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Multi-Stage Ultraviolet Photodissociation Mass Spectrometry to Characterize Single Amino Acid Variants of Human Mitochondrial BCAT2

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Abstract

Unraveling disease mechanisms requires a comprehensive understanding of how the interplay between higher-order structure and protein-ligand interactions impacts the function of a given protein. Recent advances in native mass spectrometry (MS) involving multi-modal or higher energy activation methods have allowed direct interrogation of intact protein complexes in the gas phase, allowing analysis of both composition and subunit connectivity. We report a multi-stage approach combining collisional activation and 193 nm ultraviolet photodissociation (UVPD) to characterize single amino acid variants of the human mitochondrial enzyme branched-chain amino acid transferase 2 (BCAT2), a protein implicated in chemotherapeutic resistance in glioblastoma tumors. Native electrospray ionization confirms that both proteins exist as homodimers. Front-end collisional activation disassembles the dimers into monomeric subunits that are further interrogated using UVPD to yield high sequence coverage of the mutated region. Additionally, holo (ligand-bound) fragment ions resulting from photodissociation reveal that the mutation causes destabilization of the interactions with a bound cofactor. This study demonstrates the unique advantages of implementing UVPD in a multi-stage MS approach for analyzing intact protein assemblies.

TOC Graphic

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SUPPORTING INFORMATION

Detailed descriptions of instrument modifications, experimental methods, and Figures S1-S9 including the expressed sequence of BCAT2 and structures of the PLP cofactor, schematic of the modified instrument, ppm error plots, MS1, in-source trapping, and MS/MS spectra of hemoglobin, MS1 and MS/MS spectra of BCAT2 sprayed under denaturing conditions, extracted ion chromatograms from size-exclusion chromatography of BCAT2, sequence coverage maps of the apo ions resulting from UVPD of BCAT2 monomers, and graphs summarizing the sequence coverages for the multi-stage approach. Table S1 gives the instrumental parameters used for data collection and Tables S2-S5 provide a comprehensive list of all identified HCD and UVPD fragment ions for BCAT2.

INTRODUCTION

Single amino acid variants that result in altered function are often associated with oncogenic potential.^{1–3} Precision design of small molecule inhibitors or therapeutic antibodies to target protein variants and suppress cancer growth relies on a comprehensive understanding of the functional impact of the mutation.^{3,4} Recently a proteogenomic screening of glioma stem cells from patient-derived glioblastoma tumors identified the T186R sequence variant of the human mitochondrial enzyme branched-chain amino acid transferase 2 (BCAT2) as a potential factor of resistance to standard treatments.⁵ BCAT2 exists as a 83 kDa homodimer and, along with a pyridoxal phosphate (PLP) cofactor, degrades branched chain amino acids into branched chain α -keto acids.^{6,7} Crystallographic experiments suggest R186 in the mutant repulses K59 in the other subunit of the dimer and impacts the electrostatic environment near the CXXC (C342 and C345) site. Both are perturbations which may destabilize the dimer and alter enzyme kinetics.⁸ Consequently characterization of the structural and functional differences between the wild-type (WT) and mutant proteoforms is necessary for further pursuit of the variant as a precision drug target in glioblastoma.

Mass spectrometry (MS) has emerged as a rapid and sensitive approach to characterize protein complexes^{9–11} in a manner complementary to traditional structural biology techniques that afford high-resolution three-dimensional structures, such as X-ray crystallography and NMR spectroscopy. Native MS experiments involving the transfer of intact protein complexes into the gas phase via electrospray ionization of volatile salt solutions allow the higher levels of protein organization to be probed, thus providing structural information including stoichiometry and spatial arrangement of subunits.^{9–11} Traditionally quadrupole time-of-flight (Q-TOF) instruments have been used for native MS experiments due to the efficient transmission of high m/z ions.^{12–16} However, significant inroads in characterization of protein complexes have occurred with the development of extended mass range Orbitrap mass spectrometers.^{17,18} Both proteoform differentiation and measurement of ligand binding to macromolecular complexes are possible owing to the increased effective resolution and greater m/z range of these instruments compared to previous generations of Orbitrap instruments.^{19–22} Moreover, disassembling higher-order structures using tandem mass spectrometry (MS/MS) techniques can further probe the higher-order structures of protein complexes. Electron-based activation methods, such as electron transfer dissociation (ETD)²³ and electron capture dissociation (ECD)²⁴, yield sequence fragment ions from intact protein complexes that correlate with the exposed surfaces and provide insight into the higher-order structure. Surface-induced dissociation

