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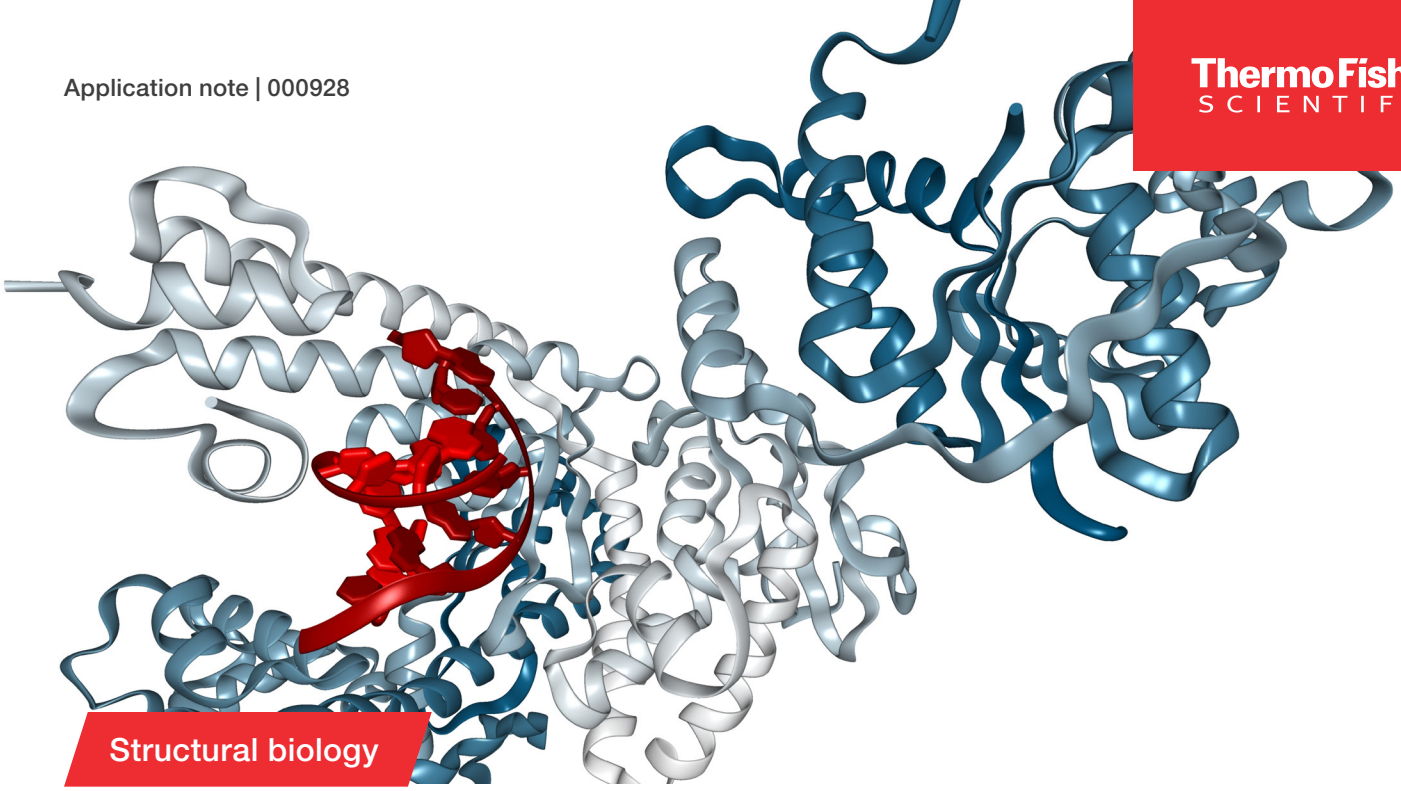

Structural biology

IMAC-enrichable crosslinking reagents for structural biology and interactomics

Authors

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Keywords

Crosslinking mass spectrometry, XL-MS, FAIMS, *E. coli* ribosome, DSSO, PhoX (DSPP), DSS, IMAC-enrichable, Proteome Discoverer software, XlinkX node, Orbitrap Eclipse mass spectrometer

Goal

Benchmarking the performance of amino-reactive, phospho-enrichable crosslinkers to study protein structures and protein-protein interactions.

