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Membrane protein analysis

Novel XL-MS analysis workflows for membrane protein characterization

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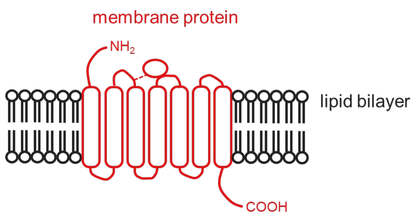
Abstract

Purpose: To develop end to end crosslinking including S-S bond mapping workflows for membrane proteins in different membrane mimetics.

Methods: Different amine-reactive, homobifunctional crosslinkers, including DSS (disuccinimidyl suberate), DSSO (disuccinimidyl sulfoxide) and tBuPhoX (tert-butyl disuccinimidyl phenyl phosphonate) were used to crosslink membrane proteins in SMALPs or detergents. Crosslinking mass spec analysis was performed on a Thermo Scientific™ Orbitrap™ Ascend Structural Biology Tribrid™ mass spectrometer with/without a FAIMS Pro™ Duo interface. Data were analyzed using Thermo Scientific™ Proteome Discoverer™ 3.2 software and XlinkX 3.2 node.

Results: With the optimized workflows, we were able to identify crosslinked peptides and provide critical information for downstream structural analysis of GPCRs, HIV proteins, and bacterial enzymes

Introduction



Membrane proteins (MPs) are involved in almost all cellular processes and comprise approximately 30% of the proteome, which makes them an ideal choice for 60% of drug targets. Although the study and characterization of MPs' structure, function, and interactions are clearly essential, they represent a challenge for current analytical methods, owing mostly to low production yields and the need for membrane mimetics to solubilize the proteins before analysis. Cross-linking mass spectrometry (XL-MS) has grown dramatically into a routinely utilized strategy for characterizing protein higher-order structure and mapping protein-protein interaction networks on a proteome-wide scale. However, the XL-MS analysis of membrane proteins is still a significant challenge due to their hydrophobic properties as well as the lipid-rich environment. In this work, we optimized sample preparation and detection of crosslinked peptides, including disulfide mapping for membrane proteins in SMALPs or detergents.

Materials and methods

Sample preparation

Different amine-reactive, homobifunctional crosslinkers, including DSS (disuccinimidyl suberate), DSSO (disuccinimidyl sulfoxide) and tBuPhoX (tert-butyl disuccinimidyl phenyl phosphonate) were used to crosslink membrane proteins in SMALPs or detergents. After crosslinking reaction, the samples were buffer exchanged into 1% DDM(SMALP) or 0.1% RapiGest containing buffers.

SMALP was removed by adding MgCl2 (50 mM stock) to a final concentration of 4 mM, incubating at 4 ºC for 1 hr, and then centrifuged at 21,000 ×g at 4 ºC for 1 hr. The supernatants were diluted in 0.1% RapiGest, reduced, alkylated and digested with either trypsin (1:20 ratio (enzyme to protein) or pepsin (1:50 ratio (enzyme to protein). tBuPhoX(TBDSPP) sample digests (60 µg) were enriched using the TiO2 Phosphopeptide Enrichment Tips according to the manufacturer's instructions[1]. For disulfide mapping, proteins were hydrolyzed in original buffer plus 25 % TFA for 10 min in a microwave - microwave-assisted acid hydrolysis (MAAH)[2] and desalted using C18 stage tips.

LC-MS/MS methods

Samples were separated by reverse phase-HPLC using a Thermo Scientific™ Vanquish™ Neo system connected to an EASY-Spray™ PepMap™ 75 um x 25 cm column over a 60 min 6-35% gradient (A: water, 0.1% formic acid; B: 80% acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The crosslinked samples were analyzed on the Orbitrap Ascend Structural Biology Tribrid mass spectrometer with/without a FAIMS Pro Duo with Instrument Control Software version 4.2. Specific MS settings are shown in Table 1. OBE-nativeMS +DMT was performed on a Thermo Scientific™ Q Exactive™ UHMR Mass Spectrometer[3]

Data analysis

The acquired spectra were analyzed using Proteome Discoverer 3.2 software using the XlinkX node 3.2 for crosslinked peptides and SEQUEST™ HT search engine for unmodified, looplinks and monolinks peptides. Data were searched against the focused database containing sequences of studied proteins and common contaminants with 1% FDR criteria for crosslink spectra matches. For data acquired using the non-cleavable crosslinkers, DSS or PhoX, and S-S bonds a search option in XlinkX-NonCleavable-open was used. Crosslinks sites were visualized in XMAS plug in for ChimeraX[4] or Pymol.

