Infrared Activation Enables Native Top-Down MS Analysis of Membrane Proteins and Protein Complexes

Joshua D Hinkle[1], Tarick J El-Baba[2], Corinne A Lutomski[2], Idlir Liko[3], Jani Reddy Bolla[2], Carol V Robinson[2], and John EP Syka[1],

[1]Thermo Fisher Scientific, 355 River Oaks Pkwy, San Jose, CA, USA 95134; [2]Physical and Theoretical Chemistry Laboratory, Department of Chemistry, University of Oxford, Oxford OX1 3QZ, UK; [3]OMass Therapeutics, The Oxford Science Park, The Schrödinger Building, Heatley Rd, Oxford OX4 4GE, United Kingdom

ABSTRACT

Purpose: Demonstrate the utility of infrared (IR) based activation for both liberating native electrosprayed membrane proteins from detergent micelles as well as improving top-down sequence coverage.

Methods: Samples containing detergent stabilized intact membrane protein complexes were analyzed on a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer modified to interface a 10.6 um infrared CO2 laser such that it irradiates ions contained within the quadrupole linear ion trap (Q-LIT) with infrared (IR) photons. IR was first used to liberate proteins from their detergent micelles, dubbed infrared demicellization (IRD). Liberated proteins were then isolated and fragmented with infrared multiproton dissociation (IRMPD) and activated ion electron transfer dissociation (AI-ETD) to assess sequence coverage and fragmentation efficiency.

Results: Results suggest that both IR-based strategies were highly advantageous for analyzing membrane protein complexes. IRD liberates proteins from bound detergent molecules in a much more controllable manner than traditional collisional strategies. Similarly, IRMPD proved to be more effective at generating robust sequence coverage than collision-based fragmentation strategies while AI-ETD enabled orthogonal electron-based dissociation for complementary sequence coverage.

INTRODUCTION

The analysis of intact membrane proteins and membrane protein complexes are uniquely challenging to analyze via mass spectrometry. Multiple aspects of these analytes present challenges to traditional methodologies aimed at either peptide or soluble proteins. Not only are these protein complexes typically very large, approaching mass range limitations to current instrumentation, but they are also particularly challenging to probe for information. Upon introduction to the instrument, unlike with the typical top-down analysis of proteins, the detergents used to stabilize the membrane proteins in solution must first be liberated from their protein-detergent micelles before precursor m/z selection is performed. This is most typically performed by using higher energy gas collisions to excite the protein-detergent complex, thereby removing the noncovalently bound detergents.[1] While this technique often works, it is limited in that the only tunable parameter is the intensity of the gas collisions. In situations where certain noncovalently bound ligands or labile post translational modifications are of interest, this lack of control can complicate removing the detergent while preserving the binding of these more fragile components of the protein complex. In addition, higher transfer energies can introduce strong m/z discrimination in the transmission of ions in the instrument complicating instrument operation

The typical charge density and resulting high mass to charge ratio also poses a problem when dissociating the proteins. The low charge density results in minimal dissociation following commercially available electron-based dissociation strategies like electron transfer dissociation (ETD).[2] Further, typical collision-based strategies are complicated by large differences in precursor and product ion m/z; these fragmentation techniques must be performed at a low precursor Mathieu *q* value, limiting product ion formation, particularly for larger protein complexes.

Here, we demonstrate that various strategies irradiating ions with an infrared 10.6 μm CO_2 laser in the q-LIT addresses all of these challenges. IRD significantly improves the ability to remove the detergent micelles by more effectively and selectively disrupting membrane-lipid interactions than typical strategies. Further, IR-based dissociation strategies address both major limitations of current top-down fragmentation strategies; IRMPD is capable of dissociating ions via slow heating but at a much lower Mathieu *q* than collision-based strategies, thereby retaining significantly more fragment ions. Similarly, AI-ETD ameliorates the charge-dependence that renders ETD inefficacious on membrane protein samples, enabling complementary electron-based fragmentation which is normally unsuccessful on membrane proteins and complexes.

MATERIALS AND METHODS

Sample Preparation

Membrane protein samples were prepared with typical methods, as described in Laganowsky et al.[1] Briefly, Ammonia channel (AmtB), Aquaporin Z (AqpZ), and components of the bacterial outer membrane β-barrel assembly machinery (BAM) complex were over-expressed in E. coli and purified by nickel affinity chromatography and size exclusion chromatography. All proteins were buffer exchanged into ammonium acetate with detergents at 2x the critical micelle concentration. For these experiments, both AqpZ and BAM complex samples were detergent exchanged into tetraethylene glycol monooctyl ether (C8E4) while AmtB samples were exchanged into n-Dodecyl-B-D-Maltoside (DDM).

