Large-scale and targeted quantitative cross-linking MS using isotope-labeled protein interaction reporter (PIR) cross-linkers

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Abstract

Quantitative measurement of chemically cross-linked proteins is crucial to reveal dynamic information about protein structures and protein-protein interactions and how these are differentially-regulated during stress, aging, drug treatment and most perturbations. Previously, we demonstrated how quantitative *in vivo* cross-linking (CL) with stable isotope labeling of amino acids in cell culture enables both heritable and dynamic changes in cells to be visualized. In this work, we demonstrate the technical feasibility of proteome-scale quantitative *in vivo* cross-linking (CL) - MS using isotope labeled protein interaction reporter (PIR) cross-linkers and *E. coli* as a model system. This isotope labeled cross-linkers approach, combined with Real-time Analysis of Cross-linked peptide Technology (ReACT) developed earlier in our lab, enables quantification of 941 non-redundant cross-linked peptide pairs from a total of 1213 fully identified peptide pairs in two biological replicate samples, through comparison of MS[1] peak intensity of the light and heavy cross-linked peptide pairs. For targeted relative quantification of cross-linked peptide pairs, we further developed a PRM-based assay to accurately probe specific site interaction changes in a complex background. The methodology described in this work provides reliable tools for both large-scale and targeted quantitative CL-MS useful for any sample where SILAC labeling may not be practical.

Graphical abstract

INTRODUCTION

Chemical cross-linking combined with mass spectrometry is an emerging structural biology tool that provides information on the spatial proximity of two amino acid residues in proteins. The insights gained from CL-MS can be translated into structural information about protein folding, architecture of protein complexes, and topologies of protein interaction interfaces[1–3]. Beyond successful applications on purified protein and protein complexes, recent developments on CL-MS technology and informatics have promoted this field to proteome-scale studies of protein-protein interaction networks in cell lysates[4–6] and living cells[7–11]. As protein structures and protein interactions are dynamic and highly dependent on their molecular and cellular environment, quantitative mapping of the edges of protein interaction networks is important for elucidating how protein conformations and interactions change in their native complex background[12].

Quantitative cross-linking MS (qCL-MS) has been demonstrated with purified proteins and protein complexes using isotope labeled cross-linking reagents such as d_0/d_4 - bis(sulfosuccinimidyl) suberate[13, 14], d_0/d_{12}-disuccinimidyl suberate[15], and d_0/d_6- biotinylated azo-lysine-targeted enrichable cross-linkers[6]. An *in vivo* qCL-MS approach based on SILAC (stable isotope labeling by an amino acids in cell culture) and the PIR technology[16, 17] recently developed in our lab, has extended applications of qCL-MS to proteome-scale[18]. This large-scale qCL-MS strategy was applied to compare changes in protein structures and interactions in multidrug-resistant human carcinoma cells[18] and probe *in vivo* conformational dynamics of heat shock protein 90 and its interactors in response to drug inhibitors[19]. For potential applications on large-scale biological samples where SILAC is not practical, we introduce the concepts and use of isotope labeled-PIR structures, which can be applied to quantification of any type of cross-linking samples, such as tissues, organs, and body fluids from plants, animals and humans. These peptide-like PIR cross-linkers are universally available at a cost-effective price as they can be easily synthesized in-house using solid-phase peptide synthesizer or purchased through any custom peptide synthesis services. In this work the heavy and light PIR pair, biotin-aspartate proline-N-hydroxyphthalimide (BDP-NHP), and its heavy counterpart, d_8-biotin-aspartate proline-N-hydroxyphthalimide (d_8-BDP-NHP), are described and used to demonstrate technical feasibility of large-scale qCL-MS by *in vivo* cross-linking of *E. coli* cells using the isotope labeled PIR pair. With careful consideration of the sites of heavy isotope incorporation in the BDP-NHP cross-linker, both heavy and light form of cross-linked peptide pair release reporter ions of the same mass upon CID MS/MS. In this way, the Real-time Analysis for Cross-linked peptide Technology (ReACT)[9], developed for assigning PIR cross-linked peptide pairs 'on-the-fly', can identify cross-linked relationships of heavy or light PIR cross-linked peptides using only a single reporter mass. Upon peptide pair identification from LC-ReACT analysis of pooled light/heavy PIR cross-linked samples, relative quantification is carried out by extracting MS^1 signals from the light and heavy PIR-cross-linked precursor

ions. Moreover, Parallel Reaction Monitoring (PRM) has evolved as a widely-available approach for quantitation in many labs to provide proteome quantitative measurements. We further demonstrate that PRM-based targeted quantification of heavy and light PIR peptide pairs has wide dynamic range, good accuracy and offers a new general capability to study protein conformational and interaction changes within a complex background that could be used in many mass spectrometry labs.