(SID) is another activation method that combined with ion mobility (IM) reveals the subunit architecture of protein-protein complexes.¹⁵ Although ETD, ECD, and SID have not yet been reported on an extended mass range Orbitrap platform, they have been demonstrated on other instruments capable of analyzing species over m/z 4000.^{15,23,24} Recently ultraviolet photodissociation (UVPD) provided feedback about both quaternary and secondary structures of multimeric protein complexes as well as limited primary sequence information, based on disassembly of the complexes when irradiated with 193 nm photons.^{25–27} Typically, separate bottom-up or top-down proteomics experiments involving digestion or denaturation of the protein complexes to further interrogate the primary sequence, localize post-translation modifications (PTMs), and identify proteoforms are necessary to provide an all-encompassing view into the dynamics and functional role of protein complexation in disease mechanisms.^{28,29}

Efforts to bridge the gap between obtaining primary sequence information and deciphering architectures of protein complexes have driven the development of new multi-modal and/or higher energy activation mass spectrometry methods.^{30–36} Recently in-source dissociation (ISD), collisional-induced dissociation (CID), and infrared multiphoton dissociation (IRMPD) were used in conjunction with ECD on a Fourier-transform ion cyclotron resonance (FTICR) MS to yield an integrated native top-down MS/MS approach.³⁶ This strategy allowed acquisition of sequence and higher-order structural information of native protein complexes. Alternatively, a multi-stage MS approach on an extended mass range Orbitrap platform has been reported that involved introducing intact protein complexes into the gas phase by native ESI methods, disassembling protein complexes into monomers using front-end collisional activation, referred to as in-source trapping (IST), isolating a specific monomer using a quadrupole mass filter, then further fragmenting the selected monomeric species with higher energy collisional dissociation (HCD). Using this platform, the resulting multiply charged fragment ions were detected at high resolution and mass accuracy in an Orbitrap mass analyzer.^{18,30,32–35} This approach has been demonstrated for model protein complexes,³⁰ applied to uncover a novel layer of regulation in a key metabolic enzyme,³⁴ and implemented in a high-throughput manner to identify endogenous protein complexes.³⁵ While these studies represent a major breakthrough, fragmentation efficiency using HCD was found to be low throughout the middle of the protein monomer sequences and bottom-up experiments were necessary to completely localize PTMs.^{30,34} In order to enhance sequence coverage in this type of advanced multi-stage strategy, integration with UVPD offers a compelling option. The high-energy excitation caused by absorption of 193 nm photons has consistently demonstrated unsurpassed levels of diagnostic backbone fragmentation for intact monomeric proteins sprayed under both denaturing and native conditions.^{27,37–41} UVPD has demonstrated improved retention of labile PTMs, including phosphorylation⁴² and sulfation⁴³, allowing better localization of modifications. Additionally, mapping holo (ligand-bound) fragment ions produced during photodissociation of native-like proteins has been used to elucidate regions of interaction with a bound ligand.^{39,44–46} These properties make UVPD an ideal activation method for characterization of protein complexes.

Here we report the use of a multi-stage native UVPD-MS approach implemented on a modified prototype of an extended mass range Orbitrap mass spectrometer instrument to

elucidate the oligomeric state, localize a single amino acid mutation, and identify the bound cofactor for two single amino acid variants of BCAT2 in a single experiment. Each variant was sprayed using native ESI to determine the oligomeric state. In-source trapping was used to disassemble the complexes into composing subunits that were isolated with a quadrupole mass analyzer and subjected to 193 nm UVPD. A previous top-down study combined ETD and CID in a LC-FT-ICR MS at 21 T to confidently localize the BCAT2 amino acid variant to its position in the sequence.⁸ In the present study, native ESI confirms that both WT and T186R BCAT2 exist as homodimers, even when the proteins are combined in solution. Gently activating the complexes in the front-end of the instrument using in-source trapping splits the dimers into monomeric subunits that are each non-covalently bound to a PLP cofactor. UVPD of the monomer affords the highest level of sequence coverage compared to HCD of the monomers. Tracking holo ions observed during photodissociation of the monomers indicates that the cofactor interacts with the loop containing K229 where the cofactor is known to bind.⁷

