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Native Top-Down Mass Spectrometry with Collisionally Activated Dissociation Yields Higher-Order Structure Information for Protein Complexes

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c06726.
Materials and methods; supplementary figures (Figures S1-S15) showing nTDMS and complex-down MS spectra, data, and protein structures of those studied; Table S1, listing information for all proteins studied (PDF)

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Abstract

Native mass spectrometry (MS) of proteins and protein assemblies reveals size and binding stoichiometry, but elucidating structures to understand their function is more challenging. Native top-down MS (nTDMS), i.e., fragmentation of the gas-phase protein, is conventionally used to derive sequence information, locate post-translational modifications (PTMs), and pinpoint ligand binding sites. nTDMS also endeavors to dissociate covalent bonds in a conformation-sensitive manner, such that information about higher-order structure can be inferred from the fragmentation pattern. However, the activation/dissociation method used can greatly affect the resulting information on protein higher-order structure. Methods such as electron capture/transfer dissociation (ECD and ETD, or ExD) and ultraviolet photodissociation (UVPD) can produce product ions that are sensitive to structural features of protein complexes. For multi-subunit complexes, a long-held belief is that collisionally activated dissociation (CAD) induces unfolding and release of a subunit, and thus is not useful for higher-order structure characterization. Here we show not only that sequence information can be obtained directly from CAD of native protein complexes but that the fragmentation pattern can deliver higher-order structural information about their gas- and solution-phase structures. Moreover, CAD-generated internal fragments (i.e., fragments containing neither N-/C-termini) reveal structural aspects of protein complexes.

Native top-down mass spectrometry (nTDMS) of gas-phase proteins yields product ions that can provide information on amino acid sequence,[1,2] sites of modifications,[3-5] and even higher-order structure.[6] Performing nTDMS with electron-based techniques such as electron capture dissociation (ECD) and electron transfer dissociation (ETD)[7-11] and photon-based techniques such as infrared multiphoton dissociation (IRMPD) and ultraviolet photodissociation (UVPD)[8,12-14] is generally favored, as it fragments the complex directly without disrupting the overall complex structure. In contrast, it has been generally assumed that collision-based fragmentation does not reveal higher-order structural information, as unfolding and ejection of monomer subunits (and ligands) occurs. However, we have found that direct fragmentation of native protein complexes with Orbitrap-based high-energy C-trap dissociation (HCD),[15] a collision-based fragmentation technique performed with higher energy on a faster time scale than conventional collisionally activated dissociation (CAD), can uncover aspects of protein higher-order structure. For a variety of protein complexes, we show here that HCD can generate *b*-/*y*-type product ions that provide information on solvent-exposed regions and subunit interfaces.

To investigate HCD fragmentation of protein complexes,[16] complex-down MS (pseudo-MS^3)[17,18] and nTDMS (Scheme 1) of yeast alcohol dehydrogenase (ADH) homotetramer (147 kDa) were compared. Complex-down MS was performed by using in-source CAD to detach a monomer from the tetramer and to subsequently activate the 12+ charged monomer with HCD. The resultant MS/MS spectrum revealed both N-terminal *b*-fragments and C-terminal *y*-fragments of ADH (Figure S1A); 24 *b*-fragments and 18 *y*-fragments

resulted in 11.8% total sequence coverage (Figure 1A). The fragmentation pattern also revealed the presence of N-terminal acetylation, a V58T proteoform, and Zn^{2+} binding. The presence of near equal numbers of abundant *b*- and *y*-fragments from the complex-down MS workflow suggests that both termini of the ADH monomer subunit are easily accessed by HCD fragmentation, i.e., the in-source CAD process releases a low-structured monomer such that subsequent HCD products yield little information about the 3D structure of the native tetramer.

