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Extracting structural information from charge-state distributions of intrinsically disordered proteins by non-denaturing electrospray-ionization mass spectrometry

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Intrinsically disordered proteins (IDPs) exert key biological functions but tend to escape identification and characterization due to their high structural dynamics and heterogeneity. The possibility to dissect conformational ensembles by electrospray-ionization mass spectrometry (ESI-MS) offers an attracting possibility to develop a signature for this class of proteins based on their peculiar ionization behavior. This review summarizes available data on charge-state distributions (CSDs) obtained for IDPs by non-denaturing ESI-MS, with reference to globular or chemically denatured proteins. The results illustrate the contributions that direct ESI-MS analysis can give to the identification of new putative IDPs and to their conformational investigation.

Introduction

Intrinsically disordered proteins (IDPs) lack a well-defined three-dimensional structure under physiological conditions of pH and salinity and in the absence of a partner or ligand. Some IDPs however fold, partially or completely, into ordered conformations upon binding to specific interactors.[1-23] The extreme structural plasticity that characterizes these proteins allows for independent tuning of affinity and specificity, recognition of multiple targets, fast association kinetics and effective regulation by post-translational modifications.[19,24] Probably due to these features, IDPs play key regulatory roles inside the cell.[10,13,14,25-28] According to disorder-prediction algorithms, ~30% of the eukaryotic proteins are mostly disordered and ~40% possess disordered regions longer than 50 residues.[29-33]

A big effort is being devoted to the investigation of IDP binding to their folded interactors. The structure of numerous complexes has been solved by X-ray crystallography, describing interaction surfaces and acquired ordered structure, although complexes where IDPs retain their flexibility have been reported, too.[18,24,34-37] To understand the mechanism of molecular recognition, it is also important to characterize the structural properties of the pre-recognition state. The flat energy landscape of IDPs in solution implies that they exist as highly dynamic and heterogeneous conformational ensembles, which escape structural characterization by conventional biophysical methods.[19,20,22,38,39] Nevertheless, progress has been made in conformational analysis of IDPs in their free state by different biophysical methods.[40] The emerging picture is that IDPs in the absence of interactors populate metastable, partially folded states with preformed elements of secondary structure (intrinsically folded structural units, IFSU) and relatively compact tertiary structure.[15-17,37,41-46] These partially folded conformers are thought to be functionally relevant, providing seeds for interaction surfaces and/or protecting IDPs from degradation and from non-specific interactions.[19,35,47-50]

Mass spectrometry offers peculiar advantages in the analysis of complex mixtures, thanks to the possibility to detect not only distinct masses, but also different conformers endowed with variable degrees of compactness in the molecular ensemble.[51-56] Direct assessment of species distributions, without averaging over the molecular population, offers a valuable tool in IDP analysis, complementary to other biophysical methods. This paper focuses on the contributions that charge-state distribution (CSD) analysis by non-denaturing electrospray-ionization mass spectrometry (ESI-MS) can give to IDP identification and characterization.

