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Probing allosteric mechanisms using native mass spectrometry

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Abstract

Native mass spectrometry (MS) and ion mobility MS provide a way to discriminate between various allosteric mechanisms that cannot be distinguished using ensemble measurements of ligand binding in bulk protein solutions. Native mass spectrometry, which yields mass measurements of intact assemblies, can be used to determine the values of ligand binding constants of multimeric allosteric proteins, thereby providing a way to distinguish, for example, between concerted and sequential allosteric models. Native MS can also be employed to study cooperativity owing to ligand-modulated protein oligomerization. The rotationally averaged cross-section areas of complexes obtained by ion mobility MS can be used to distinguish between induced fit and conformational selection. Hence, native MS and its allied techniques are becoming increasingly powerful tools for dissecting allosteric mechanisms.

Introduction

Ligand binding to one site in a protein molecule is often controlled by conformational changes that occur upon binding of ligand or effector molecules to other sites in the protein. Such control, which reflects communication between sites that are often distant from each other, has been termed allosteric regulation. Allosteric theory and models were initially formulated in the 1960's [1, 2] (and even before [3, 4]) with hemoglobin and metabolic control loops in mind. Although allosteric regulation was first discovered in cases of oligomeric proteins such as hemoglobin it is also commonly found in monomeric proteins. In recent years, there has been much renewed interest in allostery that has been driven by (i) the realization that allosteric control is crucial for the function of most cellular machines (e.g. [5]); and (ii) increasing recognition of the therapeutic potential of allosteric drugs (e.g. [6, 7]). Recent interest has also been stimulated by advances in computational structural biology [8] that have made possible to identify potential pathways of allosteric communication and novel allosteric sites which can then be tested experimentally.

The experimental analyses of multimeric allosteric systems often relies on measuring fractional saturation or initial reaction velocity as a function of ligand or effector concentration. Such plots are diagnostic as they reveal whether and to what extent the

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affinity increases (positive cooperativity) or decreases (negative cooperativity) as a function of ligand concentration. These plots, however, rarely provide insights into the allosteric mechanism for two main reasons. First, because it is most often not possible to extract from them the values of the individual binding constants corresponding to the successive ligation steps. This problem is particularly difficult in cases of large multi-site allosteric systems such as the many ring-shaped molecular machines. Second, because the measured values of the individual binding constants often correspond to an overall multi-step process that involves both binding and conformational change(s) and, thus, may be only apparent. These conformational changes can be triggered by ligand binding as in the 'induced-fit' model [2] or precede ligand binding as in the 'conformational selection' model [1]. Distinguishing between these two scenarios using kinetic analyses can be difficult for both monomeric and oligomeric proteins. It usually involves assuming that ligand binding and dissociation are fast relative to conformational changes [9*] but this simplifying assumption is not always valid, in particular, when considering multimeric proteins that undergo multiple binding events and conformational changes. In this review, we will describe advances in native mass spectrometry (MS) that now allow to determine the values of the different binding constants of a ligand for a protein with multiple ligand binding sites and to distinguish between various allosteric models. The review will focus on the thermodynamic aspects of these methods and not on the amazing technical developments that have ushered in this new phase in the study of allosteric phenomena and which have been reviewed elsewhere [10–12].

