



Mass spectrometry guided structural biology

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With the convergence of breakthroughs in structural biology, specifically breaking the resolution barriers in cryo-electron microscopy and with continuing developments in crystallography, novel interfaces with other biophysical methods are emerging. Here we consider how mass spectrometry can inform these techniques by providing unambiguous definition of subunit stoichiometry. Moreover recent developments that increase mass spectral resolution enable molecular details to be ascribed to unassigned density within high-resolution maps of membrane and soluble protein complexes. Importantly we also show how developments in mass spectrometry can define optimal solution conditions to guide downstream structure determination, particularly of challenging biomolecules that refuse to crystallise.

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Introduction

Ever since the first observations of proteins and small molecules remaining associated in the gas phase, researchers have attempted to study protein interactions using mass spectrometry. Through increased understanding of the properties of proteins in gas phase and continued instrument development, both at the solution to gas phase transition [1] and during the flight path through the mass spectrometer [2] it has now become clear that mass spectrometry (MS) can make a major contribution to structural biology. For intact protein complexes, in which protein assemblies are introduced from non-denaturing solutions and are preserved within the gas phase (native MS) manipulating pressure gradients within the instrument was

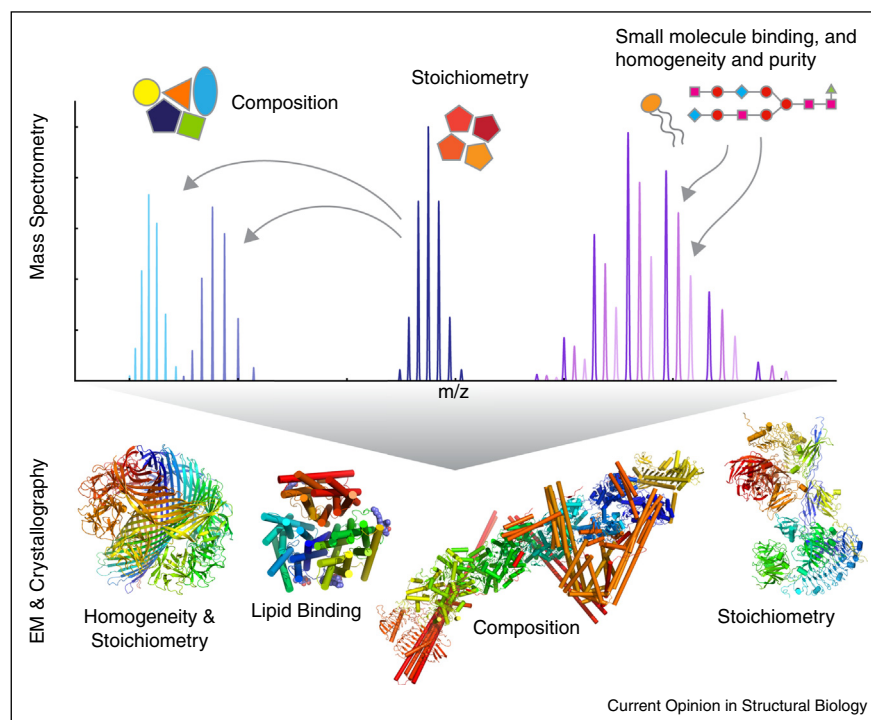
critical [3]. Whilst for membrane proteins understanding solution properties and retaining solubility was vital [4,51].

Beyond defining the subunit stoichiometry native MS has been particularly powerful in defining dynamic parts of protein assemblies that are resistant to crystallography. For example, the mobile stalk regions of ribosomes [5]; the unstructured tails of spliceosomes [6] and the dynamic subunit heterogeneity of small heat shock proteins [7]. In these native MS applications protein assemblies are introduced from non-denaturing solution conditions and transferred into the gas phase of the mass spectrometer with minimal disruption of their folded structure and interactions. These native MS experiments have recently entered a new era with the advent of high resolution mass spectrometers capable of increased mass accuracy and better separation of small molecules bound to protein assemblies [8[•],9[•]].

In addition to high resolution MS, coupling with ion mobility, which reveals a rotationally averaged collision cross section [10], enables the overall topology of protein complexes to be related to EM density [11] leading to new views of dynamic protein assemblies. These MS studies are often supplemented with chemical crosslinking, which has undergone transformative bioinformatic developments, to reveal neighbouring subunits [12] and to define conformational changes [13]. Excellent reviews of protein crosslinking within defined protein assemblies, or within the cellular context, have been presented recently [14,15].