EXPERIMENTAL

Sample Preparation

Human hemoglobin (Sigma-Aldrich, St. Louis, MO), ammonium acetate (Sigma-Aldrich, St. Louis, MO), acetonitrile (EMD Millipore, Billerica, MA), and formic acid (Fisher Scientific, Hampton, NH) were purchased. Recombinant human WT and T186R BCAT2 (Figure S1) were expressed using purchased plasmids and purified as previously described.⁸ Protein samples were diluted to 40 μ M (hemoglobin) or 20 μ M (BCAT2) of the complex in 50 mM ammonium acetate at pH 6.8 and desalted using micro bio-spin P-6 gel columns (Bio-Rad Laboratories Inc., Hercules, CA) for MS analysis.

Mass Spectrometry

A schematic of the modified prototype of a Thermo Scientific Q Exactive UHMRTM instrument (Bremen, Germany) used for all multi-stage experiments is given in Figure S2. The instrument was built on the basis of a standard Q Exactive Plus (Thermo Scientific, Bremen, Germany) and modified to optimize the analysis of ions up to 80,000 m/z , as described in references 18 and 22. Briefly, several customized boards to enable lowering of the RF frequency in the instrument's ion guides, a capillary to allow higher bath gas pressure in the HCD cell, and an S-lens exit lens with a smaller aperture (1 mm instead of 2 mm) were installed in place of standard parts. Additionally, pulsing of the electrical potentials within the injection flatapole and inter-flatapole lens allows implementation of in-source trapping (Figure S2).^{18,22,30,32–35} UVPD was implemented in the HCD cell through incorporation of a Coherent Excistar 193 nm ArF excimer laser (Santa Cruz, CA). Detailed descriptions of modifications made to the HCD cell to incorporate and trigger the laser, and all other instrumental modifications and parameters used during the multi-stage analysis (Table S1) are given in the Supporting Information.

Data Analysis

Intact molecular masses of protein complexes from MS1 and in-source trapping mass spectra were determined by taking the apex of the most abundant peak in the charge state

distribution, manually calculating the charge state based on the spacing of the peaks, and calculating the molecular mass from the assigned charge state. All MS/MS spectra were de-charged and de-isotoped using Xtract with a signal-to-noise ratio of 3, fit factor of 44%, and remainder of 25%. ProSight Lite v1.4 software was used to search monoisotopic fragment ions against the sequence of hemoglobin or BCAT2 and identify fragment ions (± 10 ppm) produced by HCD (b , y) or UVPD (a , a^* , b , c , x , x^* , y , $y-1$, z). Sequence coverage maps shown were created using ProSight Lite according to the following legend: a/x -type ions (green), b/y -type ions (blue), and c/z -type ions (red). Holo fragment ions resulting from UVPD were identified by searching the spectra for a mass shift corresponding to the heme in hemoglobin (615.170 Da) or PLP in BCAT2 (247.025 Da) fixed at the N- and C-terminus. For all MS/MS spectra, three replicates were collected. Only fragment ions (apo and holo) identified in all three replicates were considered confidently identified and reported (Tables S2-S5 and companion mass error plots in Figure S3). All raw spectra are archived and available at: <https://repository.jpostdb.org/> and accession numbers are PXD009447 for ProteomeXchange and JPST000410 for jPOST.

RESULTS & DISCUSSION

Multi-Stage Native UVPD-MS for Complete Protein Complex Characterization

The recent development of Orbitrap mass spectrometers with extended mass range capabilities has enabled improved resolution and sensitivity in the analysis of protein complexes.^{17–22,30–35} Specifically modification of the quadrupole RF drive allows selection of higher m/z species and implementation of a “trap and release” approach to convert the inject flatpole into a linear quadrupole trap affords high-efficiency desolvation and improved dissociation of assemblies into subunits.^{18,30,32–35} By leveraging these key upgrades in instrumentation, we have further advanced the utility of a multi-stage approach for analysis of protein complexes by implementing UVPD for activation/fragmentation after disassembly of the complexes. To optimize and characterize the performance of this multi-stage approach, we first analyzed the heterotetramer hemoglobin (64 kDa).