Determination of binding constants using structural MS

More than 10% of all the crystal structures of both human and *E. coli* proteins, for example, are oligomers that consist of four or more identical subunits [13]. As mentioned above, many such multimeric proteins display positive allosteric behavior that is manifested in sigmoidal plots of initial reaction velocity or fractional saturation as a function of the ligand (substrate) concentration. It is very difficult, however, to extract the values of binding constants from these plots because they are relatively insensitive to the presence of ligation intermediates, in particular, when the number of binding sites is large. Hence, methods for determining the populations of ligation intermediates are needed in order to estimate the values of the successive binding constants of a ligand for a multimeric protein. One possible approach to this problem is to use single-molecule fluorescence techniques [14] but this may require labeling the ligand with a fluorescent dye that may affect its affinity, protein immobilization and collecting data for a large number of single molecules. An alternative strategy is to use structural MS under non-denaturing conditions in which the integrity of multi-subunit protein complexes (with or without bound ligands) is preserved in the gas phase. A major advantage of this approach is that the populations of all the co-existing states [15, 16], which differ in the number of bound ligand molecules, can be determined from their corresponding intensities in a single spectrum (assuming similar ionization efficiencies, termed response factors, so that the intensity ratios correspond to the concentration ratios in solution [11]) (Figure 1A). Likewise, it is also possible to analyze simultaneously mixtures of different proteins that are in equilibrium with their respective ligands. Maintaining the integrity of the ligand-bound complexes is achieved by using electrospray (ES), a soft ionization technique, mild instrumental conditions (i.e. low interface temperatures, low

collision voltages and optimized ion guide pressures) and spraying from volatile aqueous buffers that are at close to neutral pH [10–12]. Given that many complexes of interest such as molecular machines are very large, it was also necessary to develop mass spectrometers with the appropriate m/z range and resolution. For example, the change in molecular mass upon ATP binding to the 800 kDa chaperonin GroEL is $< 0.1\%$ and the difference between ADP and ATP binding to GroEL is $\sim 0.01\%$. Such measurements of changes in mass upon binding of small ligand molecules to large complexes can be achieved by optimizing solution conditions and using extended mass range Orbitrap-based [17*] or quadrupole time-of-flight [18*] mass spectrometers as recently shown for GroEL (Figure 2).

The use of native MS to determine binding constants also has limitations [19]. One of them is that salt concentrations must be kept low [20] although they may be required as in the case of Mg^{++} -dependent ATP binding. Fortunately, however, nanoflow ES, in which the flow rate is reduced to nanoliter per minute, not only greatly enhances the desolvation process but is also often tolerant to nonvolatile salts in the mM range or even higher in some cases [21]. A second limitation is the tendency for non-specific binding to occur during the desolvation process so that the gas-phase measurements do not reflect the true stoichiometries in solution. Several approaches to overcome the problem of non-specific binding in MS studies have been suggested [22–27]. For example, blackbody infrared radiation was used for controlled dissociation of nonspecific gas-phase interactions [22] but this can cause complex dissociation when the non-specific binding is strong. Another approach involves the addition of a reference protein to monitor the appearance of non-specific complexes [23, 24] but there is uncertainty regarding the generality of the approach [25]. Mathematical models for distinguishing between specific and non-specific binding that do not involve potential perturbations (e.g. by adding a reference protein or exposing to blackbody infrared radiation) of the system under study have also been introduced. In one such model [26], non-cooperative binding of the ligand was assumed but, more recently, we suggested a method [27] that does not rely on this assumption and can, therefore, be applied to allosteric systems. The method requires prior knowledge of the total number of specific sites, N , from other data such as the crystal structure of the protein. Given N , one can calculate from the ratio of intensities that correspond to the populations with $N+i+1$ and $N+i$ bound ligand molecules, I_{N+i+1}/I_{N+i} , the value of the non-specific binding constant, K_{ns} . Given the value of K_{ns} (that is expected to be the same for different I_{N+i+1}/I_{N+i}), it is possible to determine the value of the specific binding constant for the first site, K_1 , from $I_1/I_0 (= K_{ns}[S] + K_1[S])$, where $[S]$ stands for substrate concentration) and the values of the binding constants for all the other specific sites in a similar recursive fashion. The observed binding number distribution (i.e. the populations of all the co-existing states with different numbers of specifically and non-specifically bound ligand molecules) can, thus, be converted into the binding number distribution for specifically bound ligand molecules (Figure 3).