MATERIALS AND METHODS, Cont.

Test Method(s)

Prepared membrane proteins were directly infused via static spray to evaluate IRD. Both power and irradiation time were ramped to evaluate the impact of these parameters on the intensity of liberated proteins. One hundred scans were averaged for each set of conditions prior to data acquisition.

Fragmentation strategies were evaluated by isolating and subsequently fragmenting monomer peaks to extract sequence coverage. MS2 spectra were acquired at 120,000 resolution and 100 scans were averaged prior to acquiring each spectrum. IRMPD and ETD reaction times were optimized by selecting conditions corresponding to ~80% precursor depletion. Optimal AI-ETD laser intensities for a given reaction time were determined by maximizing irradiation intensity whilst maintaining acceptably low production of IRMPD products when non-dissociative PTCR reagent ions were substituted for ETD reagent ions during the MS^2 experiment.

Data Analysis

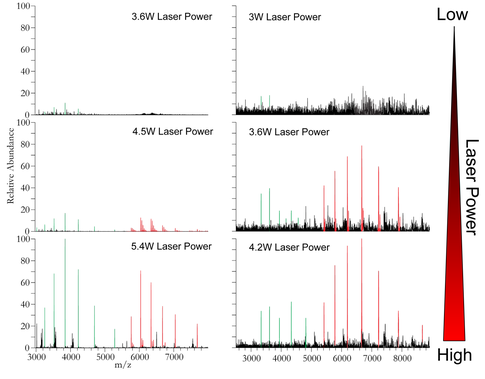
Fragmentation data was analyzed using TDValidator version 1.0 software. Spectra were first annotated using a S/N cutoff of 5, max ppm tolerance of 20 ppm, sub ppm tolerance of 15 ppm, cluster tolerance of 0.35, minimum score of 0.6, charge states ranging from 1 to the precursor charge state, using the distribution generator Mercury7. Assigned peaks were then manually inspected and incorrect assignments were removed.

RESULTS

Removal of Detergent Micelles via IRD

Performance of protein demicellization via IRD was first evaluated using the AqpZ, a ~99 kDa tetramer, and AmtB, a ~126 kDa trimer. Results suggest that a wide range of laser energies and laser irradiation times robustly generate intact protein complexes free of bound detergent. Figure 1 depicts several acquired IRD laser intensities with a fixed activation time. Below a certain laser power, no proteins are visible within the spectrum due to insufficient removal from the detergent micelle. At a certain power onset, the proteins are the sufficiently heated to reveal the signal corresponding to the protein complexes. This behavior seems extensible to other systems as the two membrane proteins were prepared in different detergent mixtures.

Figure 1: IRD Laser Power Ramp. MS1 Spectra following IRD for AmtB in DDM (Left) and AqpZ in C8E4 (right) are shown using a fixed 10 ms activation time. Intact complex peaks are denoted in red, monomer peaks are denoted in green.



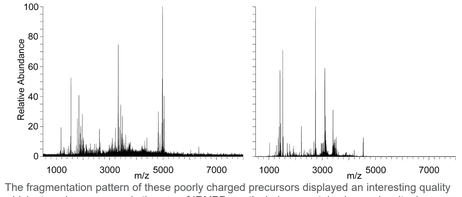
Results, Cont.

Notably, some conditions may be selected which robustly preserve some noncovalently bound species while largely liberating the proteins from the detergent micelles. This can be clearly seen by the insets in Figure 1. Using the minimum laser energy sufficient to demicellize the protein complex preserves some species corresponding to the protein complex plus one or more noncovalently bound detergent molecules. Incrementally higher energies can also subsequently remove these interactions while still retaining the intact complex. The highly tunable nature of both IRD laser intensity and exposure time makes the preservation of these ligands quite straightforward, allowing the selective preservation of particular species of interest.

Top-Down Fragmentation via IRMPD and AI-ETD

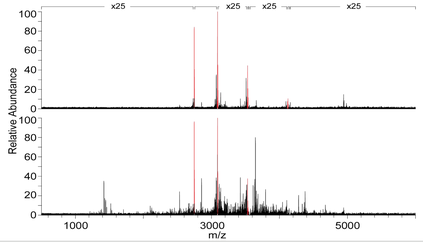
The performance of IR-based fragmentation strategies were evaluated on the proteins following demicellization. Both proteins exhibited robust fragmentation following IRMPD dissociation, as illustrated in Figure 3. Consistent with fragmentation by collisional modalities, both fragmentation spectra exhibit several sequence informative fragment ions but are significantly dominated by preferred fragmentation pathways. Nevertheless, the overall number of sequence informative ions was still substantial despite these preferred pathways.