Electrospray ionization of hemoglobin in a solution containing the volatile salt ammonium acetate allows efficient transfer of the intact heterotetramer into the gas phase (Figure S4). The 15+, 16+, and 17+ charge states of a complex comprised of two α -chains and two β -chains with each subunit non-covalently bound to a heme ligand were observed in the MS1 spectrum (Figure S4A). Increasing the applied desolvation voltage allows in-source trapping, resulting in dissociation of the tetramer into its constituent α - and β -chain monomers as well as some low abundance dimeric species (Figure S4B). Interestingly, the non-covalently bound heme is retained during this front-end low energy collisional activation, yielding the 7+ charge states of the α - and β -chains that can be selectively isolated using the quadrupole (Figure S4C, S4D) and subsequently activated with HCD or UVPD. The fragmentation patterns observed for the α - and β -chain monomers activated with HCD or 193 nm UVPD are shown in Figure S5. Important benchmark performance comparisons are facilitated by comparison of the sequence information obtained by this multi-stage approach (which allows dissection of multimers) to the sequence information obtained directly from monomeric forms of the protein. As extended mass range instruments

are designed for analysis of large, multimeric complexes, the higher mass range is optimized at the expense of the lower mass range. To ensure this did not impede the efficient transmission of monomeric proteins, the sequence analysis of hemoglobin was undertaken using similar native MS conditions on a standard Orbitrap instrument (without extended mass range) outfitted with UVPD to enable direct analysis of monomers.^{25,37–40,44–46} The 7+ charge state of α - and β -chain monomers were observed in the MS1 spectrum obtained on the Elite mass spectrometer, each of which could be isolated using the ion trap and activated with HCD or UVPD (Figure S5). Identifying the fragment ions and mapping these along the sequences confirms that the multi-stage approach on the extended mass range Orbitrap instrument yields comparable or higher sequence coverage of the α - and β -chains of hemoglobin for both HCD and UVPD (Figure S5). Additionally, holo (ligand-bound) fragment ions originated from the same regions of the protein using the multi-stage approach on the mass spectrometer with the extended mass range compared to the direct analysis of monomers on the standard Orbitrap mass spectrometer, suggesting that sufficient electrostatic contacts between the ligand and protein side-chains are maintained during in-source trapping to allow retention of binding site information. This optimized multi-stage approach involving native MS, in-source trapping, and UVPD provides an ideal platform to interrogate the differences in fragmentation of dimeric BCAT2 complexes containing the WT and T186R variants.

Native MS1 and Size-Exclusion Chromatography of BCAT2

Spraying WT and T186R BCAT2 under native conditions provides insight into the oligomeric state of each variant, thus revealing the highest level of protein structure. The MS1 spectra of WT and T186R BCAT2 indicate that each protein exists as a homodimer (Figure 1A, 1B). Although previous crystallographic experiments⁸ indicate that R186 in the mutant repulses K59 in the other subunit, this effect is insufficient to completely destabilize the dimer. Heterodimers were not observed after simply mixing the variants; a co-folding experiment would be necessary to investigate more explicitly the potential formation of WT and T186R heterodimers (Figure 1C). The observed masses of the dimers were 497 Da greater than the theoretical masses (Figure 1), suggesting that one or more ligands are non-covalently bound to the proteins. Conversely, when the proteins are sprayed from denaturing solutions, no mass shift is observed for the unfolded monomeric proteins (Figure S6). Sequence coverages were 11% (27+, denatured) and 7% (37+, denatured) from HCD, and 36% (27+, denatured) and 34% (37+, denatured) from UVPD for WT and T186R BCAT2, respectively. The mass shift for the dimers is attributed to the non-covalent binding of one PLP cofactor (247.025 Da) to each subunit of the dimer. A PLP cofactor is necessary for the enzymatic action of the protein.⁷ On-line size-exclusion chromatography was used to confirm the results observed for the MS1 spectra (Figure S7). The proteins were injected individually and as a mixture after incubation for 120 minutes. Each variant eluted as a homodimer containing two molecules of PLP. Extracted ion chromatograms confirmed that no heterodimers were observed, nor were any dimers containing a single or no cofactors (Figure S7).