Introduction

Crosslinking mass spectrometry (XL-MS), a rapid and high-resolution structural technique, has grown dramatically as a key method for characterizing protein higher-order structure and mapping protein-protein interaction networks on a proteome-wide scale. However, a significant limitation of XL-MS is the poor identification rates of crosslinked peptides due to their relatively low abundance that impedes the wider adoption of this technique for more complex protein samples. To overcome this issue, crosslinkers containing an additional affinity group have been designed to enrich low-abundant crosslinked peptides. Thermo Scientific™ PhoX (DSPP, Disuccinimidyl Phenyl Phosphonic Acid)[1] is a new amine-reactive crosslinker with a phosphonic acid group that can be enriched using immobilized metal affinity chromatography (IMAC). Traditionally used for phosphopeptide enrichment, IMAC provides highly specific and reversible capture of PhoX-crosslinked peptides.

Since these enrichment methods do not discriminate between mono-linked peptides and crosslinked peptides, additional separation methods and optimized LC-MS parameters are required to identify high numbers of crosslinked peptides. High-field asymmetric ion mobility spectrometry (FAIMS) is one method that can increase analytical performance of a mass spectrometer for complex peptide samples by incorporating gas-phase fractionation. By configuring compensation voltage (CV) settings, only specific ions dictated by the CV can pass through the electrodes into the mass spectrometer, effectively filtering out all other ions. Previous studies have demonstrated that FAIMS alone or in combination with enrichment/pre-fractionation methods significantly improves the identification rates of crosslinked peptides.[2] In this study, we optimized a Thermo Scientific™ FAIMS Pro™ interface coupled to a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer to assess the performance of novel phospho-enrichable crosslinker PhoX[1] and compared it to traditional crosslinking workflows with Thermo Scientific™ DSS (disuccinimidyl suberate) and Thermo Scientific™ DSSO (disuccinimidyl sulfoxide) for crosslinked peptide analysis of purified *E. coli* ribosomes.

Experimental

Methods and materials

Samples	*E. coli* ribosome (New England Biolabs)
Crosslinkers	PhoX (P/N A52286), DSSO (P/N A33545), DSS (P/N 21555)
Enrichment	Thermo Scientific™ High-Select™ Fe-NTA Magnetic Agarose (P/N A52284) Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (P/N A32992)
Protein and peptide concentration assay	Thermo Scientific™ Pierce™ BCA Protein Assay Kit (P/N 23225) Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (P/N 23290)
Column	Thermo Scientific™ EASY-Spray™ HPLC column, 75 µm x 25 cm (P/N ES902)
Column temp (°C)	45
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in 80% acetonitrile
Mass spectrometer	Orbitrap Eclipse Tribrid mass spectrometer, ICSW 3.5 FAIMS Pro interface
Liquid chromatography	Thermo Scientific™ EASY-nLC™ 1200 system
Data analysis	Thermo Scientific™ Proteome Discoverer™ 2.5 software and XlinkX node 2.5

Separation conditions

Retention [min]	Flow [nL/min]	%B
5.0	300.00	3.0
55.0	300.00	25.0
65.0	300.00	40.0
67.0	300.00	98.0
78.0	300.00	98.0

Mass spectrometry
Nanospray conditions

Parameter	MS
Spray voltage (V)	2,000
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	305
FAIMS mode	Standard resolution
Total carrier gas flow (L/min)	3.9
FAIMS CV (V)	-45, -60

MS conditions

	DSS	DSSO	PhoX
MS 1	**Orbitrap**	**Orbitrap**	**Orbitrap**
Resolution	60K	60K	60K
AGC	$4e^5$	$4e^5$	$4e^5$
Max. injection time (ms)	50	50	50
Charge state	3-8	3-8	3-8
MS mass range, *m/z*	380-1,400	380-1,400	380-1,400
Intensity threshold	$5e^4$	$5e^4$	$5e^4$
RF lens	30	35	30
MS^2	**Orbitrap HCD**	**Orbitrap HCD**	**Orbitrap HCD**
Resolution	30K	30K	30K
AGC	$1e^5$	$1e^5$	$1e^5$
Max. injection time (ms)	70	70	70
Charge states	3-8	3-8	3-8
First mass *(m/z)*	120	120	120
Intensity threshold	$5e^4$	$5e^4$	$5e^4$
Isolation width	1.6	1.6	1.6
NCE (%)	30	SCE 21, 26, 31	SCE 25, 30, 35
Top speed	5 s	5 s	5 s
FAIMS CV	-45, -60	-45, -60	-45, -60
Top speed FAIMS per CV	2 s	2 s	2 s

