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Native MS and ECD Characterization of a Fab-Antigen Complex May Facilitate Crystallization for X-ray Diffraction

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Abstract

Native mass spectrometry (MS) and top-down electron-capture dissociation (ECD) combine as a powerful approach for characterizing large proteins and protein assemblies. Here we report their use to study an antibody Fab (Fab-1)-VEGF complex in its near-native state. Native ESI with analysis by FTICR mass spectrometry confirms that VEGF is a dimer in solution and that its complex with Fab-1 has a binding stoichiometry of 2:2. Applying combinations of collisionally activated dissociation (CAD), electron-capture dissociation (ECD), and infrared multiphoton dissociation (IRMPD) allows identification of flexible regions of the complex, potentially serving as a guide for crystallization and X-ray diffraction analysis.

Introduction

Methods are increasingly needed to characterize protein therapeutics including monoclonal antibodies (mAbs) and their complexes. Although low-resolution circular dichroism and size-exclusion chromatography, complemented by high-resolution nuclear magnetic resonance (NMR) and X-ray crystallography, provide solutions [1–3], they are labor-intensive, require large sample size and sometimes restricted in protein size. Thus, the need increases to provide rapid approaches to high order structure (HOS).

MS can determine the molecular weight and primary structure of an antibody-antigen complex [4, 5], but more structural information can be obtained utilizing bottom-up or top-down analysis. Using the former, one can obtain peptide and even the residue-level data by protein footprinting by hydrogen deuterium exchange [6, 7], hydroxyl radical, or other labeling reactions [8–12], yielding maps of epitope binding and complementarity-determining regions (CDR).

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Supporting Information Available

Although a top-down *structural* approach with native MS affords high sensitivity, fast turnaround, and no artifacts from digestion [13–15], fragmentation to give high-order structure is difficult. The coarse-grained information from this approach identifies subunits, post-translational modifications (PTMs), and metal binding sites [16]. CAD enables good desolvation, ECD promotes backbone cleavages to identify flexible regions, and IRMPD releases fragments that had remained bound after ECD.

Results and Discussion

Making the reasonable assumption that native ESI allows a protein complex to maintain much of its native structure in the gas phase, we applied ECD, coupled with CAD and IRMPD to take apart a Fab/antigen complex. The structural information obtained from ECD pinpoints surface/flexible regions of the complex[17–23], providing information on the epitope or CDR. If the fragmentation occurs in flexible regions, as we have hypothesized, those regions that deter crystallization or are difficult to analyze by either NMR or X-ray crystallography become identifiable. The surface/flexible regions can either be within the complex or within the individual proteins. This information is alternatively available by limited proteolysis [24–26], but that requires longer preparation times and larger samples.

The system for study is an antibody Fab (Fab-1) and its antigen, VEGF, a highly specific angiogenic factor that stimulates vasculogenesis and angiogenesis[27–29]. The Fab-1 region originates from a neutralizing humanized mAb A4.6.1. We chose this system because this type is of high interest in biotechnology, and there is a crystal structure to allow comparison of results. Our objectives are to determine stoichiometry and to inquire whether flexible and dynamic regions of the complex can be located by ECD. Given that these flexible regions often stand in the way of crystallization, we are asking whether ECD information that can direct modifications of the protein to facilitate crystallization for X-ray structure determination.

We contrast the outcome of introducing the Fab-1-VEGF complex into a mass spectrometer by denaturing ESI for MW measurement and by native MS to confirm that VEGF in the complex is a covalently-linked dimer in solution (Figure S1). Denaturing ESI gives a better measurement of MW than does native MS because more solvent and salts may remain attached during the latter. Native MS preserves, at least in part, the high-order structure seen in the solid-state X-ray structure [30, 31]. From native MS, we learned the complex has a MW of 135.12 kDa and contains one VEGF dimer (monomer MW = 19.17 kDa) and two Fab-1 proteins (monomer MW = 48.40 kDa). Thus, the stoichiometry of the complex is 2:2 Fab-1:VEGF (Figure 1A; native MS of Fab-1 is in Figure S2). Although the outcome is consistent with a previously published X-ray structure of the complex [32], native MS reveals the stoichiometry of the non-covalent complex in solution with lower sample quantity than X-ray, higher accuracy than SEC and multi-angle light scattering (MALS), and faster turnaround than most other biophysical approaches[33–35].

As expected, the ions from native MS are at high m/z (5000 to 6000, from +23 to +27, Figure 1A). These data contrast significantly with the outcome of denaturing ESI for the proteins in their free forms separately, where the median charge states for the heavy chain,

light chain, and VEGF are +24, +22, +23, respectively (Figure S3–S5). The ions from native MS of the complex are less charged (+23 to +27) than those produced by denatured ESI and exist as a much smaller distribution. Native ESI preserves the tertiary structure of the complex, at least on the short timescale of MS, and introduces a stable complex that retains sufficient high-order structure to maintain intact the complex [15], whereas denaturing ESI breaks non-covalent interactions.

Successful introduction into a mass spectrometer affords the opportunity to apply MS/MS methods to the protein complex. We previously reported that a combination of CAD, ECD, and IRMPD does fragment constituents in flexible regions rather than dissociate the complex into constituent proteins as does CAD on Q-ToFs [17]. These data can serve as a guide to modify antibody-antigen complexes for X-ray diffraction analysis.