Table 1. Orbitrap Ascend MS and MS2 Settings

Settings	DSS	DSSO	tbPHOX	S-S
FAIMS CV		-50/-60		-50/-60
MS1 resolution	60000	60000	60000	120000
MS2				
fragmentation	HCD	HCD	HCD	EThcD
CE	21,26,31	21,26,31	30	Charge RT/20
AGC target,%	200	200	200	200
Resolution	30000	45000	30000	60000
Max IT	70	91	70	118

Results

Crosslinking analysis of membrane protein in SMALP

Membrane proteins are usually extracted or solubilized in detergents, nanodiscs, or styrene-maleic acid lipid particles (SMALPs), which are not compatible with downstream MS analysis. We tested two different workflows: in-gel digestion vs in-solution digestion (Figure 1) for membrane protein *S. enterica* WbaP[1]. The results are summarized in Table 2. In both workflows, we are able to achieve sequence coverages more than 80%. Importantly, SMALP was successfully removed during the in-solution workflow for the downstream LS-MS analysis. Detailed information is shown in Figure 2& Table 3 including homo-inter identified crosslinked sites/peptides. The only region that was not identified is part of the transmembrane domain of the protein. In-solution digestion provides better sequence coverage and XL identifications. Overall more crosslinked peptides were identified using DSS crosslinker, however more tBu-Phox crosslinked peptides were localized to the membrane-adjacent regions at the putative dimer interface (Figure 2 A,B&D).

Figure 1. XL-MS workflow for membrane proteins in SMALP

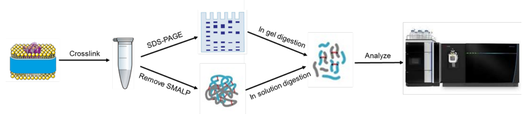


Table 2. Summary of search results from in-gel and in-solution digestion using trypsin and pepsin enzymes.

method	Seq. coverage	XL-DSS	CSM-DSS	XL-tBuPhox	CSM-tBu-Phox
In gel	82%	26	52	13	16
In solution	94%	42	116	32	93

Figure 2. XL-MS Analysis of *S. enterica* WbaP dimer: Crosslinking sites identified and visualized in XMASS with DSS(A), tBu-Phox (B) and combined (E). Histogram of Cα-Cα distances per crosslinker (C). Overlap of Identified sites (D).