Finding optimal solution conditions is critical for all types of structure determination and MS is now playing an increasing role in this endeavour. These conditions are those that yield homogeneous and stable states of proteins and their assemblies. MS is useful for identifying these conditions as sub-stoichiometric subunit binding and the presence of interacting small molecules can be detected and then minimised or enhanced as appropriate. The informative role that native MS can play in each of these applications is highlighted (Figure 1). Spectra of the intact complex immediately reveal the stoichiometry of protein assemblies, often confirmed using disassembly methods such as collision-induced dissociation in the gas-phase, to liberate individual subunits of the structure. Dissociation of structures can also inform on the arrangement of subunits within the complex via solution disruption or surface induced dissociation [16,17^{••}]. The superior resolution of MS relative to other ‘sizing’ techniques, often used in protein purification and characterisation, allows more

Figure 1



MS can be used to measure the stoichiometry and composition of protein complexes, the presence of small molecules, and to establish conditions that enable structural determination by techniques such as cryo-EM and X-ray crystallography. From left to right are crystal structures of the lysenin pore from *Eisenia fetida* (PDB 5EC5), the ammonia channel from *E. coli* (PDB 4NH2), the cryo-EM structure of the dynactin complex (PDB 5CMN), and the crystal structure of the FLRT2:Unc5D:latrophilin 3 protein complex (PDB 5FTT) all of which were informed by complementary MS.

detailed insights in how purification protocols can affect downstream structural methods. This resolution also allows the identification of small molecules bound to proteins, such as metals or lipids, to assist the assignment of ambiguous densities in crystallography.

In this review we will focus primarily on the structure elucidation of protein complexes including protein-only assemblies, protein-nucleic acid complexes and membrane protein-lipid interactions. We consider the interplay of structure determination and MS, as EM resolution increases to sub-nanometre levels and beyond, and as the possibility for resolving small molecule binding within the context of macromolecular assemblies becomes a reality.

Subunit stoichiometry of protein assemblies

Perhaps the most straightforward and important application of native MS is in the determination of subunit stoichiometry. Whilst in the majority of cases consensus is observed between MS-derived stoichiometry and that in X-ray crystal structures, exceptions have been reported, including the recent structure determination of BanLec. This lectin, which has been shown to be capable of neutralising the carbohydrate coating of the

HIV virus and is therefore a potent inhibitor of HIV replication, forms dimers during crystallography, in contrast to other lectins in the same family, which are homotetrameric proteins [18,19]. However clear evidence was obtained from MS, supported by size exclusion chromatography, multi angle light scattering and small angle X-ray scattering, that in solution BanLec possesses a tetrameric oligomeric state under a variety of ionic strengths (Hopper, under review). Whilst the discrepancy is not unheralded, presence of the dimeric form was only found by MS under very high salt conditions (4 M ammonium acetate). The tetrameric solution state of BanLec enables multiple binding events, which explain its high glycan affinity. Therefore correctly identifying the solution oligomeric state by MS was important in identifying how this protein functions.

Subunits that are present in addition to a stable core of proteins are often discovered following native MS of intact protein complexes. A case in point is the human COP9 signalosome (CSN) complex, consisting of eight subunits (1, 2, 3, 4, 5, 6, 7 and 8) that were co-expressed and purified from insect cells and for which an X-ray crystallography structure at 3.8 Å resolution was solved [20]. Interestingly, MS analysis of the endogenous intact

COP9 signalosome isolated from both human erythrocytes and HEK293 cells indicated the presence of a ninth subunit, CSN acidic protein (CSNAP), attached to the core of the eight canonical subunits. The CSN complex shares sequence similarities with two multi-subunit protein complexes: the lid component of the 19S proteasome and the eukaryotic translation initiation factor 3 (eIF3). Proteomics and chemical cross-linking experiments confirmed that the sequence and structural properties of the CSNAP subunit are similar to those of DSS1, a subunit of the related 19S lid proteasome complex (sequence similarity of 26% and identity of 18%) [21]. These MS results enabled the position of this ninth subunit to be identified on the X-ray crystal structure and to provide a complete view of the nine-component human COP9 signalosome.