Sample preparation

E. coli ribosomal proteins were prepared as described[3] and buffer exchanged into PBS, pH 7.0 using a 10 kDa centrifugal filter unit. DSS, DSSO, and PhoX solutions in DMSO were used to crosslink 8 mg/mL ribosomal proteins in PBS pH 7.0 for 1 hr at 30-100 molar excess of DSS or DSSO to protein and 2 mM PhoX. After crosslinking, reactions were quenched with 30 mM of ammonium bicarbonate, pH 8.0, for 15 minutes. The sample was reduced and alkylated with TCEP and 2-chloroacetamide at 95 ºC for 10 minutes, then cooled at 4 ºC for 5 minutes before acetone precipitation overnight with 6 volumes of ice-cold acetone at -20 ºC. The samples were washed twice with 90% acetone and the pellet was vortexed with 100 mM TEAB until re-solubilized before overnight digestion with Lys-C/trypsin (1:50 ratio (enzyme to protein). Protein and peptide concentrations were determined using the Pierce BCA Protein Assay and the Pierce Quantitative Fluorometric Peptide Assay, respectively. PhoX sample digests (700 µg of each) were enriched using the High-Select Fe-NTA Phosphopeptide Enrichment Kit or the High-Select Fe-NTA Magnetic Agarose Kits according to the manufacturer's instructions. For magnetic bead enrichment, a 1:5 ratio (magnetic bead slurry to peptides) was used. Pierce Quantitative Fluorometric Peptide Assay was used to quantitate samples before LC-MS analysis.

Data analysis

The acquired spectra were analyzed using Proteome Discoverer 2.5 software using the XlinkX node 2.5 for crosslinked peptides and SEQUEST™ HT search engine for unmodified, looplinks and monolinks peptides. Carbamidomethylation (+57.021 Da) was used as a static modification for cysteine. Different crosslinked mass modifications for lysine were used as variable modifications for lysine or N-terminus in addition to methionine oxidation (+15.996 Da). Data were searched against a database containing the Uniprot/SwissProt entries for *E. coli* or *E. coli* ribosomes with 1% FDR criteria for protein spectra matches. For data acquired using the non-cleavable crosslinkers, DSS or PhoX, a search option in XlinkX-NonCleavable-fast was used. For MS-cleavable crosslinkers, a linear-peptide search option was used for XlinkX database searching and the Uniprot/SwissProt databases of *E. coli* proteins (retrieved March 2022). Crosslinks sites were reported at a 1% FDR. Crosslinks sites in *E. coli* ribosome and protein-protein interaction networks were visualized in xVis[4].

Results and discussion

Optimization sample preparation for PhoX crosslinker

A major challenge in XL-MS is the relatively low abundance of crosslinked peptides after protein digestion. This results in very few or no crosslinked peptide identifications by MS if the sample complexity is greater than a few proteins. Therefore, enrichment of crosslinked peptides is necessary for identification in complex samples. To specifically enrich for modified peptides, affinity groups have been incorporated into the crosslinker structure. Phospho-enrichable crosslinkers are ideal due to high enrichment specificity and simple, robust protocols.

PhoX crosslinker is a trifunctional reagent with two amine-reactive NHS groups for crosslinking and a phosphonic acid as the affinity group (Figure 1) for enrichment using phospho-enrichment methods such as IMAC or TiO_2. In this study, two types of Pierce High-Select Fe-NTA agarose were used for enrichment: magnetic agarose and traditional agarose resin (Figures 2 and 3). Magnetic beads are ideal for enrichment in terms of total number of crosslinked peptides, peptide yield, ease of use, and capability for use with automated platforms. For our experiments, we used an *E. coli* ribosome sample, which was treated with a high concentration of acetic acid to remove RNA,[3] which allowed us to perform a pre-clearance step to remove endogenous phosphopeptides from the protein digest before crosslinked peptide enrichment. Using the optimized conditions and sample preparation protocol as described in the Methods section, we were able to increase the crosslinked peptide yields by 4-fold with a 2–3-fold increase in crosslinked peptide identifications. In these experiments, the Pierce High-Select Fe-NTA magnetic beads outperformed agarose beads by 10–15% as shown in Figure 3. Although both enrichment resins use the same chelator for enrichment, the magnetic bead surface and protocol results in lower background due to less non-specific binding of unmodified peptides. After enrichment, the new PhoX crosslinker significantly outperformed the traditional amine-reactive non-cleavable crosslinkers such as DSS or mass spectrometry cleavable crosslinkers such as DSSO (Figure 3).