For comparison, nTDMS results from HCD of the 25+ charged ADH tetramer were examined. Primarily *b*-products and surprisingly few peaks corresponding to released ADH monomers (Figure 1B, Figure S1B) were detected. We speculate that monomers were not ejected from the tetramer complex prior to covalent bond cleavage, i.e., the tetramer fragmented directly. To further support this claim, broadband fragmentation (of all ADH tetramer charge states) with a range of HCD energies did not yield significant levels of released monomer signals (Figure S2). nTDMS of ADH yielded 60 N-terminal *b*-fragments, but only three C-terminal *y*-fragments (17.6% sequence coverage) (Figure 1B). Numerous abundant N-terminal fragments produced by HCD resemble nTDMS products from electron-based[7,8] and photodissociation techniques.[8,12] Mapping the fragments onto the crystal structure of ADH shows that the N-terminal region is more solvent exposed than the C-terminal region, with the latter forming subunit–subunit interfaces of the complex (Figure S3). Our analysis indicates that fragments that cut at the interface of the tetramer (residues 240–310) accounted for only 8% of the fragment ion current.

To further examine how collision-based fragmentation can reveal structural information from protein complexes, intact (rabbit) aldolase homotetramer (157 kDa) was fragmented with HCD. Much like ADH, aldolase did not release monomers upon HCD, but rather *y*-fragments including an especially abundant y_{74} ion (2+ to 5+ charged) (Figure S4). At low HCD energies, a large complementary fragment corresponding to the mass of the intact tetramer losing a y_{74}-fragment, i.e., (4M – y_{74}), was observed (Figure S5 and Table S1), indicating direct fragmentation of the tetramer. nTDMS yielded 35 C-terminal *y*-fragments but only eight N-terminal *b*-fragments (11.0% sequence coverage) (Figure 2). This result differs from the complex-down mass spectrum of aldolase, which shows a nearly equal proportion of N-terminal *b*-fragments (19) and C-terminal *y*-fragments (16) (Figure S6).

The HCD fragments from the aldolase tetramer mainly cover the solvent-exposed C-terminus and are absent from the interface forming N-terminus (Figure 2). Our analysis indicates that fragments that cut at the interface of the tetramer (residues 110–224) accounted for only 1% of the fragment ion current. The relatively high proportion of C-terminal fragments present in the native HCD spectrum of aldolase is similar to that measured by ECD previously[19] and further suggests that direct HCD fragmentation of some protein complexes can reveal regions of solvent accessibility.

nTDMS with HCD was performed on several other protein complexes. Complex-down fragmentation of the glutathione S-transferase A1 (GSTA1) dimer revealed 25 N-terminal *b*-fragments and 20 C-terminal *y*-fragments (Figure S7A). In contrast, the native fragmentation spectrum of GSTA1 reveals five N-terminal *b*-fragments and 19 C-terminal *y*-fragments

(Figure S7B), consistent with the GSTA1 crystal structure showing that the C-terminus is more solvent exposed than the N-terminus (Figure S7B). For the yeast enolase dimer, 27 *b*-fragments along with 18 *y*-fragments were measured by complex-down MS (Figure S8A). nTDMS revealed 48 N-terminal *b*-fragments along with 51 C-terminal *y*-fragments without the appearance of abundant monomer ions (Figure S8B). The crystal structure of enolase (Figure S8B) indicates that both N-/C-termini are solvent exposed and are not involved in forming the dimer interface, consistent with the near equal proportion of *b*-/*y*-products measured by nTDMS.

Some complexes did not release monomers from in-source CAD for complex-down fragmentation; however, HCD of the native complexes still returned structural information. Native HCD of the creatine kinase dimer revealed nine *b*- and 38 *y*-fragments, which suggests that the C-terminus is solvent exposed and the N-terminus forms the interface of the dimer; this aligns well with the crystal structure of creatine kinase (Figure S9). Similarly, HCD of 6-phosphogluconate dehydrogenase (GND1) dimer generated 23 *b*-fragments but only six *y*-fragments, consistent with the GND1 crystal structure showing the N-termini to be solvent exposed and the C-termini forming the dimer interface (Figure S10).

