Mass spectrometry beyond the native state

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Abstract

Native mass spectrometry allows the study of proteins by probing in vacuum the interactions they form in solution. It is a uniquely useful approach for structural biology and biophysics due to the high resolution of separation it affords, allowing the concomitant interrogation of multiple protein components with high mass accuracy. At its most basic, native mass spectrometry simply reports the mass of intact proteins and the assemblies they form in solution. However, the opportunities for more detailed characterisation are extensive, enabled by the exquisite control of ion motion that is possible in vacuum. Here we describe recent developments in mass spectrometry approaches to the structural interrogation of proteins both in, and beyond, their native state.

Highlights

- Native mass spectrometry allows the interrogation of protein conformational space
- Manipulation of charge populates different minima in the protein folding funnel
- Gas-phase activation allows trajectories out of the folding funnel to be investigated
- Labelling provides an additional dimension for probing protein surfaces in vacuum
- Spectroscopy and imaging promise direct high-resolution structural information

Main text

Introduction

Native mass spectrometry (MS) is the study of proteins and other biomolecules in the vacuum of a mass spectrometer, having initially maintained their noncovalent interactions upon transferral from solution [1,2]. A variety of experimental data and simulations have shown that the native state of proteins and their complexes can persist on the millisecond timescale of MS experiments [3,4]. This provides ample opportunity for interrogation of not just native structure, but also the free-energy landscape accessible to the protein [5,6]. This can be viewed in terms of a canonical protein (un)folding funnel, where protein ions can populate states differing in their atomic coordinates and energy (Fig. 1). While the native states of proteins are relatively accessible to structural biologists and biophysicists, probing states populated only to low levels at equilibrium, or fleetingly during assembly and folding, remains extremely challenging [7]. Here we discuss how and why MS-based experiments can contribute to this endeavour.

We first consider how the protein folding funnel can be explored within the mass spectrometer, discussing the role played by protein charge state in populating different minima. We evidence how these represent starting points for exploring higher levels of the protein folding funnel by activating ions within the mass spectrometer. In the second part of this review, we describe emerging gas-phase labelling, spectroscopy, and imaging methods that are being integrated with native MS. These approaches promise to provide unprecedented detail regarding the structure of proteins in vacuum, including during excursions from the native state.

Coulombic exploration of the free energy landscape

Protein ions generated by (nano)electrospray ionisation each populate a distribution of charge states. Oftentimes the gross structure, evidenced by collision cross-section (CCS) values obtained from ion mobility (IM) measurements, appears to be unaffected by this charge state variation [8]. However, in cases where the range of charge states populated is broad relative to the average, or when the protein has an intrinsic tendency towards disorder, significant variations in CCS are observed [9,10]. This demonstrates how, as in solution [11], different charge states of a protein in vacuum correspond to different free-energy minima in the free energy landscape (Fig. 1A).

To investigate how global protein structure depends on charge state, a number of recent studies have performed IM-MS on protein ions that are selected within the mass spectrometer. Charge manipulation has been performed via ion-ion collisions with a reaction partner of opposite polarity, most commonly performed by abstraction of a proton [12,13], or transfer of an electron [14,15]. A number of studies have shown that reduction of charge on native conformers of ubiquitin and cytochrome C results in a step-wise compaction [13-18]. In other cases, increases in CCS upon charge reduction are observed [14,19]. This demonstrates the impact of charge on the initial position on the folding funnel, and that other local minima can be occupied by charge modulation. These minima therefore represent alternative starting points for exploration of the conformational landscape.

In order to navigate the protein folding funnel, the ions can be activated within the mass spectrometer (Fig. 1), typically by energetic collisions with a bath gas. By varying the amount of energy supplied, the types of bonds broken and level of structural information obtained can be controlled. At a low level of gas-phase activation, proteins first undergo structural rearrangements and unfolding due to breakage of intramolecular non-covalent

bonds [20]. IM-MS measurements have shown that this occurs via a number of discrete transitions in CCS (Fig. 2A), diagnostic of the trajectory taken by the protein as it escapes the folding funnel. The structural evolution of a protein depends strongly on its charge state [15,17,21], and tandem IM-MS experiments reveal complex conformational inter-dependencies [22], consistent with the view of different minima in the folding funnel being populated and leading to alternative pathways out (Fig. 1).