Mass spectrometry method optimization for PhoX crosslinked peptide analysis

To maximize the number of crosslinked peptide identifications, we tested different MS parameters including charge states, HCD fragmentation energy, and FAIMS CVs. Notably, we observed that including charge state +3 in acquisition methods for phosphonate enrichable crosslinkers provided the best results, which were in contrast to other crosslinkers such as DSSO and DSS. For those crosslinkers, the highest number of identifications were achieved using charge +4 and higher (Figure 4). This is most likely due to the negative charge of the phosphonate group impacting the charge state distribution of PhoX-labeled peptides.

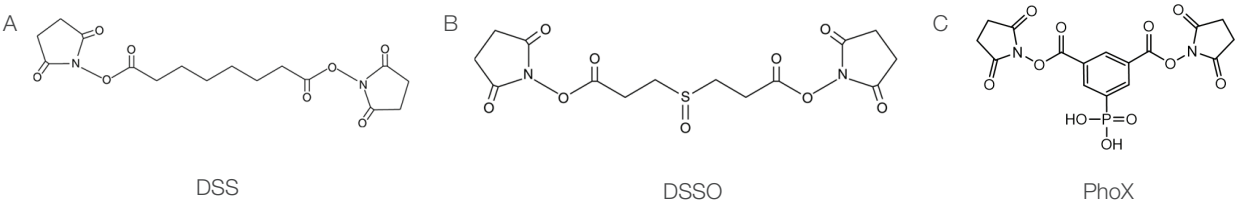

Figure 1. Structures of non-cleavable and MS-cleavable crosslinkers used in the study. (A) DSS, (B) DSSO, (C) PhoX

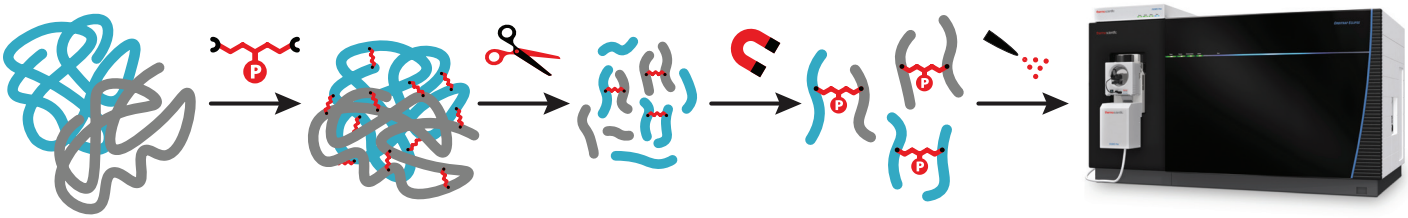

Figure 2. PhoX workflow

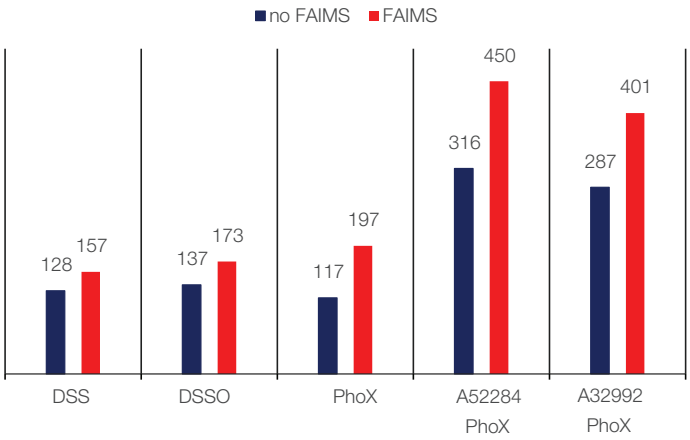

Figure 3. Unique crosslinked sites identified using DSS, DSSO, or PhoX crosslinkers with/without FAIMS

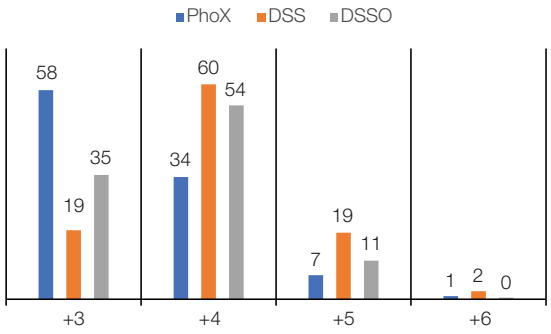

Figure 4. CSMs charge distribution (%) for DSS, DSSO, and PhoX crosslinked peptides