The values of the specific binding constants can be determined at different substrate concentrations and for each one of the different charge states, thereby reducing the errors. Non-linear regression [19] can be used for data fitting in cases of tight binding when it cannot be assumed that the free and total ligand concentrations are similar. In recent years, a range of non-cooperative ligand-protein interactions with very different chemistries have been characterized using structural MS. Examples include fatty acid binding to bovine β -

lactoglobulin [28], oligosaccharide binding to viral capsid proteins [29], the interactions of saikosaponins (glucosides consisting of a glycosyl unit and a moiety called triterpene) with cytochrome c [30] and binding of benzamidine-based inhibitors to bovine trypsin [31]. Cases of cooperative ligand binding that involve multiple equilibria have also been characterized [18*, 27, 32–34] as discussed next.

Distinguishing between allosteric models using structural MS

Several models, in particular the Monod-Wyman-Changeux (MWC) model [1] and the Koshland-Némethy-Filmer (KNF) model [2], have been developed to describe cooperativity in ligand binding by multisubunit proteins. According to the MWC model, cooperativity is due to a shift in equilibrium between two states: a so-called tense (T) state, in which all the subunits are in the same conformation with low affinity for the ligand, and a relaxed (R) state in which all the subunits are in another conformation with high affinity for the ligand. The extent of cooperativity is determined by the equilibrium constant, L , for the unligated T and R states ($L = [R]/[T]$) and the relative affinities of the ligand for these states (K_T and K_R). The allosteric transitions in this model are concerted since asymmetric states (in which some subunits in a protein molecule are in the low affinity conformation and others in the high affinity conformation) are disallowed (Figure 4A,B). In the KNF model, binding-induced conformational changes take place in a sequential fashion, i.e. asymmetric states are allowed (Figure 4C). Distinguishing between the MWC, KNF and other possible models can be important since it is likely that certain allosteric mechanisms have evolved to serve particular functions. For example, it has been suggested [35] that the allosteric mechanism of the eukaryotic chaperonin TRiC/CCT has evolved to be sequential, and not concerted as in the case of the prokaryotic GroEL, in order to facilitate domain-by-domain release and folding of its substrates and, thus, increase the folding efficiency of multi-domain proteins that are more common in eukaryotes. However as mentioned above, it is not possible to distinguish between different allosteric models from sigmoidal plots of fraction of bound sites (or initial rates) vs. ligand concentration. Moreover, even if the MWC model is assumed to be correct it is difficult to extract the values of L , K_T and K_R from such plots [36]. These impasses can be circumvented by determining the values of the binding constants K_1 to K_N for an oligomer with N identical subunits using structural MS.

Consider, for example, the ratio of intensities I_0 and I_1 that correspond, respectively, to the protein populations with zero and one bound ligand molecules. Assuming similar response factors and that non-specific binding is negligible (or has been corrected for) it follows that: $I_1/I_0 = [ES]/[E] = K_1[S]$, where E designates the oligomeric protein. In general, $I_N/I_{N-1} = K_N[S]$ and, therefore, it is possible to determine from a single spectrum the values of the binding constants K_1 to K_N that correspond to the different numbers of bound ligand molecules observed at each substrate concentration $[S]$. Measurements at different substrate concentrations can also be used to reduce the errors in the value estimates. It should be noted that a statistical correction is required to convert these measured values, K_i^{app} , into intrinsic values, K_i^{int} , in order to take into account the number of possible ways in which the i th ligand molecule can bind and dissociate from an N -mer when $i-1$ molecules are already

bound $\left(K_i^{\text{app}} = \frac{N-i+1}{i} K_i^{\text{int}}\right)$. In the case of a symmetric dimer, for example, there are two sites to which the first ligand molecule can bind and only one site from which it can dissociate, whereas there is one site to which the second ligand molecule can bind and two sites from which it can dissociate.