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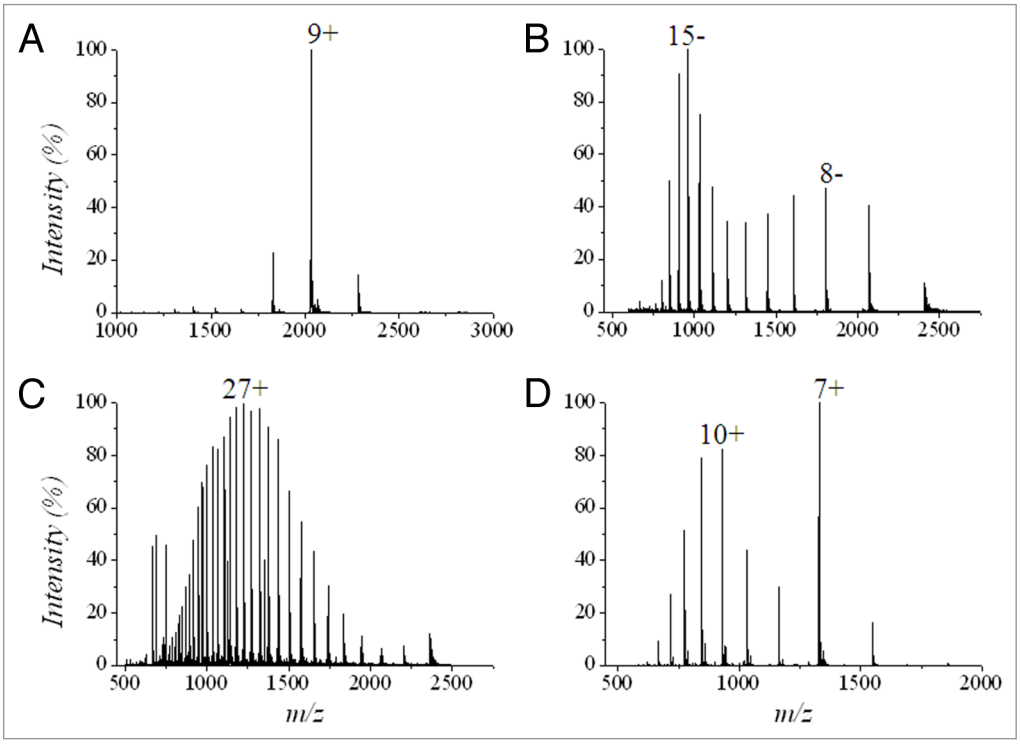

Figure 1. Examples of CSDs obtained by nano-ESI-MS under non-denaturing conditions. The spectra were recorded on a hybrid, quadrupole time-of-flight mass spectrometer (QSTAR Elite, AB-Sciex). Samples were infused at room temperature, by metal-coated borosilicate capillaries with emitter tips of 1 μm internal diameter (Proxeon). The following instrumental settings were applied: declustering potential 60/80 V, ion spray voltage 1.1/1.2 kV, curtain-gas pressure 20 psi. (**A**) 12 μM β-lactoglobulin in 10 mM ammonium acetate, pH 7.0.[100] (**B**) 12 μM α-synuclein in 10 mM ammonium acetate, pH 7.4 (negative-ion mode).[101] (**C**) 15 μM Sic1 in 50 mM ammonium acetate, pH 6.5.[102] (**D**) 10 μM Sic1$^{215-284}$ in 50 mM ammonium acetate, pH 6.5.[103]

Charge State Distributions

Mild desolvation/ionization conditions allow for maintenance of native-like protein conformations during the electrospray process.[57] Evidence for that has been obtained by experimental and computational studies. Experimental evidence is based on direct investigation of the structural properties of gas-phase protein ions by ion mobility,[58-63] electron-capture dissociation,[64,65] gas-phase hydrogen exchange[66,67] and binding analysis.[68-70] Computational studies suggest that attractive interactions inside the protein structure can compensate to a certain extent for repulsive forces introduced by protein ionization.[71-83] This effect is mainly due to hydrogen bonds and zwitterionic states, although minor contributions could also derive from dispersion forces and cation-π interactions. Altogether, intramolecular interactions of native protein structures provide a tremendous self-solvation potential that contribute to the stability of the gas-phase protein ions generated by electrospray. In particular, it has been shown that folded protein conformations tend to shrink upon desolvation, increasing the number of intramolecular hydrogen bonds and the exposure of hydrophobic residues on the protein surface.[71,73]

In order to prevent protein denaturation during electrospray, it is important to avoid the use of organic solvents and extreme pH values, and to apply mild temperature and voltage conditions to the sample source. Particularly well suited to this end are nano-electrospray devices, where a micrometer-scale capillary for sample infusion leads to smaller first-generation droplets and, consequently, more effective desolvation under soft conditions of temperature and voltage.[84,85] Nano-ESI-MS is now routinely applied to conformational studies, also offering the advantages of low sample consumption, automation, and experimental scale-up.