There are some exceptions to this pattern of *b*-/*y*-product formation directly from intact native complexes under HCD. For example, HCD of the native membrane protein, aquaporin Z (AqpZ) homotetramer,[20-22] yielded abundant monomer, dimer, and trimer products released from the intact complex (Figure S11A). This observation can be attributed to the weak hydrophobic binding interface between the monomer subunits of the AqpZ tetramer. Complexes such as aldolase and ADH are stabilized somewhat by salt bridges that strengthen greatly in the gas phase,[23,24] potentially preventing monomer ejection during HCD (Figure S12). That monomer products are released when HCD is applied to native AqpZ complexes suggests that structural information (such as the locations of solvent-exposed regions and the tetramer interface) cannot be inferred from the resulting *b*-/*y*-fragments, at least assuming that the monomers likely eject before covalent bonds cleave. This suggestion is supported by the fact that the nTDMS fragmentation pattern of AqpZ tetramers (65 *b*-fragments, 62 *y*-fragments, 38.4% sequence coverage; Figure S11A) does not differ significantly from the complex-down fragmentation pattern of isolated monomers (63 *b*-fragments, 60 C-terminal *y*-fragments, 34.6% sequence coverage; Figure S11B). Although HCD fragmentation of native AqpZ does not reveal significant higher-order structural information, it does suggest that the interaction between complex monomers in the gas phase is relatively weak.

Monomer releases during HCD are not limited to membrane protein complexes. HCD fragmentation of the hemoglobin (Hb) tetramer revealed monomer and trimer peaks in addition to 10 *b*-fragments and eight *y*-fragments from the α-subunit and seven *b*-fragments and seven *y*-fragments from the β-subunit (Figure S13A). Fragmentation of the Hb dimer also revealed released monomer peaks in addition to 11 *b*-fragments and 16 *y*-fragments from the α-subunit and 10 *b*-fragments and four *y*-fragments from the β-subunit (Figure S13B). A similar HCD fragmentation pattern can be observed from complex-down MS of individual subunits (nine *b*- and 10 *y*-fragments and six *b*- and five *y*-fragments from the α- and β-subunits, respectively) (Figure S13C). Similarly, nTDMS of human transthyretin

(TTR) tetramers by HCD releases monomer products in addition to two *b*- and 38 *y*-fragments (Figure S14A). The relative proportion of *b*-/*y*-product ions between the tetramer and monomer TTR is similar, with complex-down of the TTR monomer yielding three *b*-fragments and 41 *y*-fragments (Figure S14B). The HCD results for all of the complexes included in the study are listed in Table S2.

Lastly, we investigated the utility of internal fragments (i.e., product ions containing neither N-/C-termini that result from at least two bond cleavage events)[1,25-30] for structure determination of protein complexes. Preliminary data show that HCD fragmentation of ADH tetramers reveals numerous internal fragments spanning residues 178–236 (Figure S15A), which correspond to a solvent-exposed region (Figure S15B). More work will extend this concept further, but it demonstrates that HCD-derived internal fragments can deliver structural information on protein assemblies.

Although other studies have noted the detection of *b*-/*y*-products with concurrent subunit release from CAD[31] and HCD[16] of protein complexes, we have found that collision-based fragmentation with HCD can reveal higher-order structure information for several multi-subunit protein complexes that appear to be stabilized through the presence of salt bridges.[23] These complexes fragment directly by HCD without significant monomer release. The resulting products map to solvent-exposed areas, while regions delivering fewer fragments likely comprise subunit interfaces. Other weak gas-phase complexes eject monomers upon HCD. Nonetheless, it is currently unclear what differences between HCD and other beam-type CAD experiments are responsible for the unique fragmentation behavior.

