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Current Opinion in Structural Biology

Weighing-up protein dynamics: the combination of native mass spectrometry and molecular dynamics simulations

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Structural dynamics underpin biological function at the molecular level, yet many biophysical and structural biology approaches give only a static or averaged view of proteins. Native mass spectrometry yields spectra of the many states and interactions in the structural ensemble, but its spatial resolution is limited. Conversely, molecular dynamics simulations are innately high-resolution, but have a limited capacity for exploring all structural possibilities. The two techniques hence differ fundamentally in the information they provide, returning data that reflect different length scales and time scales, making them natural bedfellows. Here we discuss how the combination of native mass spectrometry with molecular dynamics simulations is enabling unprecedented insights into a range of biological questions by interrogating the motions of proteins, their assemblies, and interactions.

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Current Opinion in Structural Biology 2019, **54**:50–58

This review comes from a themed issue on **Folding and binding**

Edited by **Ylva Ivarsson** and **Per Jemth**

https://doi.org/10.1016/j.sbi.2018.12.011

0959-440X/

Introduction: interrogating protein structures and motions with native mass spectrometry and molecular dynamics

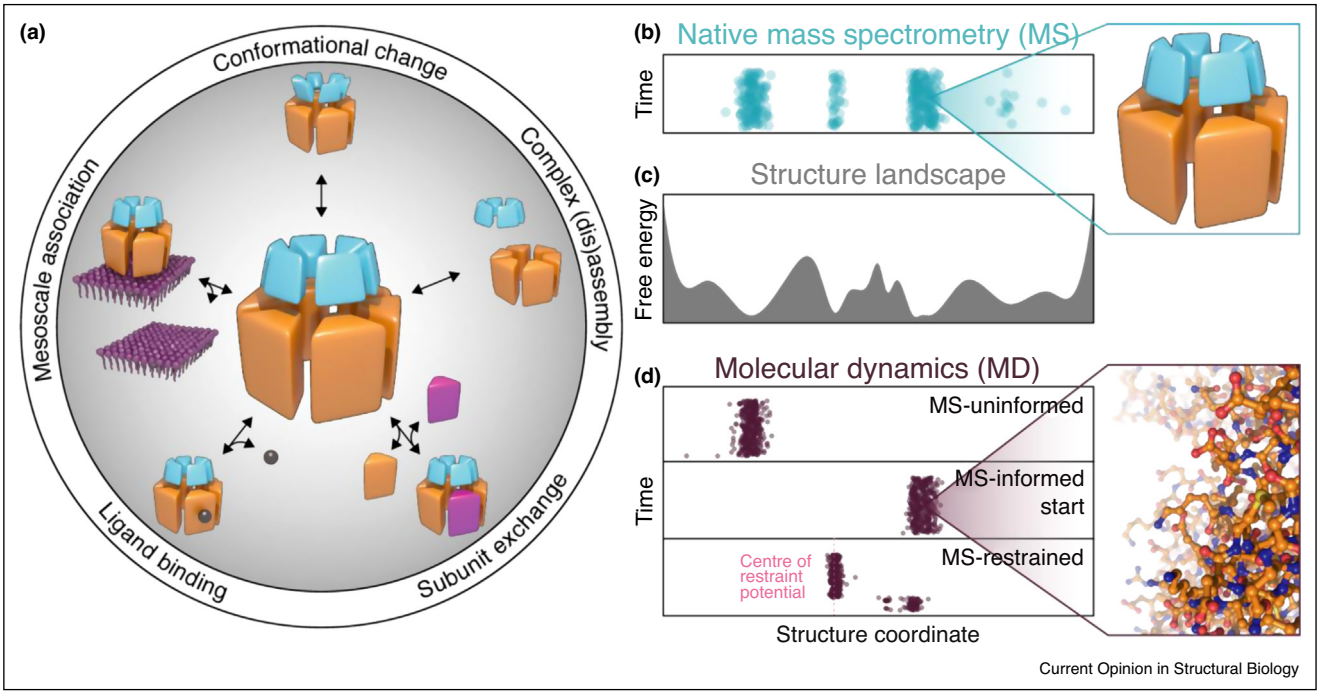
The biological functions of proteins are inherently dependent on the structure and dynamics of the interactions they make. Proteins typically populate a number of structural states, displaying a range of functional conformations, and having different binding partners depending on the cellular conditions and context [1]. These dynamics take place on many levels: fluctuations of the individual subunits' secondary and tertiary structure; the assembly, disassembly and exchange of subunits within a complex; and interactions of varying timescale with other molecules (Figure 1a). The majority of experimental techniques for interrogating the structure and interactions of proteins do not inform on the full range of these inherent dynamics [2], either reporting ensemble averages that obscure key features of the system under study [3], or by reducing the complexity through imposing or engineering homogeneity.