The growing acceptance of MS for measuring subunit stoichiometry is further highlighted in the following example, where MS was used to show and validate that the structure in the crystal form was not an artefact of crystallisation. The latrophilin (Lphn1-3) family of adhesion G protein-coupled receptors (GPCRs) contain large extracellular domains that mediate cell–cell interactions by binding ligands of the fibronectin leucine-rich transmembrane (FLRT1-3) protein family. FLRT proteins further interact with the uncoordinated-5 (Unc5A-D) family of cell guidance receptors. Using native MS Unc5D was shown to interact cooperatively with FLRT2 and Lphn3 to form ternary complexes and Lphn3 was also found to dimerise when bound to FLRT2:Unc5D, resulting in a stoichiometry of 1:1:2 (FLRT2:Unc5D:Lphn3) [22]. The MS data additionally revealed that this 1:1:2 complex further dimerises to form a larger octameric ‘super-complex’ (2:2:4) in line with the stoichiometry observed and determined crystallographically. This surprising 2:2:4 stoichiometry for the FLRT2:Unc5D:Lphn3 was originally considered a crystallisation artefact, but the MS was able to show that this also exists in solution (Figure 2a). Further support for this model came from MS determination of the effects of specific mutants on the stability of protein–protein interfaces within the super-complex. Overall this combination of MS and crystallography data showed how receptors increase their functional repertoire by forming different higher order ‘super-complexes’ with unexpected stoichiometries.

Continuing the theme of establishing subunit stoichiometry the dynactin complex, extracted from porcine brain, gave rise to a surprisingly homogeneous protein complex as assessed by native MS (Figure 2b). In this case the stoichiometry of actin related protein (Arp1) subunits was in question. When examined by MS the mass of dynactin, at ~1.0 MDa, was found to contain 23 subunits, corresponding to 11 different proteins with at least four in multiple copies. Whilst it was not possible to assign a unique subunit stoichiometry at the mass resolution obtained and with the high number of subunits and

potential for post-translational modification, native MS in combination with quantitative proteomics provided a unique solution to the subunit stoichiometry. This informed the cryo-electron microscopy analysis at 4.0 Å resolution. The resulting reconstruction revealed how dynactin is built around a filament containing eight copies of the actin-related protein Arp1 and one of β-actin [23].

Similarly MS was able to deduce the subunit stoichiometry of the histone chaperone nucleosome assembly protein (Nap1) in complex with H2A-H2B [24]. A single yeast Nap1 dimer was shown to bind to a single H2A-H2B heterodimer and to self-assemble into higher-order oligomers (Figure 3). MS was a powerful tool in this case, as it was not possible to reach a consensus on the oligomeric state and composition of the complex using techniques such as size-exclusion chromatography, sucrose gradients, analytical ultracentrifugation or small angle X-ray scattering. Such ensemble averaging techniques typically have poor compositional resolution with particles that dynamically self-assemble. MS offers unparalleled mass accuracy and enabled unambiguous determination of protein stoichiometry of this complex. The subunit stoichiometries obtained for various constructs allowed an evaluation of the relevance of the crystal structure in which six copies of the Nap1-H2A-H2B protomer formed the crystallographic asymmetric unit. This same stoichiometry was observed as the dominant state in solution using MS and in negative stain EM images, thus confirming the oligomeric state of the complex.