Table 1. MS analysis of IMAC-enriched PhoX crosslinked peptides

Sample	# XL	# CSM	# Loop links	# Mono links	# Identified spectra	% Enrichment
No enrichment	197	243	141	243	601	63%
A32992	401	664	482	871	1406	96%
A52284	450	808	486	925	1485	95%

For the analysis of DSS and DSSO crosslinked peptides, we used previously developed methods.[2] For PhoX, both single HCD (NCE =30%) and stepped collision energy (SCE) modes were evaluated with two SCE settings of 21%, 26%, 31% or 25%, 30%, 35%. Best results were obtained with SCE 25%, 30%, 35% (Figure 3). With respect to FAIMS CVs, we demonstrated that the two CV combinations: -45/-60 or -60/-75 provided similar number of crosslinked peptides. For simplicity, we selected a CV combination (-45/-60) that gave the highest crosslinked spectral matches (CSMs) for subsequent LC-MS measurements. Using the optimized parameters, we were able to identify 450 crosslinked sites (808 CSMs) in a single-shot 60 min LC-MS analysis (Figure 3). Remarkably, 95% of the peptides identified were modified by PhoX (i.e., mono-links, loop-links, and crosslinks), highlighting the excellent specificity of IMAC enrichment (Table 1).

Crosslinked sites in *E. coli* ribosome

The overall map of identified crosslinked sites in *E. coli* ribosomes using the three different crosslinkers DSS, DSSO, and PhoX are shown in Figure 5. As expected after enrichment PhoX, the most rigid crosslinker[1] with a spacer length of 4.8 Å yielded the highest number of unique crosslinking sites and loop links (Figure 5A and B). Strikingly, the most commonly used crosslinker DSS was found to have the lowest number of crosslinks, which is probably caused in part by our *E. coli* ribosome preparation (Methods). The overlap of unique crosslinking sites in all experiments combined was only ~10% (Figure 5C), but reproducibility per crosslinker was ~50%. The average Cα–Cα distances bridged by the three crosslinkers were 22 Å for PhoX, 26 Å for DSS, and 27 Å for DSSO (Figure 5D). These results were in agreement with the different spacer lengths of the three crosslinkers (Figure 1). Due to complete unfolding of ribosomes during sample preparation, we only observed a minimal number of inter-protein subunit crosslinks with almost all the crosslinks identified exceeding expected 35–40 Å distances.