As a consequence, the transitions observed and the energies at which they occur represent a characteristic signature for the protein charge state being investigated. This can be exploited as a fingerprint for comparison, enabling, for instance, distinction between different antibody structures and interactions [23-26]. Unfolding has also been actively pursued as a measure of ligand-stabilisation of protein structure [27], revealing measures consistent with solution data [28], as well as reflecting interactions that are strengthened in vacuum [29]. The number of unfolding transitions appears to reflect the number of independent structural domains seen in solution [30]. However, assignment of the transitions observed to individual regions of the protein is difficult. One way to address this is to use specific small-molecule probes, and correlate their release upon activation with the transitions in CCS (Fig. 2B)[31]. Potentially a more general strategy will come from performing electron-mediated fragmentation of the protein during its unfolding pathway [32].

Increasing activation further results in the dissociation of non-covalently attached units, protein subunits or other ligands. In general, dissociation of multimeric assemblies appears to proceed via the expulsion of single, highly charged subunits [20]. The identity of the dissociation products can yield compositional information on the complex, while the asymmetric partitioning of charge between products can be exploited to mine spectra not resolvable by MS alone [33-35]. However, fast activation, in particular by collision

with a surface, can lead to information regarding the assembly and architecture of the complex [36,37]. This can be rationalised by the activation allowing access to the higher reaches of the protein energy landscape without transitioning through lower-energy intermediates via polypeptide unfolding [38]. Remarkably, this information appears to be retained on the timescale of seconds [39].

At the highest energies accessible in the mass spectrometer, covalent fragmentation of the polypeptide backbone can be induced (Fig. 2A)[20]. This means that "top-down" proteomics is possible from the starting point of a native protein assembly [40]. Exploiting this capability enables the attribution of properties of individual protein assemblies to differences in their primary sequence [41]. A particularly attractive means for reducing native proteins into peptide fragments comes from irradiation of a protein complex using UV photons [42]. This has been shown to operate on a fast timescale, providing comprehensive sequence coverage [43,44], while retaining non-covalent interactions [43] and promoting a more symmetric dissociation pathway [45]. Interestingly, regions of enhanced fragmentation correlate reasonably well with solvent exposure in solution suggesting that aspects of the native fold are retained (Fig 2C)[46]. Similar correlations have also been found when using electron-mediated fragmentation [32,47].

Structural interrogation of native and activated states

Aside from mass and charge, the information most frequently obtained from native MS experiments is CCS, through the implementation of an orthogonal IM dimension [48]. While the CCS contains significant information on the global shape of proteins [49,50], and can be used to aid structural modelling [51,52], complementary approaches are

needed to probe structure on a local level. An attractive strategy is to perform labelling experiments analogous to those in solution [53], in the vacuum of the mass spectrometer. This provides a means for probing the surface of a native protein, mass-separated from other species in solution. Furthermore, it can be used to interrogate the structures populated along the activation pathway, allowing for a detailed understanding of surface interactions and interfaces (Fig.2A, lower panels).

Gas-phase labelling strategies have mainly involved hydrogen-deuterium exchange (HDX) reactions by collisions between the protein ion and a deuterated vapour [54]. The level of deuterium uptake, which manifests itself as a mass increase, has been shown to be sensitive to protein conformation in solution [55-57], charge state [56,58], and structural changes due to collisional activation [59]. Differences in deuterium uptake between a complex and its constituents are a measure of the occlusions and interactions at the interface. By dissociating the labelled complex to examine the partitioning of uptake over the constituents, information as to the associations made can be obtained (Fig. 2D)[60]. This experiment relies on the activation not causing significant "scrambling" of deuterium labels between the dissociation products, a phenomenon that poses a challenge for increasing the spatial resolution of solution-phase HDX [61]. However, it appears that scrambling across non-covalent interfaces is relatively minimal [62].

To extract the maximal amount of information from a labelling experiment requires localisation of the labels to individual amino acids. This is in principle possible using a top-down strategy, with the proviso that the activation that produces the peptide fragments outpaces any proton scrambling. The prospect of achieving this comes from the coupling of fast activation techniques with gas-phase HDX [63-66], but has yet to be explored fully on native proteins and assemblies.