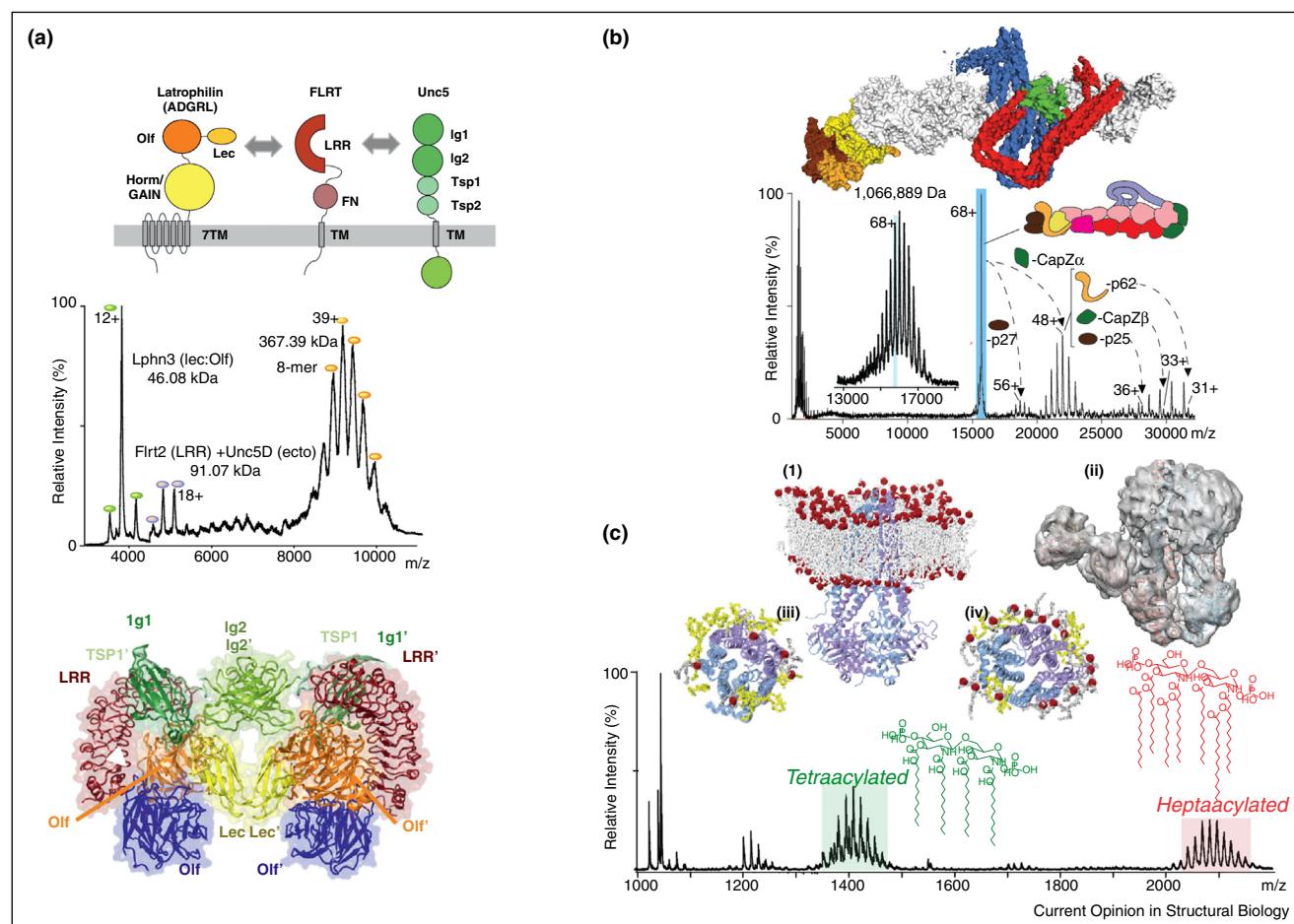
The fundamental importance of knowing the subunit stoichiometry, whilst not only informing the cases outlined above, is also providing insight into the evolution of protein complexes from subcomplexes and, when combined with bioinformatics, is leading to prediction of stoichiometries that have yet been observed experimentally [25]. Historically subunit stoichiometry of protein only complexes was one of the earliest applications of native MS but the approach is not restricted to this application as we will see in the next section.

Protein complexes involved in DNA/RNA processing

Moving into the field of protein-nucleic acid complexes brings the additional complication of nucleotide binding which in turn often carries associated cations. This therefore degrades mass spectral resolution making it difficult to interrogate the resulting native mass spectra in a detailed way. Native MS for this reason is often coupled with cross-linking or proteomics to provide additional information that is then useful in constructing models from, for example, EM data.

The subunit stoichiometry and composition of the RNA editing complexes, the CRISPR-Cas systems, were first deduced by MS [26–28]. In *Escherichia coli* the complex

Figure 2



Mass spectrometry defines subunit stoichiometry and lipid binding and thereby contributes to a variety of high-resolution structural studies from crystallography to cryo-EM. (a) The unexpected 2:2:4 stoichiometry of the Flrt:Unc5:Latrophilin protein complex was first revealed crystallographically and later confirmed by MS of the intact 8-mer complex. (b) The measured mass of dynactin (1 066 889 Da) confirmed its subunit stoichiometry with four copies of p50 and 2 copies of p24. (c) The cryo-EM structure of TmrAB revealed density of unknown identity tentatively assigned to a detergent micelle. MS also showed a series of well-defined lipids in contact with the protein and the presence of heptaacylated and tetraacylated lipid A was deduced through mass spectra at m/z 2100 and 1400, respectively.