Native mass spectrometry (MS), in contrast, yields spectra that separate (according to mass-to-charge ratio, m/z) the various constituents of the sample in their native assembled state, report their relative abundances, and allow their individual interrogation. The label-free nature, high sensitivity, and unparalleled resolution of separation has made native MS a key technology for life-science research. Coupling native MS to ion mobility spectrometry (IM) allows the sample constituents to be separated further according to the interactions they make as they drift through an inert buffer gas, a process governed by the sizes and shapes of the molecules [4]. As a result, native IM-MS not only allows for two dimensions of separation, but also provides information about the three-dimensional structures in the sample. These capabilities have led to the increasing adoption of native (IM)-MS for structural biology, including the application to proteins made challenging to study because of high levels of heterogeneity, association with membranes, and intrinsic disorder [5•,6–8].

While native (IM)-MS provides a means to probe multiple co-existing states in a single experiment (Figure 1b and c), its resolution in terms of three-dimensional structure is limited. Conversely, molecular dynamics (MD) simulations provide information that is atomically detailed but, despite considerable efforts to enhance sampling, struggle to explore the full conformational space relevant for macromolecular systems [9,10•,11] (Figure 1d). As such, the two techniques are very complementary: native (IM)-MS provides quantitative experimental information about the composition, size and overall shape for the whole ensemble of states; MD about the exact positions of individual atoms within selected states.

Because of this, MD has been used to understand MS experiments and proteins in the gas phase [12], and has become important to furthering our knowledge about how biomolecules react upon transfer into vacuum [13•,14,15,67], and when conducted in solution MD is of great value for inferring information about protein function from MS experiments. The focus of our review

Figure 1



Many levels of protein dynamics.
(a) Protein dynamics happen on many levels: conformational dynamics of subunits or oligomers, breaking and forming of protein–protein interfaces, dynamic replacments of subunits, and binding of small molecules or aggregates. **(b)** MS samples the full ensemble according to the free energy landscape **(c)**. **(d)** In absence of input from experiments, MD simulations risk sampling irrelevant parts of the ensemble. Structures and simulation conditions can be informed by MS data to enable more relevant interrogation of the system. Experimentally derived restraints can help a simulation escape local minima and focus the sampling to specific regions of interest. MS can inform about, for example topology and overall architecture, but has a limited structural resolution, whereas MD is intrinsically structurally detailed.

article is how the two techniques are increasingly being used in combination to interrogate-specific proteins involved in important biological questions, leading to findings about their structure and dynamics in the solution phase that would likely have remained undiscovered with either technique in isolation. Here, we discuss the current state of combinations of native MS and MD for the study of proteins and complexes in relation to their biological functions using recent examples from the literature, and we identify important challenges for the near future.

Integrating native MS with MD: from loosely linked to tightly knit

MD can be combined with experiments in various ways, each with their own prerequisites, limitations, and strengths. Oftentimes MD and experiment data are directly relatable, in the sense that they return the same quantities (e.g. interatomic distances), but in other cases the translation of their outputs to enable comparison is not straightforward. Furthermore, computational costs can prohibit exhaustive MD investigations, and issues of limited sampling can hinder comparison with experimental observations which themselves may suffer from errors or ambiguities [9,10•,11]. As a consequence, when the experimental information used for the MD simulations is sparse, particular care must be taken to consider thoroughly the (sometimes implicit) assumptions made about both the experiments and the simulations. Ideally, multiple hypotheses should be formulated, stated and evaluated, and replicate simulations run to assess reproducibility and confidence in the results.

In broad terms, there are three ways, in which (IM)-MS and MD are combined to provide structural and dynamical insights into proteins and the assemblies they form. The most common is the application of MD simulations (in solution or in vacuum) to aid the interpretation of experimental MS data in the context of a three-dimensional structure and its associated motions. In this practice, a structural reference state independent of the MS data (e.g. a crystal structure) is chosen as the starting point for the simulations, with the data obtained used to mutually reinforce the conclusions drawn. However, while such simple addition of MD simulations to MS data provide an attractive visual element and atomistic interpretation, it is important to recognise that there is little input into the simulation from the experiment

(Figure 1d). As such, this combination runs a significant risk of confirmation bias, and validation of the MD results should be sought to strengthen the conclusions.

