Location, Location, Location… Native Top-Down Mass Spectrometry Provides Binding Site Information for Protein-Ligand and Protein-Protein Interactions

Athanasios Smyrnakis[1]; Maria-Aggeliki Kosmopoulou[1]; Dimitris Papanastasiou[1]; Isin Sakallioglu[2], Michael Greig[2]; Matthew Kensil[3]; Joseph A. Loo[3]

[1]Fasmatech Science & Technology, Chalandri, Greece; [2]Bruker Scientific LLC, San Jose, CA; [3]UCLA, Los Angeles, CA

Introduction

Native top-down mass spectrometry (nTDMS) not only generates information on size and binding stoichiometry for macromolecule complexes, but the dissociation of the gas-phase complexes informs on sequence (and identity) and post-translational modifications. With sufficient fragmentation, obtaining binding site location of protein-ligand and protein-protein complexes is possible. Electron-capture dissociation (ECD) and electron ionization dissociation (EID) can retain ligand binding to product ions because pure trapped EXD (tEXD) processes often do not disrupt noncovalent bonds.

We show that trapped EXD-MS on the timsOmni MS system can define the amino acid residues involved in ligand binding and the contact sites of multi-subunit protein complexes. These capabilities are enhanced by incorporating multiple stage MS^n experiments to further pinpoint binding sites and protein-protein interfacial regions.

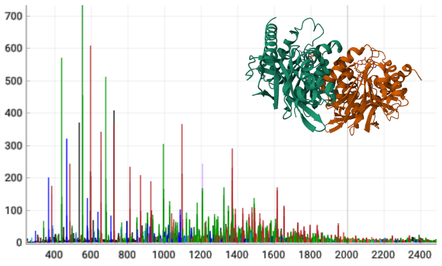

Fig. 2 MS^3 EID of the L-MDH monomer (z=10). Inset: Structure of the L-MDH homodimer.

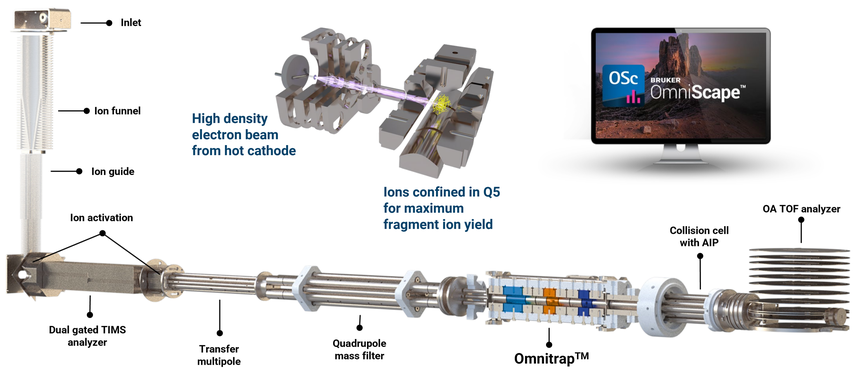

Fig. 1 Schematic of the timsOmni MS platform. A magnified view shows the external electron source coupled to Q5. The trapped EXD reaction landscape is enabled by precise control of electron energy and irradiation time. OmniScape sofware is used to expedite and simplify top-down data analysis workflows.

Instrumentation and Methods

Commercially available holo-myoglobin (Mb; horse) and L-malate dehydrogenase (L-MDH; pig) were buffer-exchanged into 150mM ammonium acetate and electrosprayed at a concentration of 5μM using Humanix Cellomics emitters with the NEOS source. Ions were activated by in-source CID (isCID) in argon gas to enhance desolvation and to dissociate protein complexes into subunits. The subunits were subsequently accumulated in the Bruker Omnitrap platform for MS^n processing following *m/z* selection using an RF generator tuned for ion isolation up to 4500 Th. Ion-activation experiments included resonance-excitation CID ($_R$CID), ECD, EID, and combinations thereof. Enhanced signal-to-noise mass spectra were obtained through ion accumulation times exceeding 100 ms and by averaging spectra over ~3 minutes, enabling deep sequencing coverage. Data processing and interpretation were performed using the OmniScape software. An instrumentation and method overview is shown in **Fig. 1**.