The discrimination between various allosteric models is based on whether the relationships between the experimentally determined intrinsic values of K_1 to K_N correspond to those that are expected for a given model (Figure 4). For example, in the case of the MWC model with exclusive binding to the R state (Figure 4A), the values of all the intrinsic binding constants are expected to be the same and equal to K_R except for the value of K_1 which is expected to differ by a factor of $L/L+1$. In the case of the MWC model with non-exclusive binding to the R state (Figure 4B), the values of all the intrinsic binding constants are expected to be different but they form a mathematical series that can be expressed as a function of L , K_T and K_R (Figure 4B). In such a case, it is possible to determine the values of L , K_T and K_R from the MS data in addition to identifying the allosteric mechanism. Knowing the values of L , K_T and K_R can then be used to determine the ligation pathway, i.e. the number of ligand molecules that need to bind to the T state in order to tip the energetic balance in favor of the R state and, thus, effect the allosteric switch. In the case of the KNF model (Figure 4C), the values of K_1 to K_N are assumed to depend on changes in the inter-subunit interaction energies during ligation. Hence, it is not possible to predict a relationship between the values of K_1 to K_N without taking into account the symmetry (or lack of) of the protein in its apo state and the order in which the subunits become occupied. In the simple case of a ring in which the ligand-induced conformational changes propagate via adjacent subunits, three values of K_1 to K_N can be expected that correspond to the initiation, propagation and termination of the conformational wave from the first to the last subunit. A recent study [18*] that employed this structural MS-based strategy to analyse the allosteric transitions of GroEL showed that they take place as proposed earlier [37], i.e. they are concerted within rings and sequential between rings. It also showed that binding of three ATP molecules is required to effect the T to R transition of a ring.

Determination of Hill coefficients using structural MS

Given the difficulties in distinguishing between different allosteric models, researchers often resort to fitting their data to the Hill equation. Such fits yield a single value for the Hill coefficient (n_H), which is a measure of the extent of cooperativity, but the value of n_H actually changes with ligand concentration as was shown many years ago for hemoglobin [38]. In cases of positive cooperativity, the value of n_H increases and then decreases as a function of ligand concentration because site-site interactions are absent at very low and high ligand concentrations. In general, determining the change in the value of n_H as a function of the ligand concentration from sigmoidal plots of fraction of bound sites vs. ligand concentration is not possible since the range of ligand concentrations for which measurements can be carried out and the accuracy of the measurements themselves are both limited. It was shown, however, that n_H can also be expressed as the ratio between the variance in the observed binding numbers and the binomial variance in the binding numbers that is expected in the absence of cooperativity [38]. The ability of structural MS to resolve

all the protein populations with different numbers of bound ligand molecules provides a way to calculate these variances from the corrected MS intensities and, thereby, determine how the value of n_H changes as a function of ligand concentration, as recently demonstrated for GroEL [18*].

Discriminating between induced-fit and conformational selection using ion mobility native MS

In addition to thermodynamic data such as binding constants, native MS techniques can also provide structural information. One such technique is ion mobility-MS (IM-MS) in which ions are separated based on their differential mobility through a gas-filled chamber under the influence of an electric field [39, 40]. An assembly with a large volume will undergo more collisions with the inert gas molecules and, thus, travel more slowly than an assembly with the same mass but a more compact structure. The migration times, therefore, provide information on the rotationally averaged cross-sectional area (or collision cross-section (CCS)) of the ions, i.e. their size and shape [39, 40] (Figure 1B). Owing to the additional dimension of separation in IM-MS, this technique can yield structural information on co-existing states and is, therefore, an excellent tool for distinguishing between conformational selection and induced-fit. According to the conformational selection model, which is essentially an extension of the MWC model [1] also for monomeric proteins, the ligand binds to one of several (or many) pre-existing conformational states, thereby shifting the equilibrium in favor of that state. In the case of conformational selection, therefore, IM-MS may reveal a number of conformational states that exist in the absence of ligand and merge into one when excess ligand is present. By contrast, only one conformational state is expected, in the absence of a ligand, in the case of induced-fit. In a recent study [41*], IM-MS revealed that the apo state of the regulatory subunit of protein kinase A exists in a large number of conformations that includes that of its cAMP-bound state, thereby demonstrating that the conformational switch of this protein occurs via conformational selection. Similarly, IM-MS was used to show that also binding of NarG (the catalytic subunit of the membrane-bound respiratory nitrate reductase complex, NarGHI) to the NarJ chaperone takes place by means of conformational selection [42]. A limitation of the IM-MS approach for distinguishing between conformational selection and induced-fit is that the various conformational states may not have CCS that are different by at least 10%, which is the current resolution of the technique. Improvements in the technique will, however, make this limitation less restrictive in the future [39, 40].