EXPERIMENTAL SECTION

Synthesis of d_0-/d_8- BDP-NHP Cross-linkers

Light and heavy forms of the Protein Interaction Reporter (PIR) cross-linkers BDP-NHP and d_8-BDP-NHP were synthesized in house according to a protocol described previously[9]. Briefly, Biotin Asparate Proline (BDP)[9] were synthesized using Fmoc chemistry and then esterified to produce activated n-hydroxyphthalamide ester. Succinic anhydride with d_4-deuterium substitution was used to synthesize the d_8-BDP-NHP cross-linker.

E. coli Sample Preparation for Large-scale qCL-MS

E. coli K12 cells were cultured in LB medium and the cell suspensions were harvested at OD 0.6~0.8. Before cross-linking, the cells were washed three times with PBS buffer and resuspended in minimum amount of 170 mM Na_2HPO_4 (pH 7.4). Subsequently, the cell suspensions were split into two equal halves, and *in vivo* cross-linking reactions were conducted by adding d_0-BDP-NHP and d_8-BDP-NHP separately into each half of the cell suspension at a final concentration of 10 mM. The *E. coli* cells were incubated with the PIR cross-linkers by constant mixing at room temperature for 1 hr. After the reaction finished, the cells were washed with PBS, lysed by cryo-grinding and ultrasonication in 8 M urea/100 mM Tris-HCl pH 8.0/150 mM NaCl. Bicinchoninic acid assay (Thermo Pierce, Rockford, IL) was conducted to determine the protein concentration of the cell lysate. Based on the measured protein concentration, the d_0-/d_8-BDP-NHP differentially cross-linked *E. coli* proteins were pooled with total protein mass ratio of 1:2 and 2:1. Each pooled cell lysate sample containing 8 mg of d_0-/d_8-BDP-NHP cross-linked *E. coli* proteins was reduced, alkylated, digested by trypsin (1:100 enzyme/protein mass ratio) overnight. The digested peptides were desalted with C18 Sep-Pak columns (Waters, Milford, MA), separated into three fractions by strong cation exchange (SCX) HPLC (250×10 mm, Luna 5 μm diameter, 100 Å pore size particles, Phenomenex, Torrance, CA). A binary solvent gradient (Solvent A, 7 mM KH_2PO_4 (pH 2.6) /30%ACN, Solvent B, 7 mM KH2PO4 (pH 2.6) /350 mM KCl / 30% ACN) with a mobile phase flow rate of 1.5 mL/min was set as follows for SCX fractionation: 0–2.5 min, 0% B; 2.5–7.5 min, 0–5% B; 7.5–47.5 min, 5–60% B; 47.5–67.5 min, 60–100%B; 67.5–77.5 min, 100%B. Samples eluted during 42.5–52.5 min, 52.5–62.5 min, 62.5–87.5 min were collected separately and concentrated by vacuum centrifugation. To further enrich the cross-linked peptides, the collected SCX fractions were incubated with UltraLink monomeric avidin (Thermo Pierce, Rockford, IL) and the cross-linked peptides were eluted from the beads in 70% ACN and 0.5% formic acid. Finally, the enriched cross-linked peptides were concentrated by vacuum centrifuge and resuspended in 0.1% formic acid for subsequent LC-ReACT MS analysis.