An assumption carried over from small-molecule dissociation studies to macroion decompositions is that, on the experimental time scale, activation from collisions always randomizes fully to steer collision-induced decompositions along the lowest energy pathways. However, those assumptions fail to consider that entropically demanding, slow rearrangements might be essential to releasing a subunit, e.g., to reposition salt bridges tethering one subunit to others.[23] In cases where the number of collisions and/or energy per collision are insufficient to stumble on the rare configuration ejecting a subunit within the experimental time frame, alternative rearrangements to eject smaller polypeptide fragments (with fewer tethers) may be competitive. Nevertheless, we show that HCD can be a powerful biophysical tool to probe the structure of proteins without the need for other electron- and photon-based activation/dissociation methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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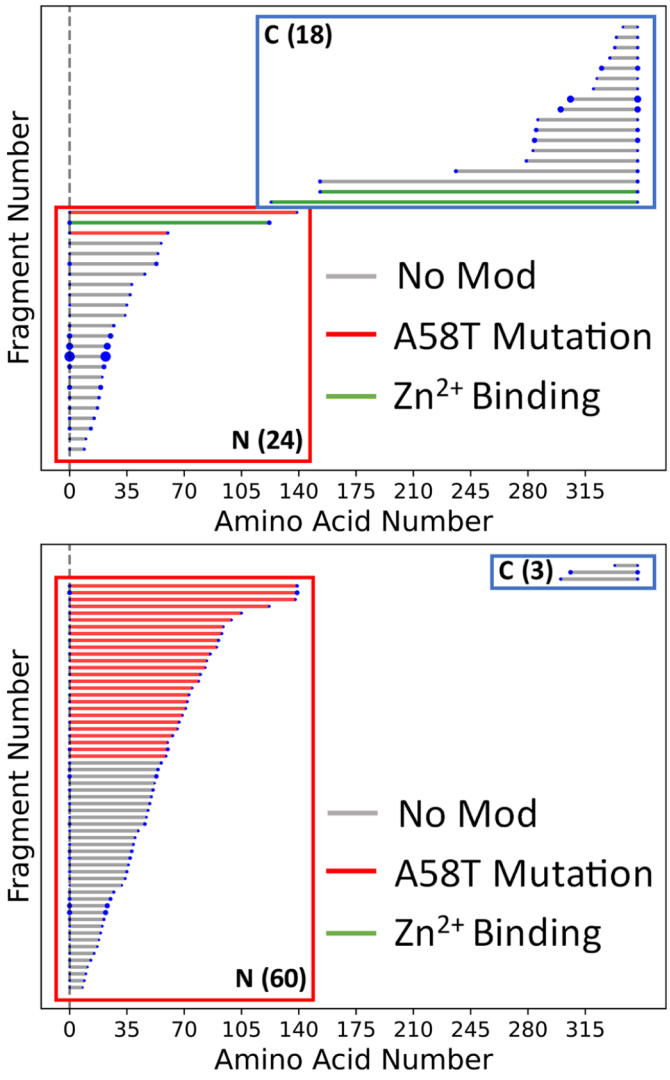


Figure 1.

Fragment location maps for ADH representing *b*-/*y*-product ions measured by (top) complex-down MS and (bottom) nTDMS with HCD. Red lines indicate V58T mutation, green lines indicate Zn^{2+} binding, the vertical dotted line indicates N-terminal acetylation, and the size of the blue dots indicates the relative intensity of each fragment. Numbers in parentheses indicate the number of product ions detected.