Structural MS analysis of cooperativity due to ligand-coupled assembly of multimeric proteins

A key feature of the KNF model [2] is that cooperativity in ligand binding results from ligand-induced changes in the inter-subunit interaction energies of the oligomer. Such changes are expected to be particularly large when ligand binding promotes subunit dissociation or association. Ligand-promoted subunit assembly, therefore, constitutes a mechanism for attaining unusually high positive cooperativity (such a mechanism cannot result in negative cooperativity). The ligand may have a higher affinity for the monomer than

the oligomer in which case ligand binding will promote dissociation of the oligomer. Alternatively, it may have a higher affinity for the oligomeric state in which case ligand binding will drive oligomer assembly. Both scenarios will result in positive cooperativity in ligand binding, with respect to ligand concentration, even if all the binding sites in the oligomer have the same affinities for the ligand. An early example for cooperativity due to ligand-promoted oligomeric assembly is the ATP- (or UTP) driven conversion of cytosine triphosphate synthetase from dimer to tetramer [43]. In later work, native MS was employed to show that NADH binding to *E. coli* citrate synthase shifts its equilibrium from dimer to hexamer but, surprisingly, NADH binding was not found to be cooperative [44]. Many ATP-dependent chaperone and degradation machines also undergo ligand-promoted oligomeric assembly. Members of the ClpB/Hsp104 chaperone family, for example, undergo nucleotide-dependent assembly reactions and display positive cooperativity in ATPase activity, with respect to both ATP and protein subunit concentrations [45].

Ligand-promoted subunit assembly is the basis for a more recently put forward model for allosteric regulation dubbed ‘morpheein’ [46]. According to this model, individual subunits are in equilibrium between two conformations (with different ligand binding affinities) and subunits in each of the two conformations assemble into different multimers. An example for such allosteric control was highlighted in a recent native MS study that showed that trimers of the *E. coli* protease DegP form either 6-mers or 9- and 12-mers depending on peptide substrate binding to the PDZ and protease domains of each subunit [47*]. Peptide binding to the 6-mer resting state of DegP generates trimers, which can then form active species such as 12-mers. By contrast, it was shown using various MS techniques that perturbations of the intra- and inter-dimer interactions of the dimeric building block of the human small heat-shock protein α B-crystallin have little effect on its oligomer size distribution [48, 49*]. This appears to be due to allosteric communication between the intra- and inter-dimer interfaces, which results in the strengthening of one type of interface when the other is weakened.

Concluding Remarks

The focus of this review was to show how native MS methods can be used to distinguish between allosteric models. Future improvements in the resolution of native MS will extend the range of potential applications of this approach for studying allostery. For example, it is of great interest in many cases (such as GroEL [50]) to determine the distribution in the number of ATP and ADP molecules bound simultaneously to the same complex but this is not yet feasible. It is also still difficult to use native MS to monitor ligand binding (e.g. ATP) to hetero-oligomers such as eukaryotic chaperonin CCT/TRiC [5] that are made up of distinct subunits with similar masses. A widely used approach to improve resolution is collision-induced dissociation during which an oligomer loses a highly charged subunit and, as a result, becomes less charged and better resolved. This strategy is, however, currently not applicable for studying allostery given the uncertainty whether the stripped subunit is ligand-bound or not [51]. In order to circumvent this problem, new activation approaches, such as surface induced dissociation [52], and computational methods should be explored. Finally, it is also important to note that allosteric models (such as the MWC and KNF models) provide frameworks for describing allosteric transitions but they do not specify at a structural level