Sample Preparation for PRM Based Relative Quantification

Two aliquots of 1 mg/mL BSA dissolved in 170 mM Na_2HPO_4 (pH 7.4) were cross-linked with 1 mM of d_0- and d_8- BDP-NHP separately at room temperature for 1hr, and then reduced, alkylated, digested. After digestion, the d_0- and d_8-BDP-NHP differentially cross-linked BSA peptides were mixed at ratios of 1:1, 1:2, 1:5, 1:10 and 1:20. The mixed BSA peptides were desalted with C18 Sep-Pak columns, dried in vacuum centrifuge, and resuspended in 0.1% formic acid prior to LC-PRM MS analysis.

4 mg of 1:1 mixture of d_0/d_8 BDP-NHP cross-linked *E. coli* sample was reduced, alkylated and buffer exchanged against 0.1 M ammonium bicarbonate using a 30 kDa molecular weight cutoff filter (Millipore, Billerica, MA). The cross-linked proteins were then enriched by incubation with monomeric avidin beads, resulting in approximately 460 μg total protein recovered from 8 M urea elution. After trypsin digestion and C18 desalting, two aliquots of each tryptic digest were spiked with 1:5 and 5:1 d_0-/d_8-BDP-NHP cross-linked BSA digests respectively, with an enriched *E. coli* protein /BSA protein mass ratio of 190:1. These samples were used to ascertain if accurate heavy:light PIR ratios could be measured for cross-linked peptides spiked into a large background of 1:1 heavy:light PIR-cross-linked *E. coli* peptides.

LC-MS Analysis of Cross-linked Peptide Pairs

Mixtures of d_0/d_8 BDP-NHP cross-linked *E. coli* peptides from each SCX fraction were analyzed in technical triplicates by a Waters NanoAcquity UPLC coupled to a Thermo Velos Fourier transform ion cyclotron resonance mass spectrometer implemented with Real-time Analysis for Cross-linked peptide Technology (ReACT)[9]. The cross-linked peptide pairs were separated by reverse phase nanoLC using a 50 cm ×75 μm C8 column packed with 5 μm diameter, 100 Å pore size MichromMagic beads with a 4 hour 10~40% B gradient elution (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile). Peptides eluted from the nanoLC column with charge states greater than 4 were subjected to CID MS/MS, and the generated fragment ions were detected by the ICR cell. The candidate cross-linked peptide ions were determined in real-time by examining the presence of reporter ion mass (m/z 752.4129) and fragment ion masses satisfying the PIR mass relationship (precursor mass = reporter ion mass + peptide α mass + peptide β mass) within 20 ppm of mass tolerance among the top 500 fragment ions in the MS/MS spectra. Upon the discovery of PIR mass relationship for a cross-linked peptide pair precursor ion 'on-the-fly', each individual peptide ion released by CID was further selected and subjected to low-resolution CID MS^3 fragment ion analysis. Two MS/MS/MS scans were acquired for each released peptide ion from one cross-linked peptide pair precursor.

Heavy-light PIR cross-linked BSA peptides and samples containing cross-linked *E. coli* peptides spiked with cross-linked BSA peptides were analyzed by a Thermo Easy-nanoLC coupled to a Thermo Q-Exactive Plus Orbitrap mass spectrometer. The cross-linked peptide pairs were separated by a 50 cm ×75 μm C8 column (5 μm diameter, 100 Å pore size MichromMagic beads) using a 2 hour 10~40% B gradient (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile, flow rate 300 nL/min). A targeted PRM method with the following settings were applied for PRM data acquisition: mass resolution

(m/z 200)17.5 k, AGC target 2e5, maximum ion injection time 100 mS, NCE 25, isolation window 1 m/z. The targeted inclusion list contains the most abundant isotope m/z of both light and heavy forms of the 13 or 15 cross-linked peptides monitored in corresponding PRM experiment (Table SI-3, SI-4).

