scale predictions of IDP interaction partners.

Materials and Methods

Meta-structure description of proteins under acidic conditions

The pH dependence of IDPs was analyzed applying the recently developed meta-structure approach. In this study, we focus on the assessment of protonation events on the meta-structural parameters of IDPs. In brief, protein 3D structural information is converted into a network structure in which a node refers to a particular residue and edges indicate the existence of neighbourhood relationships (provided that the Ca-Co distances are below a distance cutoff, typically 8 Å). Subsequently, the topological relationship between two residues is quantified by the shortest path length θ connecting these two residues in the network. The topological relationship between two residues (of type A and B) characteristically depends on the amino acid types and their primary sequence distance, I_{AB}. The statistical distribution functions ρ(θ,A,B,I_{AB}) were extracted from a selected subset of PDB structures. The statistical distribution functions ρ(θ,A,B,I_{AB}) can subsequently be used to predict topological information solely based on the primary sequence. The input primary sequence is used to predict for each possible amino acid pair (of residue type A and B and separated by I_{ab} in the sequence) in the protein and based on ρ(θ,A,B,I_{AB}) an average topology parameter, e_{ij}. Typically primary sequence separations I_{AB} > 5 are not further differentiated and considered long-range. In brief, the topological parameter e_{ij} is related to the probability of finding a shortest path length θ_{ij} = 1 (direct neighbour contact). Summation of all pairwise contributions e_{ij} leads to the compactness value C_i of residue i. Large compactness C_i values are found for residues embedded in the interior of the structure and surrounded by many neighbouring residues whereas small (even negative) values are found for flexible residues located on the surface and exposed to the solvent. The local secondary structure of proteins is assessed by using statistical distribution function ρ(θ,A,B,I_{AB}) with sequence separations I_{AB} ≤ 4. The local secondary structure parameter S_i is defined as S_i = "P_i - P_{i-b} where "P_i and "P_{i-b} are defined as: "P_{i} = N_{a} - N_{b} - N_{c} - N_{d}, N_{a} = ρ(1,i)*ρ(1,2), N_{b} = ρ(1,i)*(1.0 - ρ(2,i))/(1.0 - ρ(1,i)), N_{c} = Y_{i} - 1, N_{d} = ρ(1,i) and ρ(2,i) denote the probabilities of finding shortest path length values of 1 and 2 between residues i and j. N_{a} and N_{b} are empirical constants ensuring comparable compactness and local secondary structure values. Typically, residues located in α-helices display positive S_i values whereas for residues located in extended regions (β-strands or polyproline II helices) significantly smaller (negative) values were observed. Of particular relevance is the fact, that this sequence analysis provides quantitative information about the local secondary structure and residue compactness for each residue position.

The statistical distribution functions ρ(θ,A,B,I_{AB}) used for the calculation of meta-structural parameters were extracted from proteins predominantly crystallized under near neutral pH conditions. Under these conditions the carboxylic groups of Asp and Glu side-chains are predominantly deprotonated (negatively charged). The residue types in ρ(θ,A,B,I_{AB}) thus refer to negatively charged Asp and Glu side-chains. Because distribution functions for protonated Asp and Glu side-chains are not directly accessible through crystal structure analysis, we mimic protonation events by replacing Asp and Glu side-chains by Asn and Gln in the primary sequence. The rationale for our approach is as follows: Firstly, this amino acid replacement ensures electroneutrality (on protonation of carboxylate) of the side-chain. Secondly, the Asn/Gln amide groups are chemically comparable to protonated Asp/Glu carboxyl groups. This is due to the fact that both, amide groups in Asn/Gln and carboxyl groups of protonated (neutral) Asp and Glu, are planar and can act as hydrogen bond donors. The respective meta-structure parameters are calculated for both wild-type (negatively charged Asp/Glu side chains) and “mutated” protein (neutral Asn/Gln) forms. Subsequent differences in meta-structure derived local structure and compactness values are correlated with pH-induced alterations of local backbone geometry and compaction of the polypeptide chain.

NMR spectroscopy and signal assignment

All spectra were acquired at 298 K on an Agilent Direct Drive 700 MHz (BASP1) or Varian Unity 500 and 600 MHz (Tc4, 1 mM, pH 4.5 and 2) spectrometer using standard 5 mm 1H,13C,15N triple-resonance probe heads. NMR data for Tc4 were obtained on spectrometers at 298 K. Signal assignment was achieved as described elsewhere. PFG
NMR diffusion measurements were performed and analyzed as described elsewhere.\textsuperscript{42}

**EPR spectroscopy**

Double electron-electron (DEER)\textsuperscript{27,43,44} was applied to glassy solids obtained by freeze-quenching aqueous solutions of BASP1 double mutants (MTSL labeling at Cys-mutated residues 110 and 229 of the chicken analogue of human BASP1) after addition of 30 v/v % glycerol. More details of the experimental procedure are given in the Supporting Information.

**Acknowledgments**

M.A.H. acknowledges the support of Professor Dr. Hassan Eisa, Dr. Alaa El-Din Barghash, and Dr. Laila A. Abouzeid; Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Mansoura University, Egypt.

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