An alternative means to probing protein structure in vacuum directly is afforded by spectroscopic studies. Notably, gas-phase IR spectra display distinguishing features that mirror those obtained from measurements in solution. This has been exploited to reveal the retention of protein secondary structure in native charge states [67], and the formation of new structures upon activation [68], while measurements on green fluorescent protein allowed the role of solvent on the chromophore to be probed [69]. Spectroscopic methods also have the potential for reporting intra-molecular distances. For instance, recent data has shown that Förster resonance energy transfer experiments in the gas phase are feasible [70,71]. A related means to obtain distance measures, which has the benefit of not requiring the addition of an extrinsic fluorescent label, comes from taking advantage of excitation energy transfer [72]. By studying disulphide bond cleavage, the proximity of aromatic amino acids can be determined through residue-specific absorption behaviour [73].

Though many of the techniques discussed above push MS-based methodologies towards revealing more structural information, the ideal would be to directly image individual proteins in the gas phase, rather than inferring structure from an ensemble. The attraction from an imaging perspective is the isolation of the protein, and the control afforded in the gas phase. Recent studies show that this ideal is not unrealistic (Fig. 3). Intact protein and protein complex ions can be mass selected for “soft landing” onto a surface for imaging [74]. While imaging the native structure is of obvious interest, the technique can also report on the conformational space accessible to the proteins as a function of charge state (Fig. 3A)[75]. Landing native ions can also preserve intermolecular non-covalent interactions, with electron microscopy revealing structural differences between mass-selected species (Fig. 3B)[76]. Recently, low-energy electron holography has achieved sub-nanometre resolution on single molecules, soft-landed onto

ultra-clean graphene surfaces. Strikingly, native orientations of the landed proteins can be clearly seen (Fig. 3C)[77]. An alternative approach, that does not require deposition on a surface, is to use an X-ray free electron laser which allows for the diffraction data from individual molecules to be obtained before their destruction. The current state-of-the-art is ≈20-nm resolution, but nonetheless different orientations of reconstructed single particle images of carboxysomes show good agreement with predicted geometric models (Fig. 3D)[78]. These studies highlight how the integration of imaging approaches with the selection capabilities, and potentially ion alignment [79], of MS could enable novel methods for high-resolution structure determination.

Perspective

In the 25 years since its inception, native MS has become an important approach for structural biology, and enabled many investigations into the structure that proteins populate at equilibrium. Here we have presented the case that this approach has great potential, some of which has already been realised, for probing the conformational space of proteins. However, while the preservation of native structure in the gas phase is clear, it remains to be seen to what extent the free-energy landscape resembles that in solution. In the absence of water, and consequently any competition between protein-solvent and intra-molecular solvation, the landscape will be more rugged, with higher barriers to conformational inter-conversion [5]. However, an important question is to what extent gas-phase trajectories in the protein folding funnel resemble the functional excursions from the native state in solution. Interestingly, it has been shown that, in protein crystals, mechanics stimulated by the application of external electric fields that act on local dipoles resemble the biologically relevant motions [80]. This suggests that exploration of the protein folding funnel in vacuum, where Coulombic interactions are dominant, will likely

encompass functional trajectories. The literature we have highlighted here reveals a key aspect of native MS, namely that the control afforded by ion manipulation in vacuum allows the implementation of multiple experiments on the same ion. The versatility, and multi-pronged nature of the approach will be key in probing the free-energy landscape of proteins.

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[31] Elegant study showing the elucidation of the gas-phase unfolding pathway for a multi-domained protein, by combining ion mobility and tandem mass spectrometry measurements on chemical-probe ligands.

[46] Demonstration of the versatility and applicability of UV photo-dissociation for probing native proteins via top-down mass spectrometry. The sensitivity of the approach to both tertiary and quaternary structure is evidenced.

[60] First demonstration of the use of gas-phase hydeogen-deuterium exchange to directly report on the binding interface in a protein-ligand complex. The authors demonstrate how uptake differences can report on the global structure, and localise contributions to the individual components in a way that reflects the interactions in the crystal structure.

[67] Report describing how the secondary structure of proteins can be obtained in the gas phase by IR action spectroscopy. As in solution, spectral features differ between the β-sheet protein β-lactoglobulin and α-helical myoglobin, indicating that their conformations are retained in vacuum.

[73] Characterisation of the excitation energy transfer process for a peptide possessing Phe and Tyr residues. Understanding the energy transfer process, and its stong distance dependence, provides a valuable constraint for guiding molecular dynamics simulations.