Identification of Cross-linked Peptide Pairs

Cross-linked peptide pairs were identified by searching MS^3 spectra acquired by ReACT analysis using Comet (Version 2016.01 rev. 2)[16] against *E. coli* K12 proteome database (total 4366 target sequences, http://www.uniprot.org/) containing forward and reverse protein sequences, and mapping the identified peptide sequences to cross-linked relationships found during ReACT data acquisition. Details of the peptide pair identification process are illustrated in Figure SI-1. A comet search was conducted with the following parameters: peptide precursor mass tolerance 20 ppm, allowing for −1, 0, +1, +2, or +3 ^{13}C offsets; fragment ion mass tolerance 1.005 Da with 0.4 Da offset; static modification, carbamidomethylation of Cys (57.0215 Da); variable modifications including oxidation of Met (15.9949 Da) and BDP stump modification (197.0324 for d_0-BDP-NHP, 201.0597 for d_8-BDP-NHP) of Lys and N-termini, where 'stump' is the residual group from the cross-linker that remains on lysine side chain or peptide N-terminus after CID cleavage; considering only fully tryptic peptide sequences, and allowing for up to 3 missed tryptic cleavage. 5% false discovery rate (FDR) for peptide identification was required and only peptide sequences containing an internal Lys modified by d_0 or d_8-BDP stump were used to map back to the cross-linked relationships with a 20 ppm tolerance. The global FDR for identification of cross-linked peptide pairs was estimated to be 1.6%, from the ratio of number of decoy cross-linked peptide pairs (either one or both reverse peptide sequences passing 5% FDR threshold during peptide identification) to the total number of identified cross-linked peptide pairs.

Relative Quantification of Cross-linked Peptides

MS^1 peak intensity based large-scale relative quantification was achieved using MassChroQ (version 2.1.1)[20]. Accurate mass of the light form of each cross-linked peptide pair, charge state, and MS^2 scan number relevant to identification of each cross-linked peptide pair was input into MassChroQ for integration of MS^1 peak intensity. The mass of the heavy form of cross-linked peptides pair was defined as 8.0504 Da modification at peptide N-terminus in MassChroQ input parameter file. All the samples from two biological replicates and three technical replicates were grouped according to their SCX fraction number for retention time alignment using the Obiwarp method[21]. Within each quantification group, the areas under the curves from extracted ion chromatogram were integrated for light and heavy forms of all the identified cross-linked peptide pair with 20 ppm mass tolerance of the precursor ion using the Zivy peak detection algorithm. Peak areas output from MassChroQ were converted to $\log_2(d_8/d_0)$ ratio for each light and heavy form of cross-linked peptide pair. Further statistical processing, including removal of outlier ratios, calculation of average ratio, standard deviation and 95% confidence interval for each non-redundant cross-linked peptide pairs was carried out with functions in R. The ratios corresponding to one non-redundant cross-linked peptide pair were grouped and the outliers within each group (values smaller than ($Q_1-1.5 \times IQR$) or greater than ($Q_3+1.5 \times IQR$)) were removed. For cross-linked peptides

with multiple measurements, the mean ratios without outliers were assigned to each non-redundant cross-linked peptide pairs.

Data from targeted PRM-based quantification of isotope labeled cross-linked peptide pairs were processed by Skyline. The *m/z* of each light and heavy precursor ion and their corresponding fragment ions, including two peptide ions with the BDP stump mass on the cross-linking Lys (197.0324 for d_0-BDP-NHP, 201.0597 for d_8-BDP-NHP), two long arm peptide ions (948.4385 Da for d_0-BDP-NHP, 952.4658 Da for d_8-BDP-NHP) resulting from partial cleavage of the cross-linked peptide pair, where BDP stump and the reporter group remain attached to the cross-linking Lys, and all the predicted b and y ions from each peptide, were calculated by a in-house developed program 'ReACT2prm' (source code download: http://brucelab.gs.washington.edu/software.html, web based application: http://xlinkdb.gs.washington.edu/xlinkdb/prmTransitionForm.php) using the cross-linked peptide sequence, cross-linking site, precursor charge state, peptide modification as input. The generated PRM transition lists were input into Skyline for extraction of MS^2 peak area from each transition channel corresponding to each targeted cross-linked peptides pair. Peak areas from all relative transitions observed in both light and heavy channels were summed to provide quantitative measurement.