In-Source Trapping and UVPD for Improved Localization of T186R Mutation in BCAT2

Identification of the protein sequences requires activation and disassembly of the dimeric complexes. Irradiation of protein complexes in the gas phase with 193 nm photons has been demonstrated to release the constituent subunits as well as diagnostic backbone fragment ions.^{25,26} Isolation and activation of the homodimers of WT and T186R BCAT2 (17+ charge state with two PLP cofactors per dimer) by HCD or UVPD produces the MS/MS spectra shown in Figure 2. Both activation methods result in backbone cleavages that result in production of diagnostic sequence ions (Figure 2). All identified fragment ions are summarized in Tables S2 and S3 (for WT and T186R BCAT2, respectively). UVPD outperforms HCD for both protein variants, yielding higher sequence coverage deeper into the protein sequence. Sequence coverages were 12% and 16% from HCD, and 21% and 28% from UVPD for WT and T186R BCAT2, respectively. This improved coverage is attributed to the access to excited electronic states and thus higher energy fragmentation pathways upon absorption of 193 nm photons.^{25,26,37,38,41} Additionally, the pressure of the nitrogen bath gas in the trapping cell used for ion activation was significantly reduced during UVPD, resulting in a slower transient decay and higher resolution frequency measurements and improved sensitivity as a result of fewer collisions during ion ejection from the C-trap into the mass analyzer and during analysis in the Orbitrap.^{47,48} The use of reduced pressure has no impact on the fragmentation efficiency of UVPD but is detrimental for the performance of HCD.

When protein complexes are subjected to UVPD, sequence ions that retain the PLP cofactor or portions of other subunits are produced, in addition to the diagnostic apo (ligand-free) fragment ions. The formation of such holo (ligand-bound) fragment ions has been observed previously for UVPD.^{39,44–46} Conversely, HCD causes ejection of ligands and results exclusively in formation of apo fragment ions. In the case of the BCAT2, the sequence coverage achieved with UVPD would be even further improved if the holo fragment ions bound to the PLP cofactor could be identified and contribute in a meaningful way to the net sequence coverage. Confident identification of holo ions at this time is challenging for a protein complex of this size given the nearly unlimited array of possible fragment ion assignments that can arise from combinations of partial protein sequence segments and the PLP cofactor. In essence, “mass shifted” fragment ions could be falsely attributed to the presence of the PLP cofactor when in reality the mass shift arises from a portion of the protein from the other subunit with the same exact mass as the cofactor. By utilizing a multi-stage approach, however, monomers can be separated from the dimeric complexes at the front-end of the mass spectrometer prior to MS/MS analysis, thus alleviating the ambiguity in the origin and identities of holo fragment ions.^{30,32–35} For implementation of the multi-stage strategy, the in-source trapping parameters were optimized to cause subunit ejection and yield the mass spectra shown in Figure 3. The bound PLP ligands are retained as the subunits are disassembled. A mass shift of +249 Da corresponding to a single PLP cofactor bound to a monomeric subunit of the protein is observed for both variants. In-source trapping of the solution containing both the WT and T186R proteins yields PLP-bound monomers of each (Figure 3C).

The 12+ charge state monomer of each variant bound to a PLP cofactor resulting from in-source trapping of the observed homodimer was selectively isolated and activated with HCD or UVPD, resulting in the MS/MS spectra in Figure 4. Identified sequence ions are mapped on to the known protein sequences (Figure 4), and complete lists are summarized in Tables S4 and S5. As evident in the UVPD spectra, lower energy laser pulses (1 mJ) yielded larger fragments corresponding to cleavage in the central region of the protein. Conversely, using a higher energy per laser pulse (3 mJ) resulted in smaller fragments originating from the termini of the protein. The UVPD sequence maps shown in Figure 4 represent the combined coverage for identified apo and holo (ligand-bound) fragment ions for the spectra collected using 1 mJ and 3 mJ per pulse. For comparison, Figure S8 gives sequence coverage maps based solely on the apo fragment ions obtained from UVPD.