[77] Demonstration of the use of low-energy electron holography at good resolution, on soft-landed 'native' proteins. A powerful demonstration on how three-dimensional imaging on a truly single molecule level can be realised.

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[17] Comparison of collision cross-section distributions for the products of proton transfer reactions on ubiquitin generated from denaturing conditions compared to the native structure. This work demonstrates the significant impact charge can play on ion structure and the conformational space an ion traverses.

[29] Combined experimental and simulation study revealing the structural rearrangements made by membrane proteins upon activation in the gas phase, and the origins of lipid-induced stabilisation.

[63] The gas-phase deuterium uptake of mobility-selected peptides is localised at a residue level using electron-mediated fragmentation. Demonstration of the detailed kinetic data that can be obtained by coupling rapid fragmentation to gas phase labelling.

[55] Gas-phase hydrogen-deuterium exchange is used to monitor protein structural pertubation in solution, and is shown to be sensitive to changes in secondary structure consistent with solution measurements.

[44] Selective dissociation of compact or extended conformations using UV photo-dissociation results in differing fragmentation efficiencies. Coupling ion mobility measurements provides further evidence for this method's sensitivity to secondary structure.

Figure legends

Figure 1

Proteins exist on a rugged free-energy landscape in vacuum [5], that can be framed in terms of a protein folding funnel with the native state at the global minimum [7]. Different charge states of the protein can be considered as other minima on this surface, and thereby represent alternative starting points for exploration of the funnel. This can be achieved by gas-phase activation of the protein ions [20], enabling access to different unfolded states. At high levels of activation, dissociation of non-covalently bound units and even fragmentation of the polypeptide backbone can occur, providing information as to protein composition and identity.

Figure 2

Gas-phase activation and labelling are two orthogonal and complementary dimensions for interrogating the conformational states of proteins and trajectories in their folding funnel (Fig. 1).

A Activation provides structural information via the breaking of intra- and inter-molecular bonds. This leads unfolding, dissociation of non-covalently bound ligands or subunits, and fragmentation of covalent bonds (upper row, left to right, and Fig. 1). These processes can provide information on the identity, composition and structure of native and non-native states. Labelling instead relies on the formation of new bonds at surface-exposed sites. MS is sensitive to these reactions due to a shift in mass from the unlabelled species (dotted lines) upon introduction of the labelling agent. Labelling efficiency is sensitive to the nature of the species present and so differences can provide another means of informing on structure, across the activation spectrum (upper row, left to right).

Some of the insights that can be obtained from gas phase activation, in the absence of presence of labelling agents, are inset, together with idealised schematics of anticipated IM-MS data.

B Gas-phase unfolding pathway of the three-domain human serum albumin upon collisional activation [30]. The degree of ligand dissociation over the unfolding trajectory was determined for five ligands known to bind individual domains specifically to assign specific regions of the protein to the transitions observed. The points at which 50% of each ligand (red, domain 1; green and yellow, domain 2; blue and cyan, domain 3) is removed are overlaid on the evolution of CCS with activation. Both ligands associated with domain 2 are lost prior to the formation of the first intermediate, suggesting that the first transition is coupled to this domain. The onset of domain 3 dissociation follows next, but the persistence of one of the ligands suggests only partial unfolding has occurred during the second transition. The third and final transition is attributed to both domains 2 and 3.

C UV photo-dissociation of streptavidin leads to both dissociation into subunits, and fragmentation of the backbone [46]. Dissociation is both symmetric (tetramer into dimers) and asymmetric (into monomer plus trimer). Mapping the fragmentation yield (blue) and the solvent-accessibility (red) as a function of residue number shows significant regions of overlap (purple). This provides evidence that fragmentation of the polypeptide backbone can inform on the level of protein tertiary structure.

D Gas-phase hydrogen-deuterium exchange of the trypsin-vasopressin complex allows the interrogation of the binding interface [60]. Greater deuterium uptake is observed when the components are labelled separately (78 and 5.5 Da, respectively) relative to the

intact complex (76 Da), consistent with some exchangeable residues residing in the interface. Dissociation of the complex (spectrum shown), allowed for the individual uptake of each component to be determined, after having been labelled as part of an intact complex. The uptake differences can help inform on those residues involved in making inter-molecular contacts. (CID - collision induced dissociation).

Figure 3

Imaging methods can be coupled to native MS to provide high-resolution views of proteins separated in vacuum.