RESULTS AND DISSCUSSION

The chemical structures of the PIR cross-linkers d_0/d_8 BDP-NHP are shown in Figure 1A. Four deuterium atoms are incorporated into each arm of the MS cleavable cross-linker by d_4-deuterated succinic anhydride, leading to 8 Da mass shift between the light and heavy forms of cross-linked peptide pair in full MS scans, and 4 Da mass shift between fragment ions containing the cross-linking Lys modified with BDP stump in MS^2 scans. Figure 1B and 1C show an example of EICs, MS^1 and MS^2 spectra from a pair of 1:1 d_0/d_8 mixed cross-linked *E. coli* peptide pairs VAVI**K**AVR-EA**K**DLVESAPAALK (intra cross-link from 50S ribosomal protein). As the reporter ion composition (*m/z* 752.4129) remains unchanged for d_8-BDP-NHP cross-linked peptide pairs (Figure 1C), a single PIR reporter ion mass was searched within ReACT during data acquisition using the mass relationship (precursor mass = reporter ion mass + peptide α mass + peptide β mass). Increase in the heavy precursor mass are counter balanced in the equation by increases in both released peptide masses, due to the isotopic content of the stumps on cross-linking Lys. Co-elution of the d_0/d_8 BDP-NHP cross-linked peptide pairs (Figure 1B, top) enables high-throughput relative quantification based on ratio of peak area of precursor ions by data-dependant experiment, as well as targeted quantification of cross-linked peptide pairs by ratio from peak area of fragment ions by PRM experiment. The slight retention time shift (smaller than 5 seconds) of the light/heavy cross-linked peptide pairs does not affect the relative quantification accuracy as the integrated peak areas under the curves, instead of single spectra, are used to provide relative abundance assessment of the light and heavy pairs.

MS[1] Based Large-Scale Relative Quantification of Isotope Labeled Cross-linked Peptide Pairs

To demonstrate that large-scale chemical cross-linking by isotope labeled PIR technologies can provide useful quantitative information, we used d_0- and d_8- BDP-NHP PIR cross-linkers and applied them to *in vivo* cross-linking of *E. coli* cells. The workflow is illustrated by Figure 2. Briefly, *E. coli* cells harvested from the same batch were split into two equal halves and *in vivo* cross-linked with d_0-BDP-NHP and d_8-BDP-NHP respectively. The light/heavy PIR cross-linked cells were pooled with ratios of 1:2 and 2:1 based on total protein amounts measured after cell lysis. The pooled cross-linked proteins were digested, fractioned, affinity enriched, and analyzed by LC-ReACT. Two batches of *E. coli* cells were cultured to duplicate the biochemical process and LC-ReACT experiments were carried out in triplicate for samples from each SCX fraction. A total of 1213 non-redundant peptide pairs, including 933 identified in light cross-linking form and 813 identified in heavy cross-linking form, were fully identified by search against *E. coli* K12 database (Table SI-1). These identified cross-linked peptide pairs represent a protein-protein interaction network composed of 320 non-redundant proteins, 126 inter-protein interactions, 48 homo-dimer protein interactions, and 208 self-interactions (protein with only intra-links identified). Major hub proteins were found to have many interactors in this dataset, including D-ribose-binding periplasmic protein, outer membrane protein A, elongation factor Tu 1, which were also observed as protein interaction hubs in previously reported *E. coli* cross-linking networks[4, 6, 9] and currently existing protein interaction databases such as IntAct[22] and EciD[23]. The protein-protein interaction network obtained from this d_0/d_8 BDP-NHP CL-MS dataset is shown in Figure SI-2 and also accessible from XLinkDB 2.0[24, 25] website (http://xlinkdb.gs.washington.edu/xlinkdb, Network name 'XZEcoli07132016_Bruce'). By querying available PDB structures through XLinkDB 2.0, Euclidean Cα-Cα distances from the crystal structures were computed for 665 of the 1213 identified cross-linked peptide pairs and 90% of the distances are smaller than 35 Å (Figure SI-3).