A second combination of the approaches are cases when MS experiments are used to define starting structures and formulate-specific hypotheses that can be tested with MD simulations. In these instances, crucial information with regards to the composition and structure of the system under investigation, including the number and nature of ligands [16,17] and the conditions under which to simulate [18], is obtained from the MS data. This situation differs to the first approach, as in this case the starting structure as well as any manipulation or steering of the MD, are closely defined by experiment (Figure 1d). This justification of the starting position of the simulation not only maximises the relevance of the simulations, but also means that areas of the free energy landscape that might not have been considered otherwise are assessed. As such, both experiments and simulations play crucial roles with regards to the investigation of protein structure and dynamics. This approach is generally effective when using MD to sample motions of modest amplitude in solution, but it is limited if trying to model large-amplitude structural changes upon transfer to vacuum [14], for which iterative implementations are required [13•,15].

An integrated way of using native MS and MD is to use the former to formulate a restraint force that restricts the sampling in the latter to selected relevant regions of the conformational space (see Ref. [10•] for the case of a generalised experiment) (Figure 1d). This is an attractive means to overcome the inability of MD to fully explore the most important parts of the ensemble. Such restrained MD is rarely seen using native MS data, chiefly because of the difficulty in translating the MS-derived data into useful simulation restraints. IM-MS provides more structural information than MS alone, but is also difficult to incorporate directly into MD because the collision cross sections (CCSs) inferred from the experimental drift times cannot readily be compared to theoretical values calculated on-the-fly during a simulation due to the large computational overhead. Recent advances in CCS calculations will likely eventually change this [19,20], while other quantities which are faster to calculate, such as the squared radius of gyration (R_g^2), are sometimes used as proxies for CCS [21–23]. However, as R_g relates to CCS in a highly protein-dependent and conformation-dependent manner, caution must be taken when employing this approach. Therefore, and despite CCSs themselves being structurally ambiguous (i.e. multiple different structures can have the same value), CCS restraints for MD will be a significant breakthrough for MS-based structural biology.

Dynamics of the overall protein units

IM-MS allows the extraction of CCS distributions for each species resolvable in the MS dimension [24]. Depending on the resolving power of the experiment, and the size and shape differences between the different species, these distributions may contain multiple resolvable features. Alternatively, the presence of numerous conformations may be evidenced by the peak-width being broader than expected from the instrument resolution [24]. CCS distributions obtained from native IM-MS data of globular proteins compare well to those calculated from NMR data obtained in the condensed phase [20]. Furthermore, CCS distributions can vary with buffer conditions and modifications to the protein [25–27], consistent with the measurement being sensitive to the protein state in solution. The evidence is, however, also clear that a conformational space larger than that sampled by local thermal fluctuations in solution may be accessed in the gas phase, particularly in the case for intrinsically disordered proteins [8,28].

These further reaches of the conformational landscape can be explored by deliberate activation of the protein in vacuum, and choosing or manipulating the charge states being interrogated [29]. Comparing proteins by the evolution of CCS during deliberate gas-phase unfolding of a protein can be used to infer differences in structure not apparent from comparisons under non-activating conditions [30]. Gas-phase MD simulations have been instrumental for understanding the gas-phase unfolding process [17,31], but also reveal how its trajectory differs to equilibrium unfolding in solution due to differences in barrier heights [32] and the steering influence of charges [33]. This means that the observation of differences in stability relative to gas-phase unfolding, while necessitating cautious interpretation, do provide hypotheses for testing by MD or other methods.