Protein CSDs can deliver important structural information, thanks to the fact that protein compactness in the original solution has a strong influence on the extent of protein ionization under electrospray conditions. The higher the structural compactness, the lower the average net charge that will be observed for any given protein.[54] However, CSDs can also be affected by several other factors, such as instrumental parameters and solvent properties.[86-89] Therefore, it is important to keep the experimental conditions accurately controlled and to make sure that none of the applied instrumental settings becomes the limiting factor for CSD features. It should also be taken into account that measurements in negative-ion mode could be more exposed to the risk of electrospray-induced protein unfolding.[90] By the application of the most adequate and controlled operative conditions, conformational effects dominate protein CSDs obtained by ESI-MS. Good agreement between ESI-MS and solution methods has been shown monitoring unfolding transitions of proteins induced by acids, organic solvents and heat.[51,52,87,91-96] Nevertheless, it should also be reminded that the signal yields of folded and unfolded conformations of the same protein can differ substantially, particularly at

Table 1. Proteins analyzed in this work

Protein name[a]	Species	MW	Reference
Sic1$^{215-284}$	*Saccharomyces cerevisiae*	9293.38	103
Prothymosin-α	*Homo sapiens*	12073.85	110
Sic1$^{187-284}$	*Saccharomyces cerevisiae*	12676	98
Ataxin-3$^{182-291}$	*Homo sapiens*	13089.7	111
α-synuclein	*Homo sapiens*	14460.16	112
NTAIL	*Measles virus*	14633	109, 113, 114
NTAIL	*Nipah virus*	14949	Unpublished data
NTAIL	*Hendra virus*	15241	Unpublished data
Sic1$^{1-186}$	*Saccharomyces cerevisiae*	21593.13	98
PNT	*Measles virus*	24821	115
Sic1$^{full-length}$	*Saccharomyces cerevisiae*	33102.88	102

[a]Sic1, substrate/subunit inhibitor of cyclin-dependent protein kinase; NTAIL, C-terminal domain of the viral nucleoprotein N; PNT, N-terminal domain of measles virus phosphoprotein P.

high flow rates and high protein concentrations, rising the need of specific control experiments for quantitative analysis.[97]

Major structural heterogeneity in the molecular ensemble of a pure protein results in multimodal CSDs, where distinct peak envelopes can be resolved and quantified by deconvolution algorithms.[96,98,99] The broadness of each peak envelope, in turn, is affected by structural dynamics, with narrow profiles associated to folded structures and broad profiles associated to disordered states. Examples of CSDs obtained for IDPs by non-denaturing ESI-MS are reported in **Figure 1**, in comparison to a natively folded globular protein.[100-103] As it can be noticed, IDPs under non-denaturing conditions give rise to broad CSDs with high average charge, frequently present as distinct components of multimodal profiles. The different behavior of an IDP and a globular standard under identical conditions can be further highlighted by control experiments where the spectrum of a mixture of the 2 proteins is acquired, followed by identification of the distinct components by their specific masses.[104] The peculiar response of IDPs to electrospray offers the possibility to develop a signature for this class of proteins by non-denaturing ESI-MS, as discussed in more detail below.

Charge-to-Mass and Charge-to-Surface Relation

Besides conformational properties, also protein size has an influence on protein ionization by electrospray. Folded globular proteins follow a well-known, mass-to-charge relation, with the average experimental charge growing approximately as the ~0.57 power of the protein mass expressed in Daltons.[105-108] It has been shown that the behavior of folded and unfolded proteins can be distinguished by relating charge to protein mass, while it is unified by relating charge to solvent accessible surface area (SASA).[109] In other words, SASA seems to dictate the extent of protein ionization, regardless of the conformational state. If we relate charge to protein mass, instead, disordered protein conformations stand as clear outliers in the plot of folded globular proteins. This is true for IDPs under non-denaturing conditions, as well as for chemically denatured proteins, indicating that solvent effects play a marginal role in this regard.[109]