Figure 3: IRMPD Fragmentation Spectra of Intact Membrane Proteins. AmtB^{+12} (left, m/z=3524) and AqpZ^{+9} (right, m/z = 2747) were fragmented with 5 ms of 18W irradiation and 10ms of 10.2W irradiation, respectively.



The fragmentation pattern of these poorly charged precursors displayed an interesting quality which strongly recommends the use of IRMPD, particularly as protein charge density decreases. When fragmenting denatured proteins, fragment ions tend to cluster around precursor ion being fragmented by virtue proportionally distributed charges along the protein. Conversely, fragmenting these native electrosprayed proteins appears to show significant variability in this charge-partitioning. This leads to a large number of fragment ions possessing a m/z significantly lower than the precursor being fragmented. As a result, being able to fragment the precursor ions as low Mathieu *q* values, as is the case with IRMPD, is critical to achieving optimal results when fragmenting these proteins. For example, in the case of the above AqpZ fragmentation, the precursor ion was confined at a Mathieu *q* value of 0.1 to maintain the stability of ions larger than ~500 m/z. If precursor m/z were increased further, many of the low mass fragment ions would not remain stable within the trap. And decreasing the precursor Mathieu *q* would eject the precursor ions due to insufficient confinement to counteract the imparted kinetic energy. IRMPD avoids this limitation by allowing vibrational activation without kinetic activation.

Figure 3: ETD vs AI-ETD Spectra of the AqpZ^{+9} (m/z = 2747). Experiments are 7ms ETD reaction times with no laser irradiation (ETD, top) or concurrent 7.5W IR irradiation (AI-ETD). Charge-reduced products (ETnoD) in both spectra are denoted in red, and all peaks except for these peaks are magnified 25x.

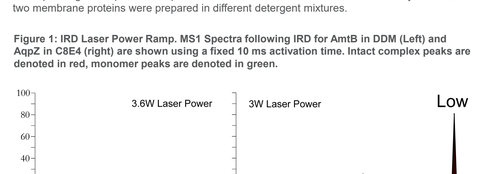


Results, Cont.

AI-ETD also proved advantageous for evaluating the two proteins. Figure 3 depicts the difference between ETD and AI-ETD fragmentation for AqpZ. The spectrum shows clear evidence of reduced electron transfer no dissociation (ETnoD) due to the reduced intensity of charge-reduced peaks (red) in the AI-ETD spectrum relative to the standalone ETD spectrum. It is worth noting, however, that the efficiency of generating these fragments was uncharacteristically low relative to fragments generated when performing AI-ETD on denatured non-membrane proteins. Most fragment ions are present at fractions of a percent relative abundance, consistent with previously reported experiments on low charge-state proteins following proton transfer charge reduction.[3]

Combining the identified fragment ions from each set of spectra leads to good sequence characterization of the proteins, particularly AqpZ. Figure 4 depicts the sequence coverage maps of both proteins when combining the results from IRMPD and AI-ETD fragmentation. AqpZ in particular achieves substantial sequence characterization. The overall sequence characterization of AmtB was sufficient to clearly identify the sequence and largely generate cleavages across the length of the molecule, but the overall performance was somewhat lower than AqpZ. This may be due in part to two current limitations. Primarily, AmtB monomers are quite a bit larger than AqpZ. This leads to individual fragment ion intensities being diluted across more fragmentation pathways and more numerous isotopes. As a result, many generated fragment ions may have simply fallen below the electronic noise, particularly for AI-ETD given the aforementioned poor fragment intensity. Strategies that can boost precursor ion populations like multiple fills will likely improve these results in the future.[4] IRMPD coverage may have also been somewhat limited due to charge-remote fragmentation given the lower charge state. This can lead to fewer random cleavages across the sequence, which limits total sequence coverage output. A solution may exist, however, in that large fragment ions can undergo secondary reactions with longer IRMPD reaction times. This would suggest that multiple reaction times may cover complementary regions of the protein sequence, improving the overall sequence coverage.

Figure 4: Combined Sequence Coverage of AmtB (Left) and AqpZ (Right). Ions generated by AI-ETD are denoted in red and ions generated by IRMPD in blue.