A recent study that highlights both the diagnostic utility of IM-MS, under both native and activating conditions, and the insight brought by MD, concerns the plant photoreceptor protein UVR8 — a regulator of adaptation to UV radiation [34••]. UV light causes UVR8 dimers to dissociate, which in the cell triggers numerous downstream responses. The authors used a modified instrument, where the sample was irradiated at 280 nm immediately before injection into the mass spectrometer, to investigate the structural dynamics surrounding the photoactivation. Using native IM-MS and comparing with CCSs calculated from MD trajectories they found that, in contrast to the commonly used truncated core domain, the full-length protein exhibits notable conformational dynamics, most likely localised to the N- and C-terminal tails. Using gas-phase unfolding complemented with MD simulations, the C-terminal tail, when in an extended conformation, was shown to destabilise the core domain fold. Surface-induced dissociation revealed that the more extended dimer conformation dissociates more easily, strongly suggesting the extended C-terminal tail also facilitates the dissociation. These observations,

considered in the light of existing literature, led the authors to hypothesize that the extended structure is not only primed for dissociation, but also for more productive interaction with its binding partners in the regulatory chain, thanks to its altered core domain structure. This study shows how combining MS and MD can reveal insight into functional, stimulated motions of proteins.

Dynamics at protein–protein interfaces

Native MS can be used to monitor complex formation, which is inherently difficult with many other techniques since assembly intermediates can be transient and scarcely populated. The assembly and subunit dynamics of filaments [35,36], and protein cages [37–39] are particularly interesting applications because of the many coexisting intermediates at any given time. In these cases, IM-MS can be used to provide additional information about the overall shape of the intermediates, and separate architectural isomers. The extraction of CCS can be very useful for structural investigations to facilitate model building, and as a means to filter structural models [13•,37,40].

The ability of native MS to quantify the abundances of assembled and disassembled forms of biomolecular complexes allows the strength of the interfaces in terms of free energies or separate enthalpic and entropic contributions [41•,42,43]. These important thermodynamic parameters have great utility for formulating hypotheses that can be tested with MD. We recently used a combination of native MS and MD to explain the incompatibility of two classes of small heat-shock proteins [44••]. The two classes have arisen from a gene duplication event >400 million years ago, and the many members of each class can co-assemble with other members of the same class into hetero-dodecamers from dimeric building blocks. However, co-assembly is not possible between members in different classes, a segregation that results in functional benefit to the organism [45]. As part of this study, we used native MS to showed that dimerisation was class-selective, but the crystal structures of both homo-dimeric proteins and 2-μs MD simulations of them and a hetero-dimer (created *in silico*), showed that their dimer interfaces were essentially identical (Figure 2a), and revealed no clue about selectivity.