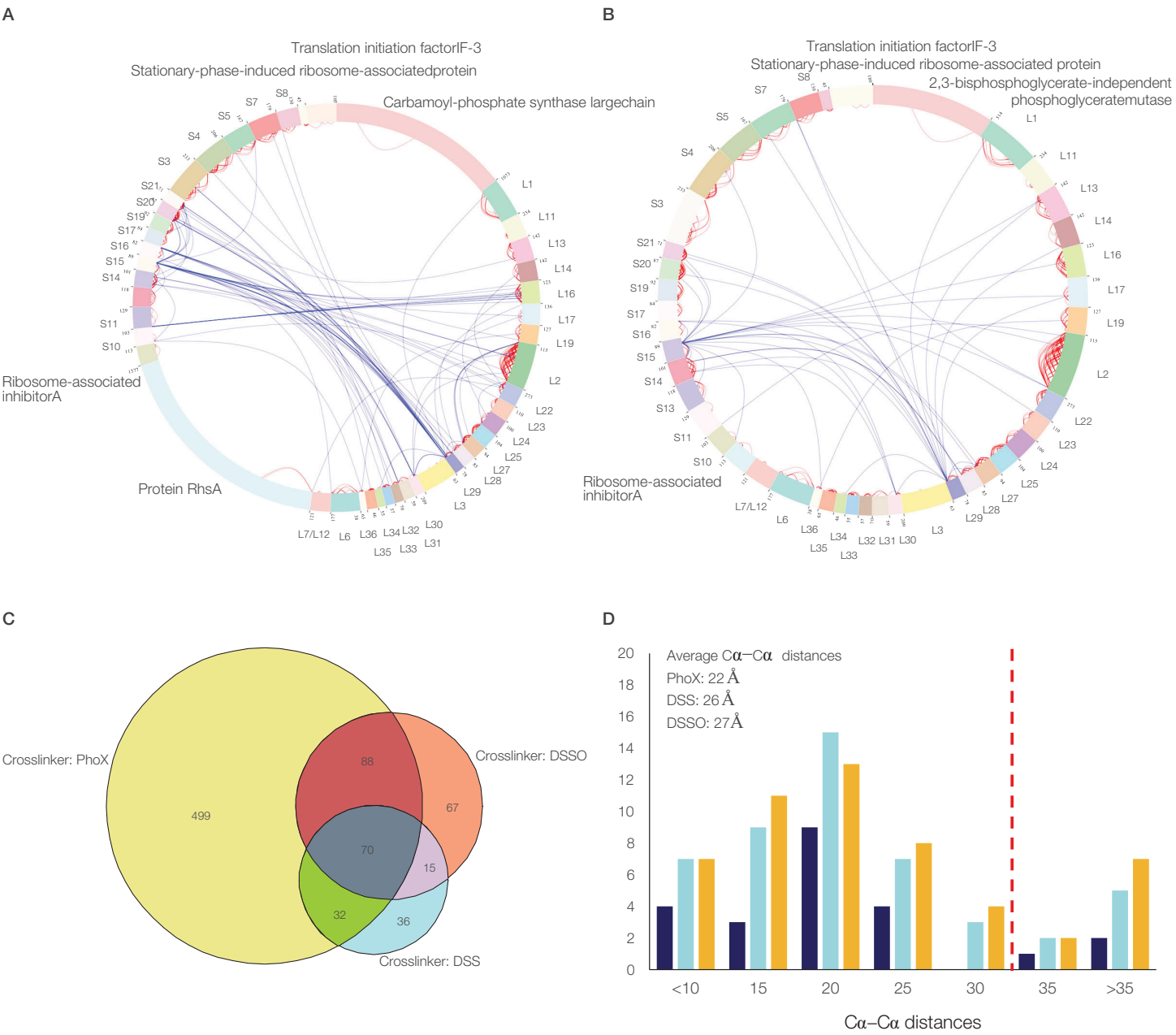

Figure 5. Crosslinking sites identified in *E. coli* ribosome. (A, B) Crosslinking sites identified in the *E. coli* ribosome after enrichment are presented as circular plot with xVis (https://xvis.genzentrum.lmu.de). Intermolecular crosslinks between different ribosomal subunits are shown in blue; intramolecular crosslinks within one ribosomal subunit are shown in red. L: large subunit; S: small subunit. (C) Venn diagram showing the overlap of crosslinking sites identified using all three crosslinkers. (D) Histogram of Cα-Cα distances in E. coli ribosome proteins for the three crosslinkers DSS (dark blue), DSSO (sky blue), and PhoX (yellow) used in this work for S20, L27, and L33 proteins. The red dashed line represents the expected max Ca-Ca distance for DSS, DSSO, and PhoX crosslinkers.

Conclusions

- The IMAC-enrichable crosslinker PhoX (DSPP) in combination with our integrated sample preparation workflow represents a highly promising approach for the identification of protein-protein interactions and the refinement of protein structural models.
- Our optimized enrichment workflow for crosslinked peptides resulted in 2-3x more crosslinked peptide identifications compared to traditional crosslinking workflows with unenrichable crosslinkers in a complex sample.
- Using optimized MS parameters, the FAIMS-XL-MS workflow resulted in an additional 1.5-fold increase in the number of unique crosslinked peptide identifications for both magnetic and non-magnetic agarose-enriched PhoX samples.

References

1. Steigenberger, B.; Pieters, R. J.; Heck, A.; Scheltema, R. A. PhoX: An IMAC-enrichable cross-linking reagent. ACS Central Science 2019, 5(9), 1514–1522.
2. Schnirch, L.; Nadler-Holly, M.; Siao, S.; Frese, C.; Viner, R.; Liu, F. Expanding the depth and sensitivity of cross-link identification by differential ion mobility using high-field asymmetric waveform ion mobility spectrometry. Analytical Chemistry 2020, 92(15), 10495-10503.
3. Cannon, J.R. et al. Ultraviolet photodissociation for characterization of whole proteins on a chromatographic time scale. Anal Chem. 2014, 86(4), 2185-2192.
4. Grimm, M.; Zimniak, T.; Kahraman, A.; Herzog, F. xVis: a web server for the schematic visualization and interpretation of crosslink-derived spatial restraints. Nucleic acids research 2015, 43(W1), W362–W369.

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