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Tandem Mass Spectrometry Defines the Stoichiometry and Quaternary Structural Arrangement of Tryptophan Molecules in the Multiprotein Complex TRAP

Margaret G. McCammon, Helena Hernández, Frank Sobott, and Carol V. Robinson*

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K.

Received December 15, 2003; E-mail: cvr24@cam.ac.uk

Mass spectrometry (MS) is now well established for the characterization of biomolecules and their noncovalent interactions in macromolecular complexes.[1,2] As the size and complexity of noncovalent assemblies increase, mass measurement alone is often insufficient to delineate all possible combinations of proteins and their ligands. Tandem MS, in which ions at defined *m*/*z* values are isolated for collision-induced dissociation (CID), could in principle overcome these difficulties. When applied previously to small complexes containing up to 6 subunits, excitation by CID or blackbody infrared radiation results in dissociation of single subunits,[3−6] indicating the potential for determining stoichiometry. Here, we employ tandem MS and CID to determine the stoichiometry of complexes of TRAP (tryptophan RNA-binding attenuation protein) with up to 46 protein and ligand molecules.

TRAP from *Bacillus subtilis* has been shown to self-assemble into highly stable oligomeric ring structures[7] in which the stoichiometry is largely unknown. X-ray analyses of the crystal structures of noncovalent TRAP complexes in the presence of trp showed a single ring of 11 subunits and indicated 11 trp binding sites.[8] Under certain conditions, however, it has been proposed that TRAP assembles into higher oligomers including a 12-mer,[9] 22-mer,[8] and, in the presence of RNA, a 44-mer.[7] Using MS, we probe the stoichiometry of assemblies formed with different concentrations of trp in solution.

To examine the interactions between TRAP and trp, we first investigated TRAP with a 4-fold excess of trp per monomer of TRAP in aqueous buffer under MS conditions optimized for noncovalent interactions,[10,11] Figure 1a. Three species are observed, tentatively identified as single, double, and triple ring arrangements of TRAP, respectively. The broad width of peaks observed in the spectrum, attributed to buffer molecules adhering to gas-phase complexes,[12] prevents unambiguous determination of stoichiometry. To reduce this peak broadening, we optimized desolvation conditions by increasing the acceleration of ions in the source and recording the MS (Figure 1b). Splitting within peaks assigned to the single ring is observed for the most intense charge state, revealing the presence of $TRAP_{11}$ and a second species corresponding in mass to $TRAP_{12}$ (Table 1). The latter species has not been characterized previously, and its interactions with trp are unknown. We investigated the stoichiometry and trp binding to $TRAP_{12}$ using a quadrupole time-of-flight instrument capable of tandem MS of high *m*/*z* species.[13]

A MS/MS spectrum of the 12-mer (23+) subjected to a collision cell voltage of 50 V yields peaks at higher *m*/*z* than the isolated ions, assigned to loss of positive ions by charge stripping[14] (Figure 1c). In the MS/MS, these peaks contain pronounced splitting indicative of three discrete populations. Initially, on the basis of measured masses, we assigned these species to fully liganded $TRAP_{12}$, apo $TRAP_{12}$, and an assembly containing half the full complement of trp (F, A, and H, respectively). However, the mass