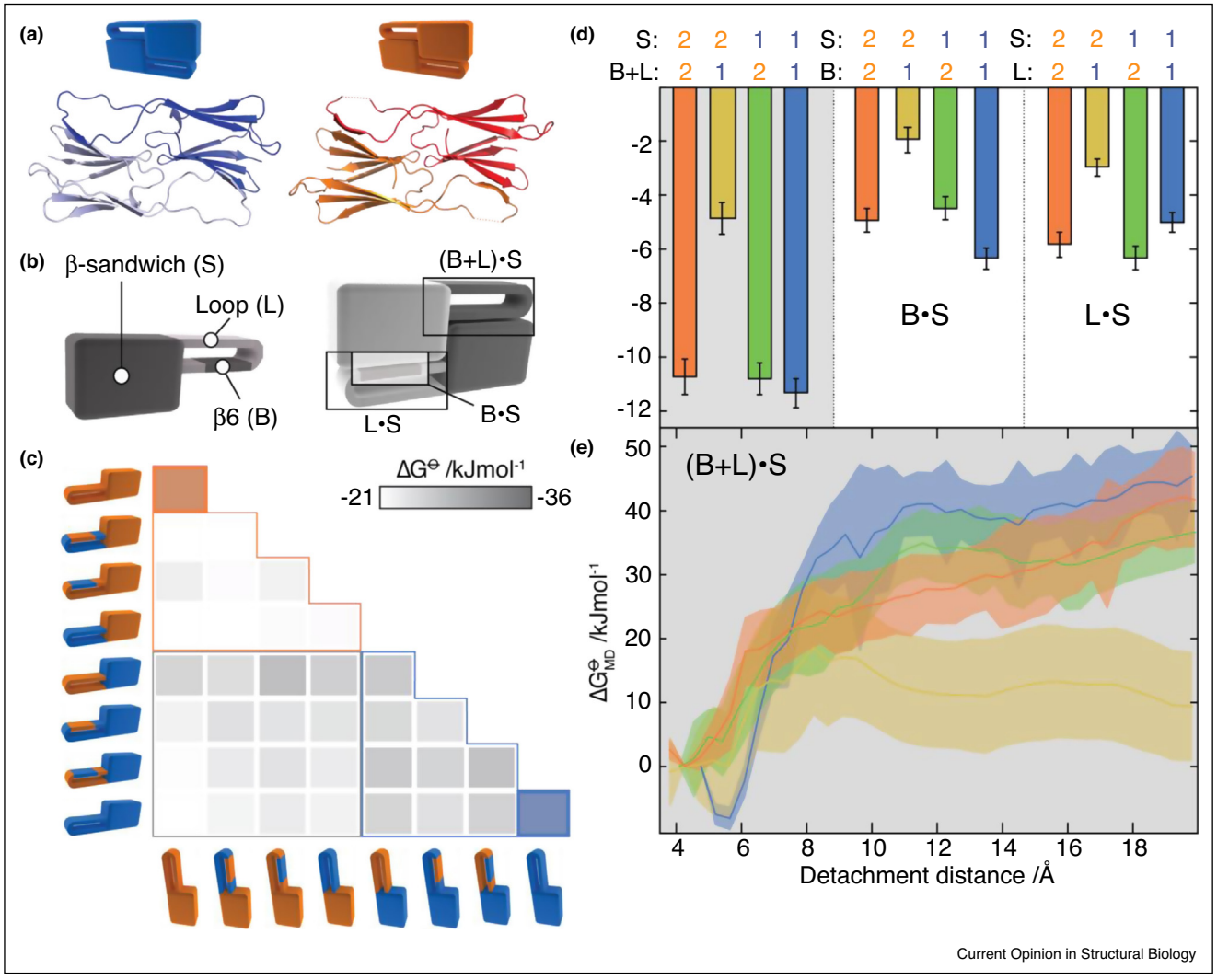
We generated chimeric constructs by swapping corresponding segments between the two classes, and performed native MS titration experiments on all possible binary mixtures (Figure 2b and c). This allowed us to quantify how the individual segments of the proteins contribute to the binding free energy, and we found that, surprisingly, non-interfacial parts were the strongest determinant of selectivity (or the lack thereof). This motivated us to perform steered MD and umbrella sampling to obtain the free-energy profiles for breaking selected parts of the homo-dimer and hetero-dimer interfaces, finding that the combination of contacts apparently weakest in experiment indeed had a considerably flatter barrier. To understand this selection mechanism, we ran 2-μs simulations of each monomer, and found that their differences in structure and dynamics could be projected onto a coordinate in which the two wild-type monomers represented extremes, and the chimeric monomers intermediate thereto. Again, non-interfacial loops turned out to be the major determinants for the monomer structure and dynamics. This combination of native MS and MD enabled a very powerful interrogation of a complex protein system, allowing us to propose a thermodynamic argument for how the abundances of heteromeric and homomeric complexes in the known structural proteome have evolved. This study is a striking example of how exploiting cross-talk between native MS and MD can lead to deeper insight than each method allows alone.

Dynamic interactions with small molecules and membranes

Native MS, aided by recent technological and methodological developments [46–48], is an ideal means to discern heterogeneous interactions between proteins and small molecules. Complex stability and topology can be probed through activation and dissociation, and IM-MS can inform additionally about ligand-induced structural changes or, with complementary modelling, the location of the ligand. MD simulations can also shed light on such interactions, pinpointing binding modes and locations. A recent example of this is the interrogation of BanLec, an anti-viral lectin, and the interactions it makes with N-glycans from HIV [49•]. The authors established a preference of BanLec towards oligomannose, and that this glycan-binding is indeed important for HIV neutralisation *in vivo*. Using native MS, they then found that BanLec is tetrameric, and revealed it to have an architecture similar to other lectins of the jacalin family. Each monomer presented two accessible glycan-binding sites on the tetramer surface, with MD simulations in implicit solvent revealing that oligomannose binds simultaneously to both binding sites. This observation prompted additional native MS experiments showing that mutation of either binding site virtually abolished the binding of oligomannose, corroborating the bidentate binding mode seen in the MD.