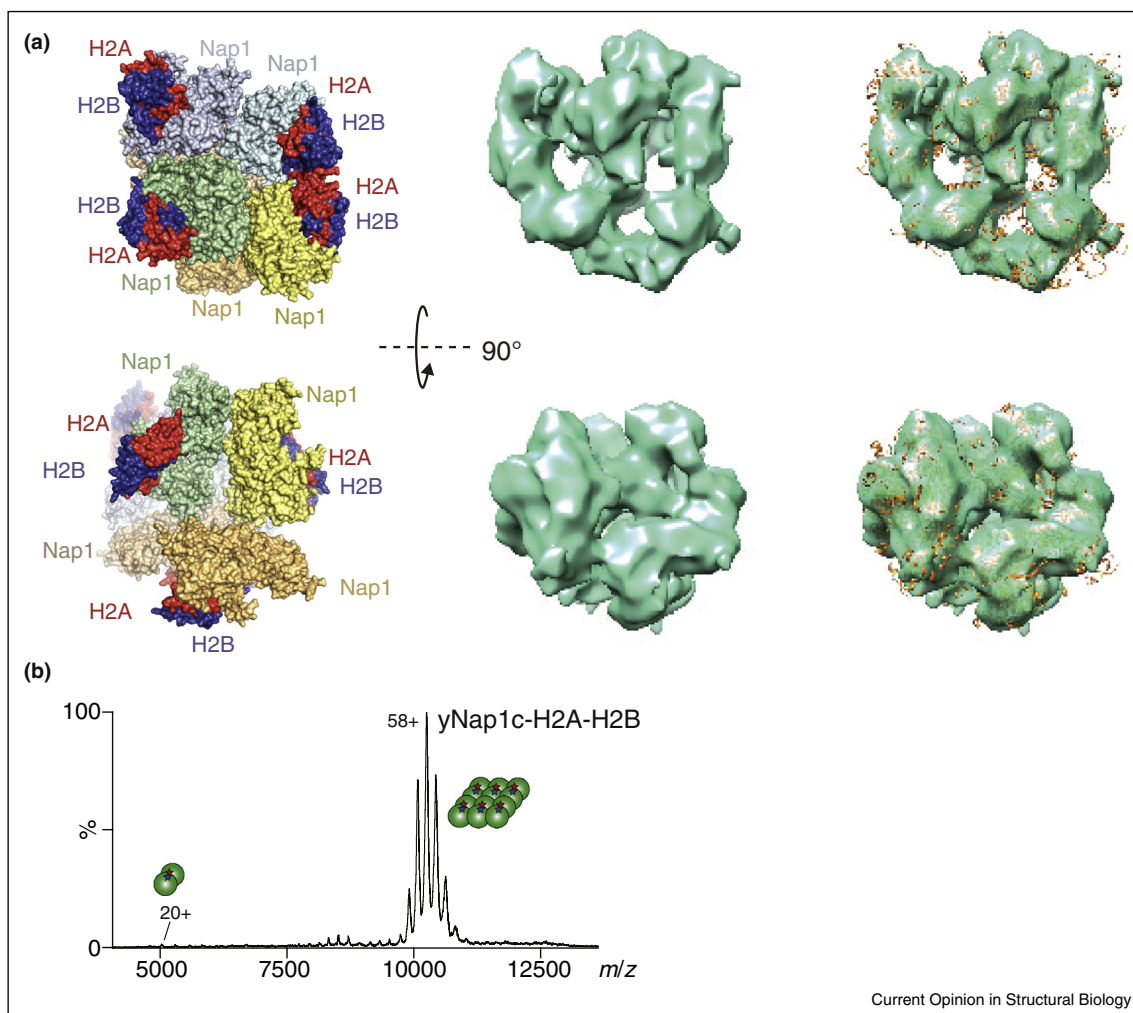
was shown to consist of five functionally essential CRISPR-associated (Cas) proteins (CasA1, B2, C6, D1 and E1) and a 61-nucleotide CRISPR RNA (crRNA). In *Sulfolobus solfataricus* and *Thermus thermophilus* eight different subunits, many of which are similarly sized, complicated determination of the stoichiometry. This was resolved by generating multiple sub-complexes in solution and characterising using native MS, allowing definitive assignment of the subunits. This arrangement was validated in the near-atomic resolution cryo-EM reconstructions of native complexes from *T. thermophilus* in the absence and presence of target RNA [29].