A Scanning tunnelling microscopy (STM) images of deposited cytochrome C ions shows a difference in unfolding as a function of charge state, with the highest charge states being almost completely extended [75].

B Transmission electron microscopy (TEM) images of mass-selected and soft-landed *apo*- and *holo*-ferritin [76]. The crystal structure of the spherical ferritin cage is shown for comparison, and the cavity occupied with the iron core is illustrated.

C Low-energy electron holograms of bovine serum albumin after soft-landing, reveals images of individual molecules that correspond to the native structures [77].

D Reconstruction projection images of carboxysomes generated from single-particle diffraction patterns from an X-ray free electron laser (XFEL). Corresponding icosahedra are shown alongside in projection-matching orientations [78].

Figure 1

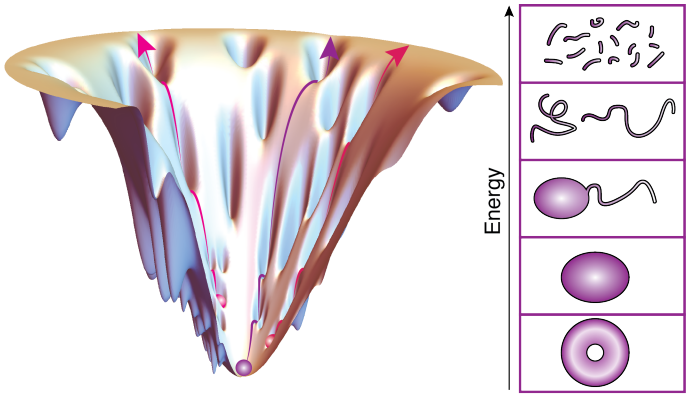


Figure 2

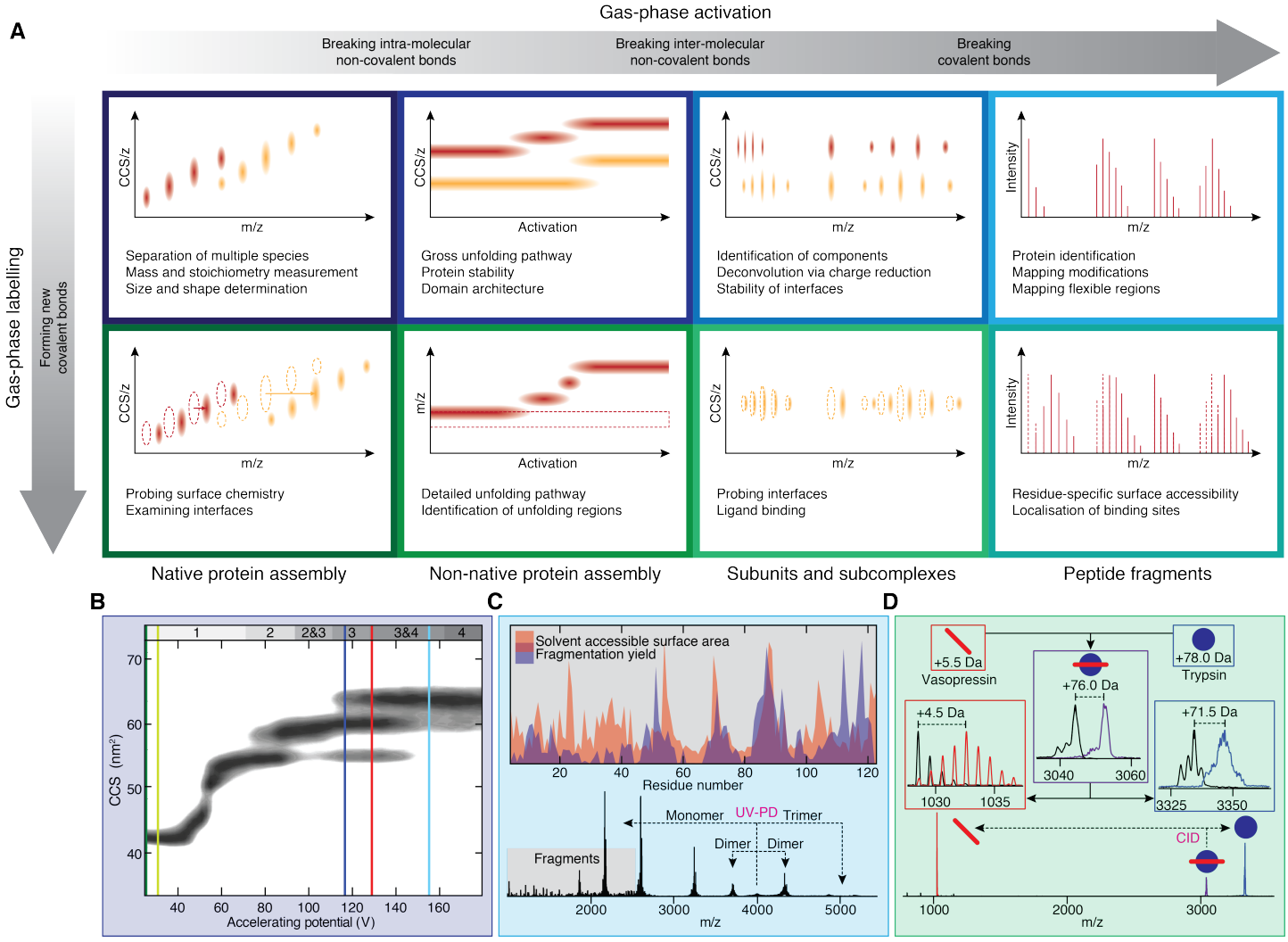
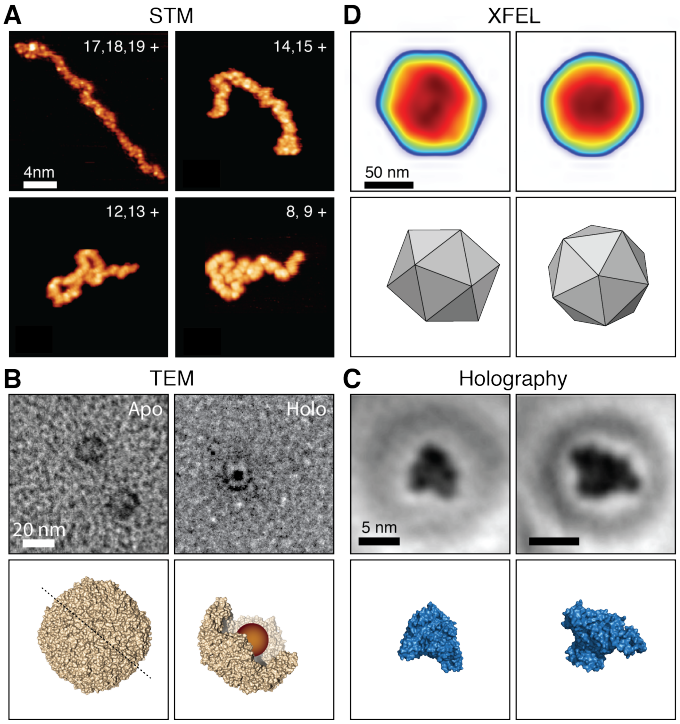

A
Gas-phase activation
Breaking intra-molecular non-covalent bonds
Breaking inter-molecular non-covalent bonds