UVPD affords higher sequence coverage (45% and 37% for WT and T186R BCAT2, respectively) compared to HCD (13% and 18% for WT and T186R BCAT2, respectively) and more effectively spans the central region of the protein where the mutation is located. These sequence coverages obtained by UVPD are higher than or comparable to those found for denatured BCAT proteins, as reported above for the 27+ and 37+ charge states. HCD of the monomers ejected from the dimeric complexes afforded only a 1–2% increase in sequence coverage compared to HCD of the dimers. UVPD of the monomers ejected from the dimers give significantly higher sequence coverage compared to UVPD of the intact dimers (e.g. increasing from 21% to 45% for WT BCAT2). The holo (PLP-containing) sequence ions contributed significantly to the gain in sequence coverage achieved by UVPD. Figure S9 summarizes the sequence coverage afforded by HCD and UVPD. In general, substantial improvements in sequence coverage and localization of the mutation in BCAT2 were obtained by disassembling the dimer and selecting a single charge state of the monomer for subsequent UV photoactivation.

Mapping UVPD Holo Fragment Ions to Examine the Cofactor Binding Site along BCAT2

Identifying PLP-bound holo ions produced upon photodissociation not only adds to the sequence coverage but also sheds light on the binding site based on the pattern of apo versus holo fragment ions. The high energy deposited during absorption of 193 nm photons by the protein backbone favors preferential cleavage of backbone bonds rather than disruption of the electrostatic interactions with a bound ligand.^{39,44–46} Figure 5 gives the sequence of BCAT2 with the regions of the protein demarcated where backbone cleavages occurred to produce holo fragment ions upon UVPD. For both variants, the majority of holo ions are bi-directional (i.e. holo ions for which both N-terminal and C-terminal fragment ions retain the ligands and share overlapping residues) and cover regions in the middle (V205–Y233) of the protein. Those residues which are consistently included in bi-directional holo fragment ions expected to be those that interact with the cofactor.^{39,44–46,49} To aid in visualization, space-filling models of the crystal structures of WT (PDB ID: 5CR5)⁵⁰ and T186R (PDB ID: 5MPR)⁸ BCAT2 with the residues corresponding to holo fragment ions produced by UVPD represented as colored spheres are shown in Figure 5.

BCAT2 is dependent on a PLP cofactor to catalyze the transfer of an amino group from the donor branched-chain amino acid to α -ketoglutarate. This ligand is known to bind at active

site residue K229.⁷ The observation that the bi-directional holo ions generally contain K229 is consistent with the prevailing understanding of the structure of BCAT2. Furthermore, the N-terminal holo ions are only observed C-terminal to the first loop that is in proximity to K229 (F29-H37) and the active site (Figure 5B, 5C). Both variants yielded holo ions from the same regions with the exception of P144-L146 and P158-A161 which were only observed for the wild-type protein. The T186R substitution occurs in this region suggesting that the mutation may destabilize electrostatic interactions between amino acids along that area of the protein and the PLP cofactor, a hypothesis that merits further confirmation using ancillary structural and biophysical methods, as well additional supporting examples from future UVPD-MS studies. This finding agrees with previous electrostatic free energy calculations suggesting that the substitution of Thr for Arg at residue 186 induces a sufficiently large pKa shift of nearby Cys residues in the other subunit of the dimer that influences the Cys protonation state, and prevents the substrate and PLP cofactor from orienting correctly.⁵ Obtaining this type of insight on the ligand binding sites by mapping holo fragment ions is an advantage of UVPD in a multi-stage MS approach.

CONCLUSION

In addition to establishing T186R BCAT2 exists as a dimer in solution and identifying a bound PLP cofactor, our study demonstrates the general utility of incorporating 193 nm UVPD into a multi-stage MS approach for complete characterization of protein complexes from the oligomeric state down to the primary sequence. The first stage (MS1) provided a comprehensive view of the oligomeric state adopted by each BCAT2 protein and aided in the identification of a bound PLP cofactor based on the measured intact mass. Direct interrogation of the BCAT2 dimers by UVPD afforded moderate sequence coverage and yielded holo product ions that could not be confidently assigned owing to the large number of possible compositional combinations. To mitigate the ambiguous identification of key PLP-containing holo fragment ions, in-source trapping was used to eject a PLP-bound BCAT2 monomer which was subsequently subjected to UVPD. The ability to confidently identify holo ions contributed significantly to the sequence coverage achieved, and mapping the origin of observed holo ions along the sequence provided insight into the binding of PLP in the T186R mutant of BCAT2 compared to the WT protein. In summary, by increasing the diversity of sequence ions observed and producing assignable holo ions that reveal information about bound ligands, the incorporation of UVPD advances the utility of a multi-stage MS approach for complete characterization of intact protein complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ESI mass spectra (m/z 1,000–10,000) of (A) WT, (B) T186R, and (C) WT+T186R BCAT2 sprayed in 50 mM ammonium acetate (pH 6.8). Each species observed is identified by colored circles in an expanded view (insets) of the 17+ charge state of the homodimer (m/z 4,850–4,950). The peaks denoted by diamonds (♦) represent the corresponding Na-adducted species. The experimentally measured masses are 497 Da larger than the expected masses for both WT and T186R BCAT2. This mass shift is attributed to a PLP cofactor bound to each subunit of the dimer. As demonstrated in (C), combining the protein variants in solution