Often structure determination is hampered by inherent protein flexibility in the absence of nucleic acid and the heterogeneity of subunit composition. The heterotetrameric capping enzyme catalyses the capping process,

which is the first step in pre-mRNA processing, during which both of its domains undergo conformational changes. The enzyme is functionally and physically coupled to the transcriptional activity of RNA polymerase II. MS was employed to define the stoichiometry of the capping enzyme bound to a transcribing Pol II complex [30]. Combining this information with chemical cross-linking and cryo-EM images of the Pol II complex and with the capping enzyme bound at 7.4 Å and 17.4 Å resolution respectively, revealed the location of the capping complex, which spans the end of the RNA exit tunnel for sequential binding of exiting RNA.

As shown above for protein-only complexes, often the complete composition of protein nucleic acid assemblies is unclear. In some cases additional subunits are anticipated, for example there can be electron density

Figure 3



Combining MS with EM and X-ray crystallography to define the oligomeric state in the chaperone mediated histone assembly complex. Nap1 is a histone chaperone involved in the nuclear import of H2A-H2B and nucleosome assembly. MS was used to confirm the stoichiometry observed in the crystal structure of a Nap1 construct bound to H2A-H2B and to inform the interpretation EM density maps. **(a)** Crystal structure of the Nap1-H2A-H2B complex in two different orientations, 3D reconstruction of the complex from negatively stained particles (green) and fit of the crystal structure into the EM map. **(b)** Mass spectrum of the yNap1c variant in the presence of H2A-H2B.

evidence, but without confirmation of identity the missing subunits cannot be modelled. This was the case for the telomerase complex, which is composed of RNA and three protein subcomplexes. Two newly identified and functionally distinct RPA-related complexes were reported: Teb1-Teb2-Teb3 (TEB) and p75-p45-p19, which are connected to the TERT-TER-p65 catalytic core by p50. The presence of Teb2 and Teb3 was confirmed by MS based proteomics. With this information it was possible to use cryo-EM, NMR and X-ray crystallography to piece together the structure of the *Tetrahymena* telomerase complex. This breakthrough structure also revealed the path of the RNA component in the telomerase catalytic core [31[•]].

Linking eukaryotic initiation factors (eIFs) to the ribosome was facilitated by combining a cryo-EM structure at 28 Å resolution with chemical cross-linking and MS [32]. This combination provided a large number of low-resolution restraints that could be integrated into a detailed interaction map of eIF3 on the ribosome. The arrangement of eIF3 subunits surrounding the 40S subunit ensures that the factors required at both the mRNA channel entry and exit are assembled simultaneously. Studies of the initiation factors themselves also revealed novel and unexpected stoichiometries, including a decameric eIF2B complex for both human and yeast forms that were identified by native MS [33,34]. Subsequently this unexpected stoichiometry was also observed using

X-ray crystallography [35]. Conformational changes induced by binding of t-RNA to eIF2 were also revealed using a comparative cross-linking strategy [36].

Although more complicated than the protein only case studies, and despite the general phenomenon of the heterogeneity of nucleotide binding, considerable progress have been made since the early days of the MS of ribosomes and viral particles. The ability to define the RNA content of viral capsids with masses of 4 MDa and beyond is testament to the advances on a variety of MS platforms including improvements to time-of-flight and Orbitrap detectors, as well as new charge based detectors [37]. In this regard developments in charge detection MS of virus capsids of up to 25 MDa [38] hold great promise for defining the precise stoichiometry of large protein RNA virus structures.