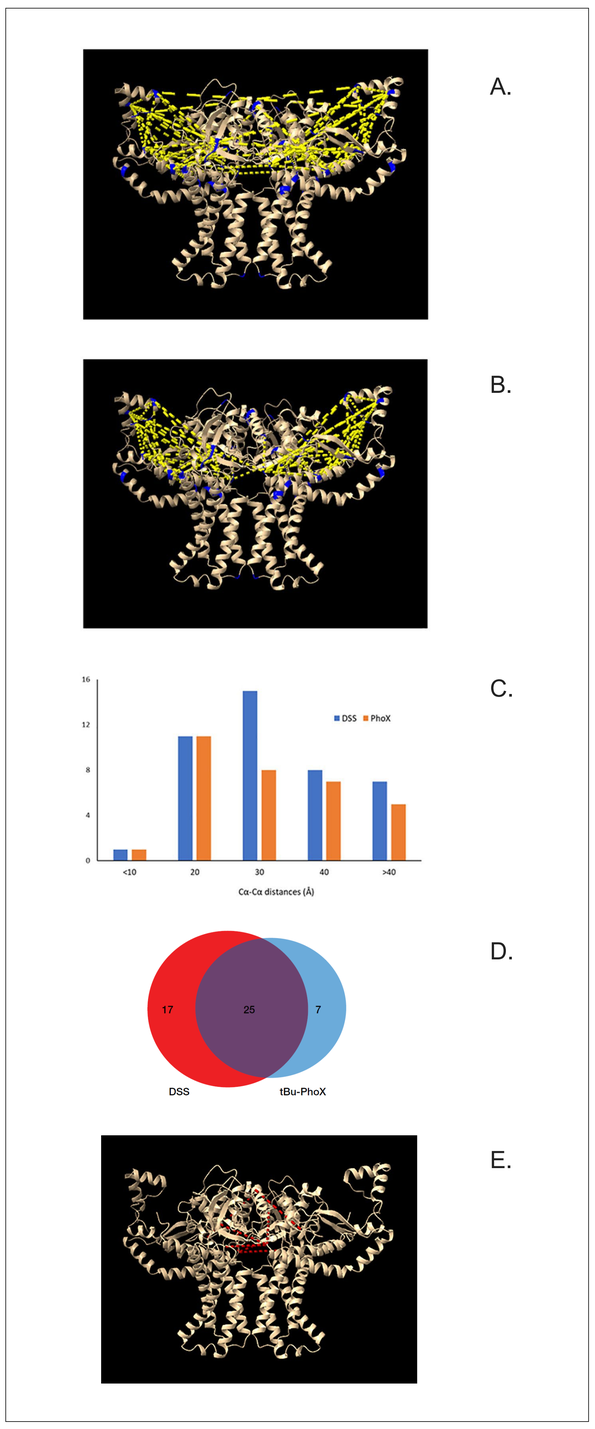


Table 3. Homo inter-crosslinks confirming homodimer structure of WbaP. A number in bold indicates that the distance is within the maximum distance of the crosslinker.

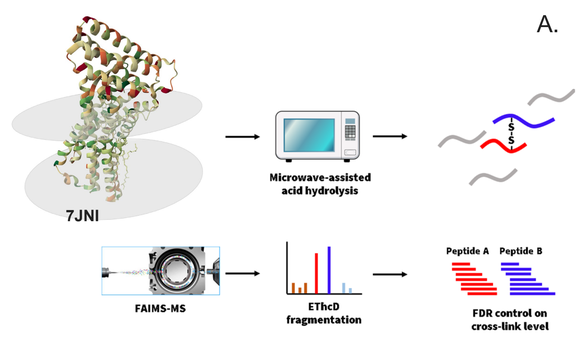
Residue 1	Residue 2	Distance intra, A	Distance inter, A
Lys 81	Lys273	**28.4**	**20.7**
Lys148	Lys273	43.2	**20.8**
Lys232	Lys273	46.3	**13.0**
Lys222	Lys422	42,2	**26.6**

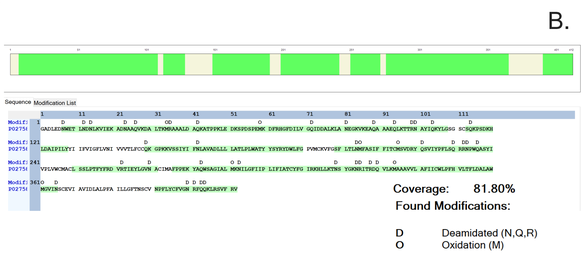
Results

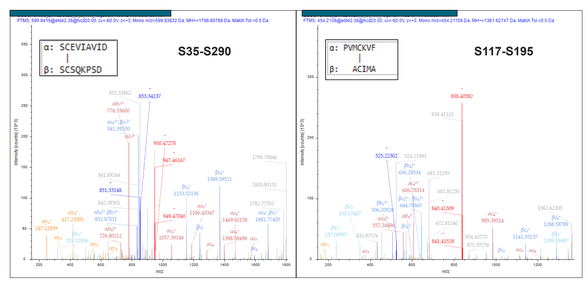
Disulfide mapping of membrane protein in detergent.