Breaking covalent bonds
Gas-phase labelling
Forming new covalent bonds
CCS/z
m/z
Separation of multiple species
Mass and stoichiometry measurement
Size and shape determination
Activation
Gross unfolding pathway
Protein stability
Domain architecture
Identification of components
Deconvolution via charge reduction
Stability of interfaces
Intensity
Protein identification
Mapping modifications
Mapping flexible regions
Probing surface chemistry
Examining interfaces
Detailed unfolding pathway
Identification of unfolding regions
Probing interfaces
Ligand binding
Residue-specific surface accessibility
Localisation of binding sites
Native protein assembly
Non-native protein assembly
Subunits and subcomplexes
Peptide fragments
B
1
2
2&3
3
3&4
4
CCS (nm²)
Accelerating potential (V)
C
Solvent accessible surface area
Fragmentation yield
Residue number
Monomer
UV-PD
Trimer
Dimer
Dimer
Fragments
m/z
D
+5.5 Da
Vasopressin
+78.0 Da
Trypsin
+76.0 Da
+4.5 Da
+71.5 Da
CID
m/z

Figure 3


A
STM
17,18,19 +
14,15 +
4nm
12,13 +
8, 9 +
D
XFEL
50 nm
B
TEM
Apo
Holo
20 nm
C
Holography
5 nm