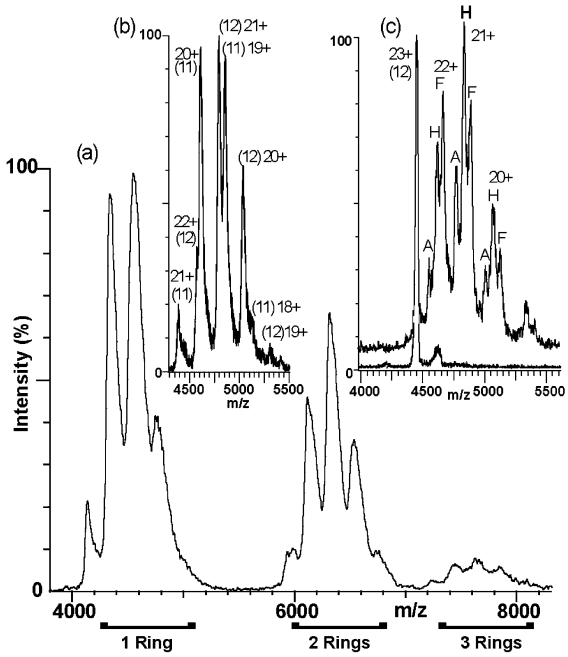

Figure 1. (a) Nanoflow electrospray MS for a solution of TRAP:trp (20 μM) with a molar ratio of 1:4 per monomer of TRAP in ammonium acetate, 20 mM, pH 7.0 at a cone voltage of 90 V. This TRAP:trp ratio was employed throughout unless otherwise stated. (b) Expansion of the MS of TRAP:trp (1:2) assigned to the single ring structure (cone voltage 140 V) with charge states labeled and number of subunits in parentheses. (c) MS/MS spectrum of the *m*/*z* region 4400−4500 encompassing the 23+ charge state of the $TRAP_{12}$ at collision cell voltages of 30 and 50 V (lower and upper traces). Numbers of trp molecules associated with $TRAP_{12}$: A = apo (0), H = 6 trp, and F = 11 trp. Experimental conditions: cone voltage 110 V, capillary voltage 1600 V, extractor cone 5 V. Pressures were as follows: ion transfer stage 5.7×10^{-3} mbar, collision cell 2×10^{-2} mbar.

difference between F and A corresponds to 11 trp, implying that the full complement of 12 trp does not bind to $TRAP_{12}$. Moreover, mass differences between F and H and H and A correspond to 5 and 6 trp molecules, respectively, consistent with binding of 11 trp molecules.

Procedures analogous to those used to examine single ring assemblies were applied to double ring structures. Ions in the *m*/*z* range 6350−6550 were isolated and subjected to CID with increasing collision cell voltage (Figure 2a−d). At low voltage, the spectrum shows isolated 32+ ions with charge states formed by charge stripping, 31+ and 30+. At intermediate voltage, additional charge states (29+, 28+) are observed as well as two species at low intensity corresponding in mass to loss of TRAP subunits from the oligomer. At *m*/*z* 1000−2000, a single TRAP subunit is also evident. At high voltages, two further series are apparent corresponding to losses of two and three TRAP subunits.

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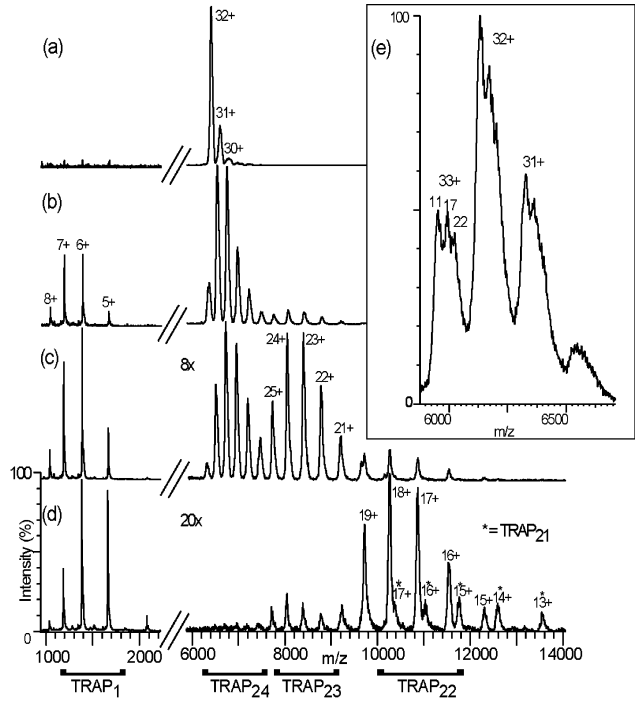