Assigning unknown density to lipids

Whilst it was anticipated that viruses can survive and even be transmitted through the atmosphere much less clear was the fate of membrane proteins in the gas phase of a mass spectrometer. Since the early days it was clear that these complexes would only survive the phase transition if sufficiently protected in detergent micelles. These developments that allow membrane protein interactions to survive are particularly timely given that some of the most dramatic inroads into high-resolution structure determination have arisen from developments in cryo-EM technology and its application to membrane protein complexes [39]. Taking advantage of the improved technology there are now structures of the transient receptor potential (TRP) channels [40], the ABC transporter TmrAB [41], the voltage-gated calcium channel Cav1.1 [42] and the rotary ATP synthases [43]. Even with these high-resolution structures, distinguishing lipids from detergent remains problematic from electron density alone.

In the case of the ABC transporter TmrAB, which was studied using cryo-EM a collar of electron density was assigned to a DDM detergent micelle [41]. Native MS however was able to show the presence of a defined annular belt of lipids that were also critical for the ATPase activity of the complex. The cryo-EM density could be attributed to phospholipids as well as to the presence of lipid A identified by MS. The discovery of this collection of tightly bound lipids through MS led to the proposal that TmrAB is extracted with an annular lipid belt, that the lipids which compose this belt are important for its function as an efflux pump, and that this function may also include a lipid flippase activity [44].

Whilst not forming an annular belt, lipids in the 3 Å X-ray crystal structure of MscS were revealed via electron density to occupy defined pockets [45]. Lipid exchange between the pockets and the bilayer was proposed as a mechanism to mediate channel opening. This idea was

supported by molecular dynamics simulations, which showed that as MscS opens, the lipid content of the pocket decreases, by approximately one lipid per pocket. The mass spectrum of heptameric MscS was consistent with a series of phospholipids molecules bound to the protein, corresponding to the density observed in the crystal structure.

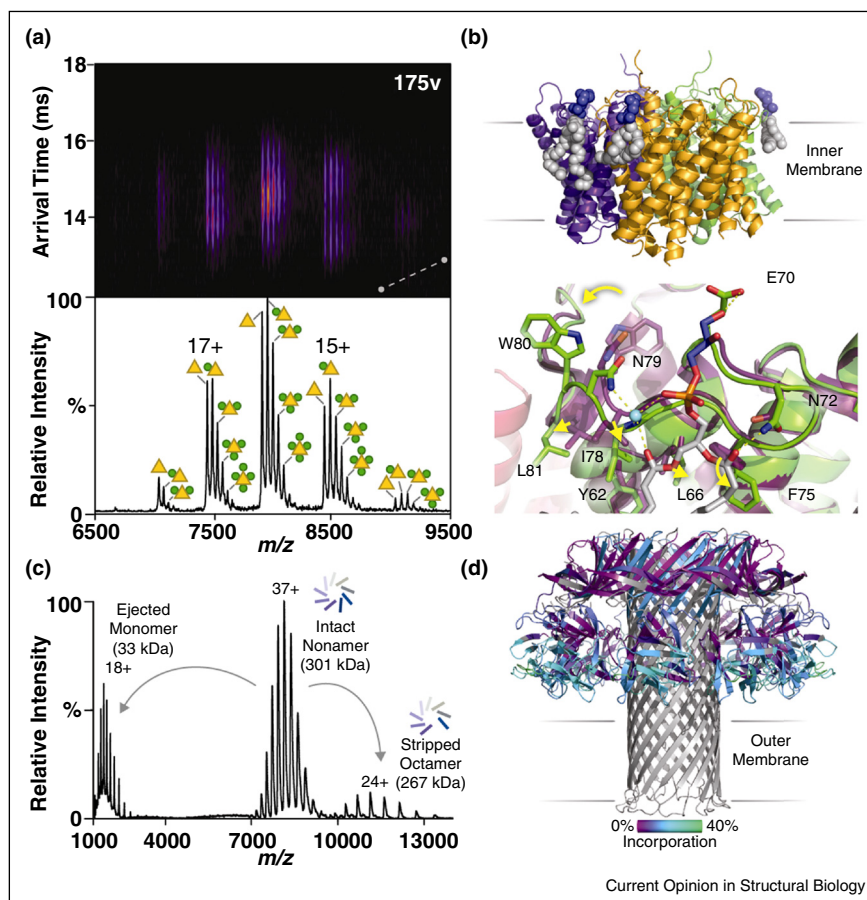
Whereas the presence of lipids in rotary ATP synthases as lipid plugs was anticipated [46], the discovery of lipids between cytochrome C oxidase (CcO) subunits to modulate dimer formation was less well established [47]. In a recent MS study of CcO, purified from bovine heart tissue, the complex was found to be dimeric and to contain a large population of lipids. Given that CcO is an integral membrane protein complex, comprising of 13 transmembrane proteins per subunit, this represents the largest integral membrane protein complex that has been transferred intact into the gas-phase. Lipids were revealed in subunit interfaces in the X-ray structure, and were found to form a 'tailored' plug between the two subunits. Interestingly, phosphorylation and acetylation sites of CcO were also identified in this study and located within the peripheral subunits and the dimer interface respectively. These distinct locations suggest roles for regulation and stabilisation of the dimer through modulation of lipid binding.