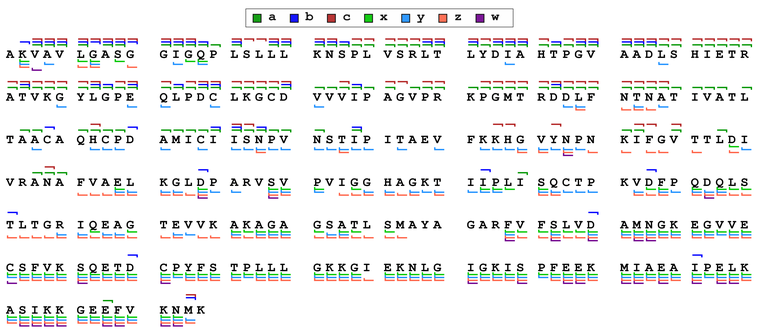

Fig. 3 Combined sequence map of MS2 experiments on the L-MDH monomer z=10+ and dimer 17+ providing 92% total Sequence Coverage.

Results and Discussion

L-Malate Dehydrogenase

MS^2 $_R$CID of the 17+ L-MDH 66 kDa homodimer yields 44% sequence coverage (SC). The majority of the detected y-ions cover the C-terminus, consistent with its structure showing a solvent exposed C-terminus. MS^2 ECD and MS^2 EID (32 eV) of the 17+ dimer both generate 49% coverage (separately), also primarily from the solvent exposed C-terminal end. Further nTDMS of the released monomer from in-source CID (MS^3 ECD, EID and ECciD) provided access to the N-terminus, with MS^3 EID **(Fig. 2)** getting deeper into the monomer core and yielding the highest SC of 63%. A total SC of 92% (**Fig. 3**) was provided by combining MS^2 experiments on both the monomer z=10+ and the dimer z=17+. Importantly, the M227-R233 dimer interface region did not produce any MS^2 fragments, which suggests that this stretch is protected and involved in tight protein-protein interactions as probed by nTDMS.

Holo-myoglobin

Myoglobin is involved in oxygen- and iron-binding through its interaction with protoporphyrin IX (heme). The holo-myoglobin gas-phase complex is relatively fragile, as heme is readily lost from collisional activation. $_R$CID of 8+ holo-Mb yields 68% sequence coverage and loss of heme. Intact heme and fragments of heme are consistent with its structure (**Fig. 4**). Loss of one or both carboxymethyl groups from the propionate side chains are observed at ~557 Th and 498 Th, respectively, as well as methyl group losses from the porphyrin periphery (420-490 Th).

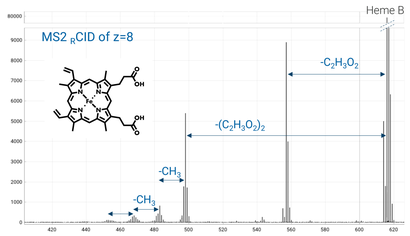

Fig. 4 Heme fragmentation in MS2 $_R$CID of holo-myoglobin.

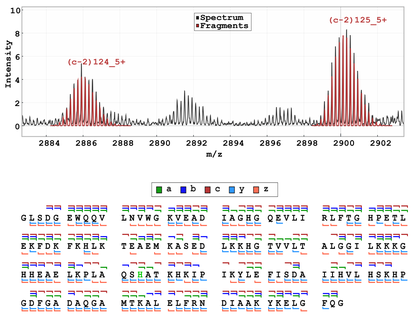

Fig. 5 a. c-fragments exhibiting extended isotopic distributions shifted by two hydrogen atoms. **b.** Combined sequence map of MS^2 $_R$CID, MS^2 ECD and MS^3 $_R$CID of holo-myoglobin.

MS^2 ECD of 8+ holo-Mb generates structure-consistent c-/z-product ions between H64 and H93 with and without bound heme group, showcasing that it can remain attached on either of the two histidines after dissociation.

In addition, ECD yields charge-reduced radical intact holo-Mb; MS^3 CID of the 7+ radical holo-Mb produces fragment ions (c, z, y mostly) in the region H64-H93, not accessible by MS^2 ECD, but heme-loss is observed. Interestingly, fragments near both termini (c120-150 and z110-150) remain attached to the heme group indicating that heme loss is size-dependent and less efficient when greater size fragments are formed. Most of these heavier c-fragments exhibit heterogeneous populations with extended isotopic distributions shifted by two hydrogen atoms (-2H), which is likely related to the oxidation state of Fe-heme following collisional activation (**Fig. 5a**). Overall, the combined data show SC=99% (**Fig. 5b**), and that heme can remain attached on either of the two histidines after ECD.

- timsOmni is a flexible MS^n platform combining CID and tEXD to provide a comprehensive fragmentation toolkit
- These multimodal fragmentation capabilities combined with nTDMS provided unique data for mapping specific sites of ligand- and protein-binding

Proteomics: Intact Proteins and Top Down Analysis II

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Conflict of Interests
MK, IS, MG, AS and DP are employees of Bruker, which develops and manufactures advanced mass spectrometry platforms for life science applications. This presentation includes data and findings related to Bruker's new timsOmni product.