only yields homodimers. SEC confirms that these are the oligomeric states that exist in solution (Figure S7). The table in (D) summarizes theoretical and measured masses for the dimers.

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Figure 2.

HCD and UVPD mass spectra of the 17+ charge state of the homodimer of (A) WT and (B) T186R BCAT2. Expanded views (m/z 1,255–1,280) in panel (A) show selected fragment ions labelled. The precursors are indicated with a star in the MS/MS spectra. Sequence coverage maps for (1) HCD and (2) UVPD of (A) WT and (B) T186R BCAT2 are shown beneath the spectra. Sequence coverages were 12% and 16% from HCD, and 21% and 28% from UVPD for WT and T186R BCAT2, respectively. The Thr residue mutated to an Arg is shaded in gold in panel (B). Tables S2 and S3 contain lists of all identified fragment ions.

Figure 3.

In-source trapping mass spectra (m/z 1,000–10,000) of (A) WT, (B) T186R, and (C) WT + T186R BCAT2. Each species observed is identified by colored circles in an expanded view (insets) of the 12+ charge state of the monomer (m/z 3,420–3,500). The experimentally measured monomeric masses are 249 Da larger than the expected masses for both WT and T186R BCAT2. This mass shift is attributed to a PLP cofactor bound to each monomer. Given that in-source trapping occurs at the front of the instrument, each monomeric species of interest can be isolated and subjected to MS/MS analysis (Figure 4). The table in (D) summarizes theoretical and measured masses for the monomers.

Figure 4.

HCD and UVPD mass spectra of the 12+ charge state monomer of (A) WT and (B) T186R BCAT2 observed after in-source trapping. Expanded views (insets) in panel (A) show selected fragment ions labelled. Holo fragment ions that contain the PLP cofactor are denoted by a diamond. The precursors are indicated with a filled star in the MS/MS spectra. The unfilled star denotes the charge-reduced precursor (11+) as the PLP cofactor is ejected during HCD. Sequence coverage maps for (1) HCD and (2) UVPD of (A) WT and (B) T186R BCAT2 are shown beneath the spectra. The UVPD sequence maps represent the combined coverage for identified apo and holo fragment ions for the spectra collected using 1 mJ and 3 mJ per pulse. Apo ion only sequence coverage maps are given in Figure S7. Sequence coverages were 13% and 18% from HCD, and 45% and 37% from UVPD for WT and T186R BCAT2, respectively. The Thr residue mutated to an Arg is shaded in gold in panel (B). Tables S3 and S4 contain lists of all identified fragment ions.

Figure 5.

(A) Sequence of BCAT2 (X160 = T for WT or R for T186R in bold font) with the observed N-terminal (blue), C-terminal (red), and bi-directional (green) PLP cofactor-bound holo fragment ions observed during UVPD of the 12+ monomer (combined from 1 mJ and 3 mJ) mapped above for WT (solid line) and T186R (dotted line). Bi-directional fragmentation indicates complementary N- and C-terminal ions occurring at the same backbone position. The known binding site of the PLP cofactor is K229 shown highlighted in yellow. Note that the numbering system used to refer to the variant (T186R) and PLP binding site (K229) accounts for the 27 amino acid transit peptide that is not included in the expressed chain. (B, C) Space-filling models of the crystal structures of WT (PDB ID: 5CR5) and T186R (PDB ID: 5MPR) BCAT2 with the residues corresponding to holo fragment ions produced by

UVPD represented as colored spheres. The PLP cofactor (purple) and residue K229 (orange) are shown as sticks and labelled in (C).

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