The ability of native MS to interrogate membrane protein complexes has enabled the study of interactions with lipids in the membrane [6]. This is an important feature, since interactions with lipids can influence, or determine, the functions of membrane proteins [50]. At the same time, such interactions are often transient, heterogeneous, and display varying degrees of selectivity, which effectively places them out of reach of most other techniques for structural biology. The integration of native MS with MD simulations has proven very fruitful in this area, providing information as to likely lipid binding sites

Figure 2

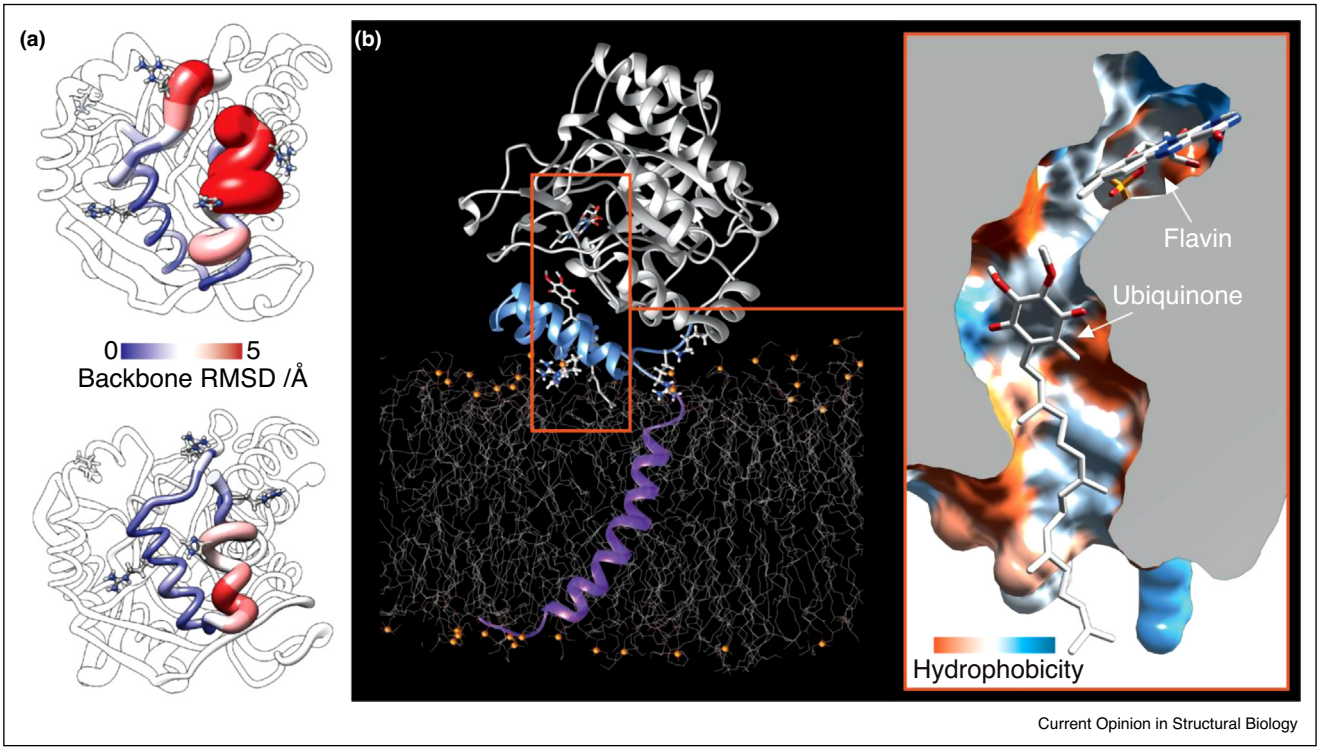


Combining native MS with MD to elucidate interface selectivity.
(a) The structures of the core domain of two *Pisum sativum* small heat-shock proteins from different evolutionary classes appear virtually identical. **(b)** To determine the sequence-determinants of selectivity, we created chimeras by swapping different segments B, S, and L (left), to ascertain the impact on dimerisation (right) **(c)**. Native MS titrations of all pairwise combinations allowed the determination of interface free energies for each case (darker grey, stronger interface). **(d)** Thermodynamic modelling of the global dataset allowed us to extract the microscopic contributions to the overall free energy, and revealed the surprising result that non-interfacial residues contribute as much to interface stability as interfacial residues do. **(e)** Steered MD with umbrella sampling revealed that the predicted weakest interface in (d) — mustard — has the smallest barrier to rupture. Adapted from Ref. [43].

[17,51], if and how the lipids stabilise protein–protein interfaces [52,53], influence of charge on lipid binding [54], and structuring of the annular lipids around membrane proteins [55].