The dissociation methods produce several species, as seen in the production spectrum of Fab-1-VEGF complex (Figure 1B and Table 1). A dominant dissociation of the complex is to charge-reduced ions in the higher m/z region (i.e., lower charge states) than those of the precursor. Although these reveal little about structure, several ions within this m/z region can map the Fab-1-VEGF interaction. The top-down mass spectrum shows formation of ions with masses of the complex minus one Fab-1 (2 VEGF + 1 Fab-1) and of the Fab-1 alone (Figure 1), indicating step-wise dissociation of the Fab-1 from the complex. The process whereby both Fab-1 molecules are expelled from the complex is not dominant. More importantly, our results indicate that, in the complex, each Fab-1 protein interacts with one core dimeric VEGF, as shown in the X-ray structure [32]. In addition, we observe ions that represent the heavy chain (HC) and the light chain (LC) from the Fab-1. There is only one inter-chain disulfide bond between HC and LC; thus, the structure of Fab-1 is easier to dissociate upon activation than is the full antibody. The outcome provides confirmation that the Fab-1 lies on the perimeter of the complex (all the information from the subunits is summarized in Table 1).

The product ions exist in the low m/z region and provide additional structural detail. We identified three series of fragment ions from the three distinct species, the HC and LC from the Fab-1 (Figure 2A), and from VEGF (Figure 2B). We observed c_3 - c_{10} and z_2 - z_7 ions from HC (E1 to G10 and S225 to L231, respectively), c_3 - c_9 (except c_7 because the proline-8 of the Fab-1 LC cannot be cleaved) from LC (D1 to S9), and c_3 - c_{12} from VEGF (A1 to H12). Some of the small c and z ions occur in congested region and are difficult to identify. In addition to these c and z ions, there are several b and y ions from both HC (b_4 and y_4 , L4 and K228, respectively) and LC (b_4 to b_7 , L4 to S7) likely formed in IRMPD (Figure S6).

Although these fragments delineate part of the sequences of Fab-1 and VEGF, they also indicate that the corresponding regions either lie on the surface and/or are highly flexible, and can be excluded from any binding interface. Indeed, the crystal structure indicates that these regions do not include the CDR or epitope-binding residues.

In X-ray crystallography, flexible regions of a protein often hamper crystallization and are candidates for removal to facilitate crystallization. Prior identification of such flexible regions relies on limited proteolysis by nonspecific or combinations of specific proteolytic

enzymes, requiring time and sample [36]. Native ESI and activation can provide similar information from a single experiment, with low sample consumption (μg) and fast turnaround (up to 2 h).

Some of the fragment ions represent surface residues (c3 to c10 for HC, and c3-c9 for LC), but most of them are from regions either not seen in the X-ray structure or deleted to obtain the crystals. For example, of the 231 residues of the HC, the X-ray structure reveals only residues from E1 to K224. The ECD of the Fab-1 gives z2-z7 ions from the HC (Figure 2A), indicating that region S225-L231 is highly flexible, dynamic, and appropriate to remove for X-ray crystallography. Similarly, the receptor-binding domain of VEGF that was included for X-ray crystallography began at G8 to D109 but the structure is seen starting at E13, indicating that the first 12 residues (A1 to H12) are highly flexible and do not diffract well. Indeed, we observe abundant c3 to c12 ions (Figure 2B), corresponding to residues A1 to H12 of VEGF. Thus, those regions of the C-terminus of the Fab-1 HC and the N-terminus of VEGF fragment readily, but they do not show in the X-ray structure.

We emphasize that this approach contrasts significantly with top-down approaches using denaturing electrospray, where the protein charge is higher and the fragmentation is more efficient as needed for sequencing. This approach was previously used for mAbs [13, 37, 38], where the goal was to provide high sequence coverage. Our strategy is to keep together the complex by native MS to provide binding stoichiometry, flexible-region location, and even partial sequence, which cannot be obtained by denaturing ESI.

Methods

Methods and instrumentation are described in detail in the supplementary information. The Fab-1-VEGF complex was prepared with a 2:1 molar ratio, purified by size exclusion chromatography, and re-analyzed to insure purity. The proteins were buffer-exchanged into 200 mM NH_4OAc (pH 6.8), centrifuged, and washed to complete desalting.

Native MS and activation were on a Bruker Solarix 12 T FT-ICR, as described previously [16]. All products were produced by a combination of CAD, ECD, and IRMPD in series. Denaturing ESI, as described previously [7], followed sample denaturation with urea, reduction with DTT, acidification with HCOOH , and separation on a C4 analytical column prior to MS analysis in the positive-ion mode of a MaXis 4G quadrupole time-of-flight mass spectrometer (Bruker, Bremen, Germany). Product ions were identified by using Biotoools software; assignments were accepted if experimental m/z was within 5 ppm of theory, had a signal-to-noise ratio greater than 5:1, and were validated manually.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Native MS (A) and B) product-ion spectrum of the complex produced by combining CAD, ECE, and IRMPD. “Heavy Chain/Light Chain” highlighted regions show peaks corresponding to the mass of heavy chain (HC) and light chain (LC) from Fab-1. “Fragments” highlighted the region of fragment ions from both VEGF and Fab-1.

Figure 2. Zoom-in region of the native MS upon activation by combination of CAD, ECD and IRMPD (Figure 1B) of m/z 200–1250. The assigned fragments (c and z ions) are highlighted in different colors according to their source. The unassigned peaks may represent internal fragments, which cannot be identified by current software.

Table 1

Identified components in native ESI upon activation of the Fab-1-VEGF complex by CAD, ECD, and IRMPD.

Protein	<i>m/z</i>	Deconvolved aver. mass (theory) in kDa
Intact complex (2VEGF + 2Fab-1)	5000–10000	135.12 (135.11)
2VEGF + 1Fab-1	5500–8000	86.72 (86.72)
Fab-1	3000–7000	48.40 (48.39)
HC and LC of Fab-1	1900–2500	24.96 (24.96); 23.44 (23.43)
Fragments	200–1300	see Figure 2