Defining conditions for crystallography

Whilst MS is still a relative new comer to the study of membrane proteins, progress in its utility in defining lipid binding is gaining momentum. Identifying lipids that form strong interactions with membrane proteins is important, primarily for structure and function, but also for defining conditions for crystallography. In the first example of such a study, MS was used to study the effects of lipid binding to membrane proteins [48•]. The lipids were selected and ranked based upon their ability to help the protein resist unfolding in the gas phase [49]. The optimised choice of detergent and lipid in MS was then used as the basis to set up crystallography experiments, which led to a high-resolution X-ray crystallography structure of the ammonia channel AmtB bound to lipids [48•] (Figure 4). This was the first crystal structure of AmtB with bound lipid, and was notable for the novel conformational change the lipid binding induced.

Similarly, for the lysenin pore, which has been a target for crystallography for more than two decades, MS enabled the optimisation of the detergent extraction and delipidation protocol of the pore from liposomes. By screening using MS, at each stage of the protocol, optimal conditions were found that enabled native MS analysis to reveal an unexpected nonameric oligomeric state. Critical to this study was the feedback loop in which each stage of the extraction and purification could be monitored, by way of spectral quality, until the stoichiometry of the

Figure 4



Mass spectrometry informs the conditions for crystallography. (a) and (b) Mass spectrum of AmtB with bound lipid and the crystal structure of AmtB in a lipid environment. Crystallisation conditions were identified from MS screening for detergents and ranking of lipids based on their ability to prevent unfolding in the gas phase. (c) Mass spectrum of the lysenin nonamer, showing collision-induced dissociation into monomer and octamer species. (d) H/D exchange mass spectrometry mapped onto the structure of the lysenin pore crystal structure identifies the more exposed regions of the complex.

protein could be unambiguously assigned. When combined with H/D exchange MS the dynamic regions were located which surround this long pore-forming channel that likely undergo conformational change to move the channel within the membrane environment (Figure 4).

Concluding remarks

Through this series of examples we highlight recent advances in structure determination, focussing on those that have been guided by MS. From the early days of native MS, where some scepticism was expressed to its current use prior to, and often parallel with structure elucidation, there is a panoply of case studies demonstrating not only its competence, but also its importance. The opportunity to establish links with new cryo-EM technology are particularly promising when the subunit stoichiometry is in question, the preparation of homogeneous protein complexes is often desirable, and where the identity of small molecules may remain elusive. Moreover

with the advent of increasing resolution for cryo-EM, the presence of small molecules bound to protein complexes will become increasingly apparent; and as such the capability to define them using high resolution MS will follow.

In summary it is intriguing to see how a technique, initially controversial, is now informing a growing number of structural biology studies. The very first examples, in which established crystal structures were used to inform MS [3], have led to acceptance of the GroEL_{14-mer} as a defining study for native MS. Over the intervening years, MS can be considered to have gone full circle and is now informing on a wide range of protein complexes from the subunit heterogeneity of molecular chaperones [24], to the stoichiometry of dynactin and the latrophilin family of adhesion GPCRs as well as various initiation factors. Looking to the future, new ways of generating subcomplexes, through surface induced dissociation or laser activation, are coming to the fore [17^{••},50]. Particularly

powerful also will be the elucidation of different lipid binding properties as illustrated for the ABC transporter TmrAB, CcO and MscS. The different roles of lipid binding, and their effect on stability and subunit interactions, will be critical for membrane protein structure determination, either via crystallography or EM. Importantly, the examples presented herein represent just the inception of these partnerships. We envisage that with advances in all techniques these synergies will continue to form formidable combinations and contribute to future structural biology investigations.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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