A recent work of ours demonstrates the applicability of MS and MD to peripheral membrane proteins [56••]. Using native MS we found that the mitochondrial dihydroorotate dehydrogenase (DHODH) protein — part of the nucleic acid synthesis pathway and a potential anticancer target — displays a preference for binding phospholipids with exposed charges, such as the PE lipids abundant in the mitochondrial membrane. Spectra recorded at excess concentrations of PE showed that the protein only makes limited contacts with the lipid membrane. We performed MD simulations of DHODH in solution and in a membrane system, with and without the C-terminal helix that spans the membrane. The simulations without the C-terminal helix enabled the protein to move more freely on the membrane, and suggested that the lipid interactions are quite unspecific. We found that the C-terminal helix helps position

Figure 3



Combining native MS with MD to elucidate ligand binding.
(a) Root-mean-square deviation of the backbone relative to the starting structure derived from MD simulations of DHODH in the absence (upper panel) and presence (lower panel) of a membrane. Without a membrane, the domain is very flexible, similar to the crystal structure, whereas membrane association confers considerable stabilisation. **(b)** MD simulations reveal how the C-terminal membrane-spanning helix keeps the globular domain oriented such that the Q10 binding pocket faces the helix. Notably, the positioning of the binding pocket means that Q10 gets pulled up from the membrane, which might be an important factor in the mechanism for selectivity (inset) The binding pocket forms narrow tunnel, sealed by the hydrophobic tail of the Q10 coenzyme. Adapted from Ref. [55].

DHODH such that the acceptor-binding site is facing the membrane, which stabilises the electron-acceptor binding site and places it at a slight angle and distance from the membrane, so that it must 'pull up' its Q10 coenzyme from the membrane to the substrate in an unexpected manner. We speculated, based on similarities with the binding-site architecture of respiratory complex I, that this contributes to the specificity for Q10. This work demonstrates how atomic-level mechanistic insights into the activity of proteins can be obtained when MD and MS are combined.

Concluding thoughts

The examples we have described in this review demonstrate the great utility and rapid growth of combining native MS with MD for the study of proteins and their various interactions. The inherent suitability of native MS for interrogating structurally dynamic and heterogeneous systems gives it a unique position within structural biology, and it benefits greatly from the addition of MD as a 'computational microscope' that can inspect closely regions of interest in the structural ensemble. We expect the use of native MS to continue to grow in the fields of structural biology and biophysics, addressing new and challenging applications in academic and industrial research, and argue its integration with computational methods will be a key aspect of its impact.

While we have focussed here on the combination of native MS with MD, other MS-based approaches have a significant role to play in structural biology. Most notably, these include various labelling approaches (hydrogen/deuterium exchange, chemical-crosslinking, footprinting) followed by proteolysis and bottom-up 'proteomic' MS. Recent studies have exploited the information these methods provide, in combination with MD or molecular modelling, to study protein structure, dynamics and interactions [57–60]. In most examples in the literature where MS experiments are used in conjunction with MD to obtain structural insight, MD is performed subsequent to MS; hypotheses are rarely formulated based on the simulations for subsequent testing in the mass spectrometer. We believe that this is something of a wasted opportunity, and that the most

profitable route is when cross-talk between the two is embraced, with simulations inspiring new experiments and *vice versa*, providing a joint route towards new exciting discoveries. To achieve this aim, and usurp the dominant 'MS-then-MD' workflow, the field needs to refine a common grammar for the two techniques, with software that allows the comparison of MS-derived data to atomic coordinates and electron densities describe macromolecular structures an important step on this journey [19,20,59,61,62].

We anticipate that MS-based approaches will continue to ultimately target measurements *in vivo* [63], or in conditions that mimic the (sub-)cellular environment closely [64,65••]. Accordingly, as in these surroundings multiple protein complexes interact with one another and with other biomolecular superstructures, there will be a demand for MD simulations going beyond the typical time and length scales. Mesoscale MD approaches have been used for the simulation of micron-sized protein-containing membrane systems for milliseconds [66]. It will be interesting to see what these methods, when combined at this level, can do for our understanding of the cellular functions of proteins.

Acknowledgements

We thank Michael Landreh (Karolinska Institutet) for help with Figure 3. EGM holds a Marie Skłodowska Curie International Career Grant, which is jointly funded by the Swedish Research Council and the European Commission (2015-00559). EGM and JLPB thank the EU COST action BM1403.

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