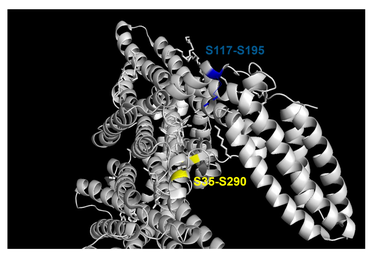
Angiotensin II subtype -2 receptor or AT2R is a G protein coupled receptor (GPCR). It contains 7 transmembrane domains and 13 cysteines. According to its crystal structure there are 2 disulfide bonds and 3 surface exposed cysteines[5] . Using newly developed MAAH[2] based workflow we were able to confirm 2 expected S-S bonds and exposed cysteines (Figure 3). The best benefit of MAAH workflow is that protein can be digested and analyzed without any buffer exchange and in presence of detergent. We were able to achieve high sequence coverage (>80%) without peptide clean up step using FAIMS-LC-EThcD MS/MS.

Figure 3. GPCR- AT2R analysis using MAAH-FAIMS-LC-EThcD workflow(A): Sequence coverage (B); Identified spectra (C.) and Pymol Visualized (D.) S-S bonds.





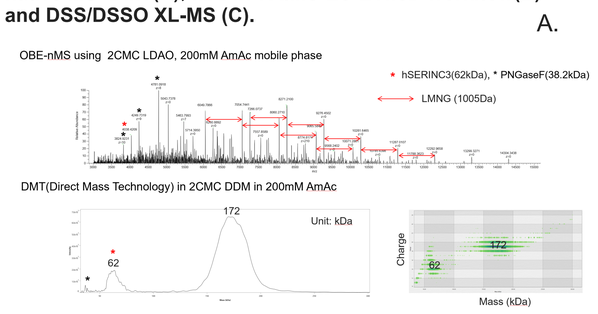




MS analysis of SERINC3 membrane protein in detergents.

The host protein SERINC3 is HIV-1 restriction factor that reduce infectivity when incorporated into the viral envelope by integrating serine into phospholipids. Because of its function there is a substantial interest to study structure of SERINCs. The ~50 kDa integral membrane protein, has ten transmembrane domains and a single N-glycosylation site. Its cryoEM structure was reported in 2023[6]. The goal of this study was to test newly developed methods and improve structure resolution for dynamic regions using XL-MS. We fully characterized, PNGase F treated hSERINC3 in 0.02% DDM/0.004 CHS or 0.003%LMNG using previously developed OBE-native MS +DMT[3], MAAH and XL-MS methods (Figure 4, Table 4). The cryoEM map shows that hSERINC3 is comprised of two α-helical bundles:H5, 6, 7, and 10(1), and another bundle contains H1, 2, 3, and 9(2). The two bundles are connected by a diagonal "crossmember" α-helix (H4). H4 is paired with H8, which has an ill-defined density in the full-length WT map attributed to conformational variability. Using XL-MS we were able to confirm these bundles and interactions between H8 and cytoplasmic side of bundle 2 and between 2 bundles.

Figure 4. hSERINC3 analysis in DDM/ CHS or LMNG using native MS+DMT(A); MAAH-FAIMS-LC-EThcD workflow(B) and DSS/DSSO XL-MS (C).



Conclusions

- End to end XL-workflows for membrane proteins in detergent or SMALP were developed and evaluated using three membrane permeable crosslinker**s** DSS, DSSO and tBuPhoX.
- The in-solution workflow provides a better sequence coverage and more XL identifications for membrane proteins.
- MAAH-FAIMS-LC-EThcD workflow enables disulfide mapping and high sequence coverage of membrane proteins without buffer exchange.
- Using optimized bottom up and native MS+DMT fully characterization of membrane proteins in SMALP or detergents can be achieved.
- We obtained better XL-MS results in LMNG detergent vs DDM/CHS and demonstrated dynamic nature of H8 domain of hSERINIC3.

Table 4. Summary of XL-MS results from in-solution digestion using trypsin enzyme.

sample	Seq. coverage	XL-DSS	CSM-DSS	XL-DSSO	CSM-DSSO
DDM/CHS	78%	1	3	2	2
LMNG	79%	13	132	1	2

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