how allosteric communication actually takes place [53]. Information regarding the structural changes that occur during an allosteric transition can be obtained using, for example, MS techniques (which are not reviewed here) for monitoring hydrogen/deuterium exchange [54], radical footprinting [55], comparative chemical crosslinking with and without ligand [56] and limited proteolysis [57*].

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Figure 1. MS techniques for studying allosteric mechanisms.

Schematic representation of the native MS (left) and IM (right) approaches. Native MS is based on the ability to transfer intact protein complexes to the gas phase, while maintaining weak non-covalent interactions between protein subunits and bound ligands. Insight into allosteric mechanisms can be gained by determining the protein populations with different numbers of bound ligand molecules (bottom left panel). IM measures the time it takes for an ion to travel through a tube filled with an inert gas. The ion's transit time depends not only on mass and charge, but also on its overall shape: an assembly with a large volume will experience more collisions with the gas and, therefore, travel more slowly than a complex with the same mass but a more compact structure. The measured drift times (dt) can be converted into collision cross-sections which, in turn, can be related to the conformation of

the analyzed assembly. IM can be used to characterize structural changes in the conformational ensemble upon ligand binding.

Figure 2. Electrospray ionization MS can reveal the number of ATP molecules bound to GroEL.

(A) Superposition of spectra acquired on a modified high mass QToF instrument in the presence of different concentrations of ATP (the region of the 57⁺ charge state is displayed). The peaks are labeled according to the number of bound ATP molecules to which they correspond. The increase in the number of bound ATP molecules as a function of ATP concentration reflects the step-wise manner of ATP binding to GroEL (reproduced with permission from [18]). (B) Mass spectra acquired on an extended mass range Exactive Plus Orbitrap showing the 70⁺ charge state of GroEL unbound (black) or incubated with ADP (red) or ATP (blue). The high mass resolution allows the number of bound nucleotides (ATP or ADP + Na⁺, m/z 6.4 or 7.6 Th) to be counted, as indicated (reproduced with permission from [17]).

Figure 3. Recounting protein populations according to specific binding.

The illustration shows for a dimer with two specific and three non-specific ligand binding sites how the populations with different numbers of bound ligand molecules are counted before (A) and after (B) taking into account non-specific binding. The protein populations with 0, 1 and 2 ligand molecules bound at specific sites are shown in red, blue and green respectively. Contributions from both specific and non-specific binding are included in each population with a given number of bound ligand molecules before the correction for non-specific binding is implemented. Following the correction, all the protein molecules with a given number of specifically bound ligand molecules are counted together regardless of the number of non-specifically bound ligand molecules. Listed below each pole of the abacus are the relative weights of the different populations that it counts.

Figure 4. Scheme describing the concerted (MWC) and sequential (KNF) allosteric models for a tetramer.

According to the concerted model, the oligomer in its apo state is in equilibrium between low (T) and high (R) affinity states for the ligand ($L = [R]/[T]$). In the case of the concerted model with exclusive binding of the ligand to the R state (A), the values of the intrinsic binding constants corresponding to all the successive binding steps will be the same except that of the first site which will differ by a factor of $L/(1+L)$. In the case of the concerted model with nonexclusive binding of the ligand to both the T and R states (B), the values of the intrinsic binding constants corresponding to the successive binding steps will all differ

from each other but they will form a series that can be expressed as a function of L and the respective ligand binding constants of the T and R states, K_T and K_R . In the case of the sequential model (C), a simple relationship between the values of the N binding constants is not necessarily expected.