Figure 2. MS/MS of the 32+ charge state of the double ring TRAP assembly at collision cell voltages 45 (a), 75 (b), 85 (c), and 105 V (d). The stoichiometry and highest intensity charge states have been labeled. (e) Expansion of the MS of the double ring structure recorded at a cone voltage of 140 V with numbers of trp molecules shown for the 33+ charge state. Experimental conditions: capillary voltage 1600 V, cone voltage 110 V, and extractor cone 5 V. Pressures were as follows: ion transfer stage pressure 4.9×10^{-3} mbar, collision gas 2×10^{-2} mbar.

Mass analysis demonstrates the presence of dissociation products containing 23, 22, and 21 subunits, confirming that the species that was isolated is composed of 24 TRAP subunits. Close examination of these products reveals differences in mass for each charge state within a series, attributed to variations in numbers of associated trp molecules. For this reason, it is not possible to determine precisely numbers of trp molecules from these MS/MS spectra. Optimization of desolvation conditions for the fully liganded 24-mer, however, effected by acceleration in the source, exposes splitting within these peaks (Figure 2e). Measurement of the mass differences between the three species reveals populations containing 5 and 6 trp molecules, similar to that observed for dissociation of $TRAP_{12}trp_{11}$ and consistent with the loss of trps from one ring of $TRAP_{24}$. Moreover, the observed mass for fully liganded 24-mer is lower than that calculated for $TRAP_{24}trp_{24}$ and is consistent with $TRAP_{24}trp_{22}$.

Given that binding of 22 trp molecules results in self-association to $TRAP_{24}$ and that X-ray analysis has shown trp binding to one face of the TRAP 11-mer,[7] three arrangements are possible for the double ring complex: two symmetric associations and one asymmetric model. For both symmetric associations, all trp molecules are either buried or exposed, we would predict populations containing 22, 12, and 0 trp molecules due to dissociation of 10 and 12 ligands by analogy with the single ring where dissociation of 5 and 6 trp molecules is observed. For the asymmetric structure, in which only 11 trps are exposed, we anticipate populations containing 22, 17, and 11 trp molecules due to dissociation from one ring of 5 and 6 trps. The data reveal 22, 17, and 11 trp molecules bound to the $TRAP_{24}$ (Figure 1e) consistent with dissociation from one ring in the asymmetric arrangement. Moreover, observation of the triple ring species (Figure 1a) is consistent with asymmetric stacking of TRAP rings.

We have shown the presence of TRAP oligomers with half the full compliment of trp molecules during gas-phase dissociation of fully ligated forms. This raises the question as to whether such species are formed as a result of activation in the gas phase or preexist in solution. Because it is difficult to conceive of a gas-phase mechanism in which 5 then 6 trps are lost sequentially from the single and double ring structures, rather than successive ligands, we propose that these differences are related to the structures in solution and are revealed by gas-phase dissociation. A model consistent with these data would involve a subset of 6 trp binding sites that are more stable to dissociation than the other 5, presumably occupying alternate protein subunits. Such a model implies that trp binding induces conformational changes, perturbing binding sites in neighboring subunits. We have also shown that, in addition to 11-mer, $TRAP_{12}$ as well as 24-mer can assemble, the latter formed by stacking of rings such that 11 trps are buried and 11 are exposed. The fact that neither $TRAP_{12}$ nor 24-mer binds to 12 or 24 trps, respectively, but rather 11 and 22 molecules, allows us to propose that changes induced by trp binding also destabilize the fully liganded structures. More generally, these results illustrate how tandem MS is a powerful adjunct to existing structural biology techniques, revealing not only the stoichiometry of a 46-component complex but also quaternary structural arrangement and subtle differences in trp binding within individual rings.

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Supporting Information Available: Calculated and measured masses of all species observed. This material is available free of charge via the Internet at http://pubs.acs.org.

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