Figure 2 shows the charge-to-mass plot for several IDPs that have been analyzed by ESI-MS. These proteins were either already known as IDPs, or identified as such in our laboratory by independent evidence, like circular dichroism (CD) and nuclear magnetic resonance (NMR). Their names, sources and masses are listed in **Table 1**.[98,102,103,109-115] The data reported in **Figure 2** summarize the ionization behavior of these IDPs by nano-ESI-MS under non-denaturing conditions. For those cases yielding mass spectra with bimodal distributions, the values of average charge have been calculated on each distinct component. The data are plot with reference to the curves of natively folded, globular proteins under denaturing or non-denaturing conditions. Two observations are straightforward: (1) IDPs have at least 1 component that is a clear outlier relative to the reference curve of globular proteins measured under non-denaturing conditions and, rather, approaches the behavior of chemically denatured proteins, and (2) many IDPs have 1 component that is surprisingly close to the reference curve of folded, globular proteins. The relative amounts of the different forms, shown by the color code in the figure, can vary significantly, depending on the protein and the environmental conditions. These results are consistent with a remarkable conformational heterogeneity of IDPs in solution under non-denaturing conditions. The strong deviation from the typical ionization behavior of globular, folded proteins measured under the same conditions indicates that CSD analysis by ESI-MS offers a tool to identify putative IDPs. At the same time, these results illustrate that ESI-MS can capture compact states populated by IDPs, even if highly dynamic and poorly represented. We discuss below the available evidence that supports the interpretation of these spectral components in terms of IDP conformational states.

High-Charge Component

As shown in **Figure 2**, the high-charge component is typically predominant in ESI-MS spectra of IDPs with bimodal distributions. As generally seen for chemically denatured proteins, it is also quite broad, reflecting further heterogeneity due to structure dynamics. Most importantly, when this component is analyzed as a function of mass, it follows the same power law as the denatured conformation of natively folded proteins. Furthermore, its intensity varies in response to solvent composition, typically accompanied by opposite changes in the low-charge component. These observations strongly suggest that the experimental CSDs reflect the dynamic conformational ensemble of IDPs in solution.

Altogether, this evidence supports identification of the high-charge component as a highly disordered conformational state of IDPs, characterized by low structural compactness. The presence of such a component under non-denaturing conditions can be considered as a signature of IDPs and could be used for fast

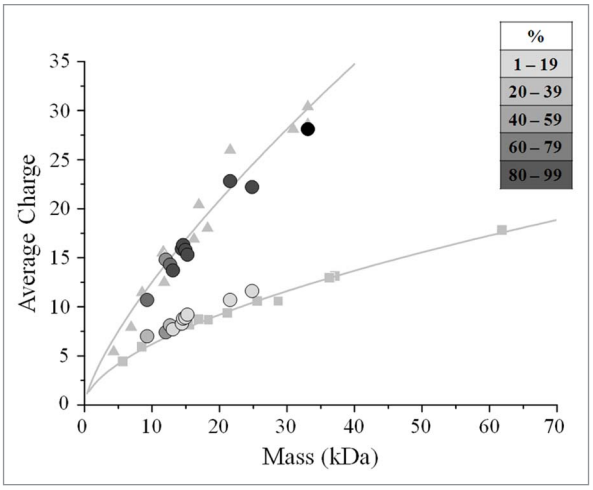

Figure 2. Charge-to-mass plot for distinct components of some IDPs analyzed by non-denaturing ESI-MS. In the case of bimodal distributions, each component is represented by a circle, colored according to the relative abundance as specified in the inserted table. For unimodal distributions (unique case of full-length Sic1), the global average charge is considered and the symbol is colored in black. The gray small squares and triangles represent data for globular proteins, respectively under non-denaturing and denaturing conditions. The gray lines are interpolations by power-law functions.

screening of putative new members of this conformational class. It is important to underscore that the anomalous ionization behavior of IDPs is evident also when the protein contains only a disordered tract, together with a normally folded domain, like in the case of Ataxin-3.[104] Furthermore, it has been observed in either positive- or negative-ion mode.[101,112]

Low-Charge Component

In most of the considered IDPs, a sharply bimodal CSD suggests that a small fraction of the molecular population exists in a highly collapsed state. It is surprising that such a component approximates the ionization behavior expected for folded, globular proteins of the same size. Nevertheless, it is known by several other independent methods that IDPs in solution often populate partially structured states and can collapse into compact globular structures.[22,116] These states are potentially relevant for protein function. The challenge in trying to characterize these states is that they are highly dynamic and poorly represented in the molecular ensemble, easily escaping characterization (and even detection) by biophysical methods. It is, therefore, extremely attractive to directly visualize minor, structured components, out from the background of the predominant conformers, thanks to the ion-sorting properties of MS measurements. At the same time, it is important to collect evidence supporting the interpretation of these spectral components in terms of protein conformation.