Top-Down Identification of BamABCDE Complex

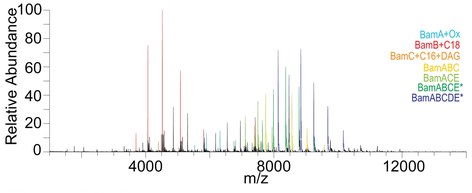
Having validated the utility of IR-based techniques for native membrane protein analysis, these techniques were then applied to the β-barrel–assembly machinery (BAM) complex to identify subunit components and modifications within the molecule. The 200 kDa protein complex was first demicellated via IRD. The resulting MS1 spectrum was highly complex, containing several combinations of intact and partially intact complex components, as seen in Figure 5. These species were then subsequently probed via the combination of IRMPD and AI-ETD in order to assign the subunits within each of the charge-state distributions generated.

N-terminal modifications were identified via MS/MS. All components except for BamA are known lipoproteins and many were identified with bound N-terminal modifications. N-terminal cysteines on Bam components B, C, and D were found to contain concurrent diacyl glycerol and saturated fatty acid modifications. BamB and BamD contained C18 fatty acids while BamC contained a C16 modification. Combinations of protein subunits were identified to contain various combinations of these protein subunits, all with concurrent modifications. BamABC and BamACE complexes were identified via MS/MS fragment ions from all substituent components. Conversely, BamABCE and intact BamABCDE complexes were only assigned via intact mass corresponding to combinations of the individually sequenced subunits; MS/MS experiments could not be performed because all charge-states for these subcomplexes were >8000 m/z, which represented the upper limit for isolating on the instrument.

Results, Cont.

The success in identifying the BAM protein complex peaks strongly recommends the use of these IR-based modalities for sequence characterization. Not only was the sequence coverage via IRMPD sufficient to identify peaks from each BAM complex subcomponent, but several peaks were uncharacterizable by alternative strategies. It was found that for most precursors above ~6000 m/z, trap-type collision induced dissociation (CID) and high-energy collision-induced dissociation (HCD) were entirely unsuccessful at fragmenting the protein. Using activation Q values for CID to retain the sequence informative fragment ions simply ejected the precursor from the trap, and even maximum HCD energies were unsuccessful at dissociating these large subcomplexes. Comparatively, IRMPD and AI-ETD enabled the near-complete characterization of the entire complex. These results suggest that, given improvements in the ability to isolate high mass ions, IR-based dissociation strategies should enable this kind of analysis to become a routine procedure.

Figure 5: Identified Protein Subcomplexes in BamABCDE. Peaks are color coded denoting the subcomplexes they represent. Peaks denoted with asterisks (*) were only assigned via intact mass.



CONCLUSIONS

- IRD is highly effective at liberating membrane proteins from detergent micelles.
- The inherent tunability of IRD in terms of both energy and irradiation time is highly effective at preserving labile noncovalent interactions while removing the detergent micelle.
- IRMPD appears particularly effective at generating comprehensive sequence coverage, often circumventing limitations inherent to collision-based modalities.
- AI-ETD allows the generation of c/z fragment ions from large, poorly charged membrane proteins that do not normally fragment when performing ETD without concurrent IR.
- Combining IRD, IRMPD, and AI-ETD is uniquely capable of characterizing large multi-protein membrane protein complexes.

REFERENCES

1. Laganowsky, A., Reading, E., Allison, T. M., Ulmschneider, M. B., Degiacomi, M. T., Baldwin, A. J., & Robinson, C. V. (2014). Membrane proteins bind lipids selectively to modulate their structure and function. Nature, 510(7503), 172-175.
2. Riley, N. M., Westphall, M. S., & Coon, J. J. (2015). Activated ion electron transfer dissociation for improved fragmentation of intact proteins. Analytical chemistry, 87(14), 7109-7116.
3. Hinkle J, Mullen C, Lodge JM, Huguet R, Westphall MS, Coon JJ, Syka JEP,. Activated-Ion Electron Transfer Dissociation Enables Electron-Based Dissociation Following Proton Transfer Charge Reduction. ASMS Annual Conference 2020. Online
4. Earley, L., Anderson, L. C., Bai, D. L., Mullen, C., Syka, J. E., English, A. M., ... & Compton, P. D. (2013). Front-end electron transfer dissociation: a new ionization source. Analytical chemistry, 85(17), 8385-8390.

TRADEMARKS/LICENSING

PO66095 EN0921S