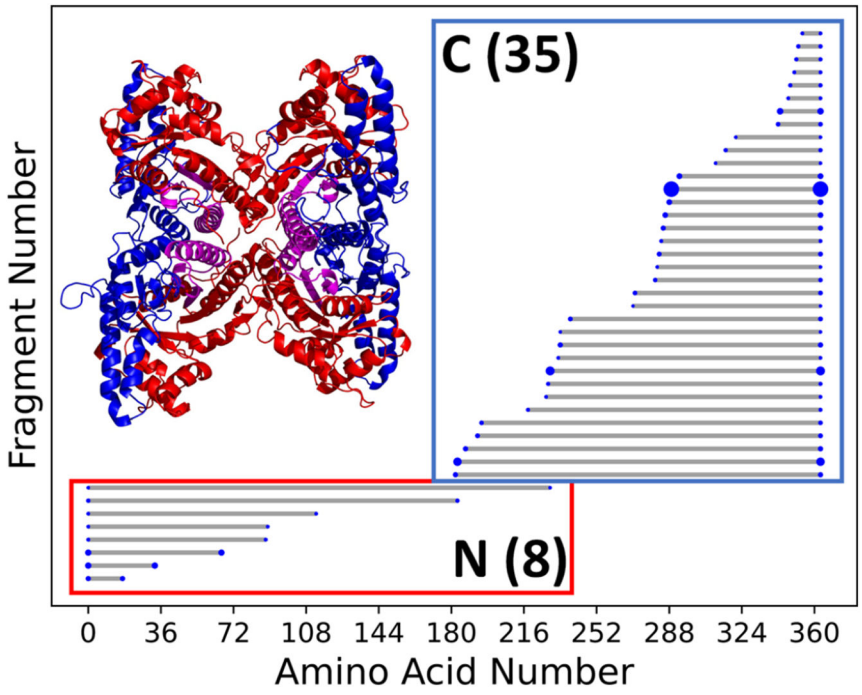
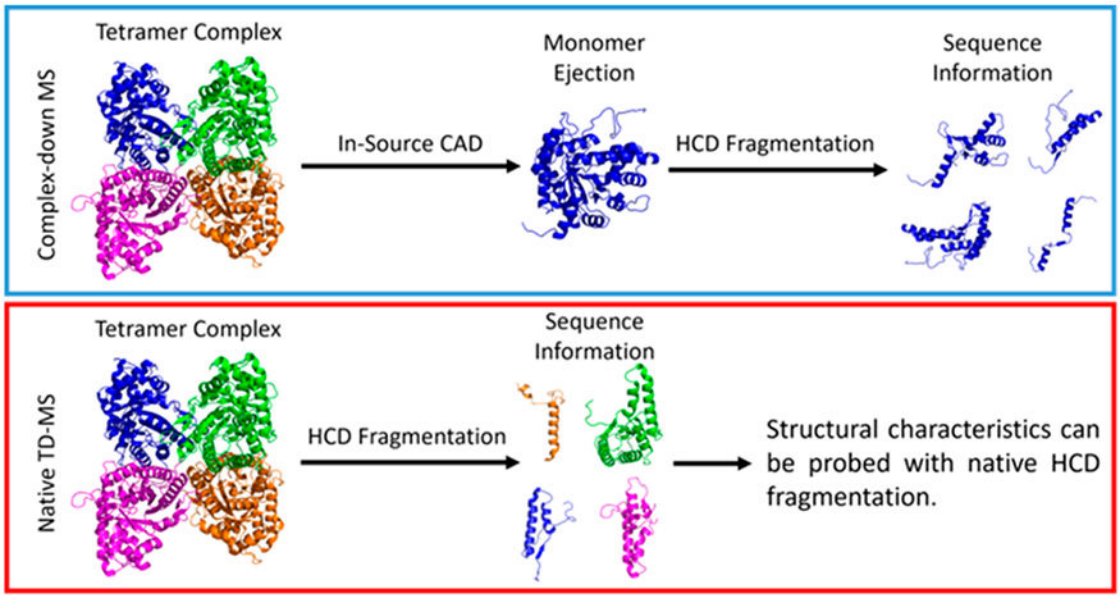


Figure 2.

Fragment location map for nTDMS products of the 25+ charged precursor of aldolase homotetramer, with the size of the blue dots corresponding to the relative intensity of the fragments The crystal structure shows that most cleavage sites lie on the solvent-exposed C-terminus (blue), rather than the interface forming N-terminus (red). The purple region is covered by both N-terminal and C-terminal fragments.



Scheme 1.
Complex-Down MS and nTDMS Workflows Used in This Study.