One of the most important evidence is that these components can be progressively depleted by varying the solvent properties, and that this response is protein-specific. For instance, the low-charge component of Sic1 kinase inhibitory domain (KID) is selectively lost by acidification whereas it accumulates in response to the same treatment in the case of α-synuclein.[101,103,112] Furthermore, such a transition can be quite cooperative, as in the case of the pH-dependence of Sic1-KID compact form.[103] Thus, it seems that IDPs compact states can be "denatured" by particular agents, in a way that is not so dissimilar from unfolding transitions of natively folded proteins. It is also important to point out that these transitions are characterized by changes in the relative amounts of the different components, as expected for an interconversion process, and not by progressive shift of a given peak envelope in the spectrum, as more typical for the effects of solvent composition or parameter setting on ESI-MS data.[86]

Furthermore, IDPs compact conformers corresponding to the low-charge components have been isolated and identified in gas phase by ion-mobility methods.[58-62] This technique adds a second dimension to the ion sorting mechanism of MS measurements, based on drift time through a buffer gas. Since ion mobility depends on collisional cross section, compact conformers will be faster and will be separated from elongated conformers inside the drift cell. Thus, thanks to the survival of non-covalent interactions responsible of protein compaction, the distinct physical properties of the desolvated ions can be highlighted by a criterion that is orthogonal to the *m/z* measurement of conventional MS analyses.

Another important evidence is given by the specific ligand-binding properties of these components. Such a behavior has been observed, for instance, in the case of copper binding by α-synuclin.[101] Complexes with a 1:1 stoichiometry could be identified by ESI-MS upon metal binding. However, CSDs analysis revealed that the bound form is mainly detectable in the low-charge component of the protein spectrum. This result further supports the hypothesis that such a component corresponds to a distinct conformer of the protein ensemble, displaying higher compactness and higher propensity for metal binding. The relative amount of the compact form also increases upon copper addition, indicating that the protein undergoes a process of induced folding promoted by copper binding.

Finally, computational studies can provide further insight on compact conformers of IDPs in solution. Structural models have been developed by molecular-dynamics simulations for the compact states of Sic1-KID.[117] Although these methods are not adequate to describe the actual equilibrium between compact and extended conformations, they effectively model the forces responsible for protein compaction and can, therefore, generate putative structures for IDP compact states. The most probable structures generated by simulations on Sic1-KID display IFSUs and considerable degree of compaction. The computational results find support in the available experimental data. For instance, intramolecular interactions in the computational models were found to be prevalently of electrostatic nature, with minor contributions of hydrophobic interactions.[117] This result is in agreement with the observed strong effect of acids and negligible effect of organic solvents on the ESI-MS spectra of this protein.[103,117] Furthermore, the SASA estimates based on the computational models (53–65 nm^2) are in good agreement with those derived by the ESI-MS data for the low-charge component of the CSD (59.78 nm^2).[103,109]

These studies strongly suggest that the low-charge components frequently detected in the ESI-MS spectra of IDPs under

non-denaturing conditions correspond to metastable compact conformers with specific structural and functional properties.

Conclusions

The ionization properties of IDPs under electrospray conditions reveal significant and systematic deviations from the reference behavior of folded, globular proteins. These differences emerge by measurements under non-denaturing conditions and analysis of average charge by the charge-to-mass relation. Thus, CSD analysis offers an effective tool for high-throughput conformational screening aimed at the identification of putative new members of this structural class. Confident protein classification will then require further analysis by other techniques, such as CD, size-exclusion chromatography and NMR.

Non-denaturing ESI-MS is also a powerful tool for the identification of partially structured states of IDPs, even if highly dynamic and scarcely populated. The generally applicable charge-to-surface relation, which holds independently of the conformational state, allows extracting useful structural information by SASA calculation for each detectable component. This information can be used as a constraint for computational modeling, possibly integrating other kinds of experimental measurements.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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