MassChroQ was employed for peak area integration as it simply requires peptide precursor mass, charge state and retention time as the input information, performs peak alignment among replicates, and analyzes isotope labeled data. Out of the 1213 non-redundant peptide pairs identified from the combined biological replicates, 941 non-redundant peptide pairs (77.5%) were quantified, including 825 non-redundant peptide pairs quantified in the 1:2 (d_0/d_8) mixing samples, 771 quantified in the 2:1 (d_0/d_8) mixing samples, and 655 quantified in both inverse labeling experiments (Table SI-2). Figure 3 shows the histogram plots of measured $\log_2(d_8/d_0)$ ratios of cross-linked peptide pairs from the 1:2 d_0/d_8 mixing sample and 2:1 d_0/d_8 mixing sample, with the medians centered at $\log_2 = 0.81$ and -1.24 respectively, interquartile range (IQR) of 0.87 and 0.94 respectively. For cross-linked peptide pairs quantified multiple times, scatter plot of 95% confidence interval versus the mean $\log_2(d_8/d_0)$ value is shown in Figure SI-4. The reproducibility of large-scale relative quantification is also assessed by correlation of light/heavy cross-linked peptide ratios measured in the inverse labeling replica experiments (Figure SI-5), and distribution of the differences and associated 95% confidence intervals of the light/heavy ratios measured from the inverse labeling experiments (Figure SI-6). Although outlier ratios always exist due to the low MS[1] peak intensity of some cross-linked peptide pairs and spectral interference from

co-eluting species, the overall quantification results obtained are consistent with the expected ratios and the variability is comparable to results from our previous SILAC qCL-MS study of human carcinoma cells (IQR of $\log_2$ Ratio=1.09, N=1054)[18]. Comparison of results generated using a single cross-linking reaction with mixed light/heavy SILAC-labeled cells[18] with those generated here revealed the present approach with two separate cross-linking reactions using light and heavy cross-linkers prior to sample mixing did not significantly increase observed ratio variance.

Targeted Relative Quantification of Isotope Labeled Cross-linked Peptide Pairs by PRM

Targeted PRM is commonly considered as an accurate and sensitive approach for peptide and protein quantification and often applied to confirm the protein abundance changes found from large-scale discovery experiments. As a proof-of-principle experiment for targeted quantification of isotope labeled cross-linked peptide pairs, d_0- and d_8- BDP-NHP cross-linked BSA digests were mixed with ratios of 1:1, 1:2, 1:5, 1:10, and 1:20, and a total of 13 light/heavy cross-linked peptide pairs were targeted and quantified by PRM (Table SI-3). Predicted transitions for each form of cross-linked peptide pair, including singly and doubly charged peptide precursors, b and y ions from each peptide precursor were extracted using Skyline. Figure 4A shows PRM transitions of the light and heavy forms of cross-linked BSA peptide pair SLGKVGTR-LSQKFPK from the 1:1 (d_0/d_8) mixing sample. The two released peptide ions with BDP stump and two long arm peptide ions with BDP stump and reporter group, are frequently observed to be the most abundant fragment ions generated by HCD. The measured $\log_2(d_8/d_0)$ ratio for each light/heavy cross-linked peptide pair was calculated based on peak areas from summed EIC of all the transitions present in both form of the cross-linked peptide pair. As shown in Figure 4B, the observed average $\log_2(d_8/d_0)$ ratios calculated from the 13 light/heavy pairs of cross-linked peptides measured by PRM in duplicates agree well with the expected ratios and the response appears linear over the range studied (Table SI-5).

To mimic probing conformational or interaction changes of a specific protein through cross-linked peptide quantitation from a complex sample background, we spiked 1 part of light/heavy BDP-NHP cross-linked BSA digests (d_0/d_8 1:5, 5:1) into 190 parts of tryptic digests from *in vivo* cross-linked *E. coli* (d_0/d_8 1:1) proteins and developed a PRM assay to quantify the changes of cross-linked BSA peptide pairs in the background of cross-linked *E. coli* samples. Three representative BSA light/heavy cross-link pairs were monitored to quantify the changes of BSA (Table SI-3). Meanwhile, twelve frequently observed light/heavy cross-linked *E. coli* peptide pairs from the major protein interaction hubs and their interactors were also quantified simultaneously to provide a reference of measurement variation for those cross-links whose relative responses are not expected to change significantly (Table SI-4). Figure 4C presents the PRM quantification results from triplicate measurements of each *E. coli* sample spiked with cross-linked BSA (Table SI-5). The d_8/d_0 fold changes of the three BSA cross-linked peptide pairs monitored were close to the expected value of 1:5 and 5:1(error within 33%, N=3), while the relative quantity of twelve pairs of *E. coli* cross-linked peptide pairs remained close to 1:1 or $\log_2$=0 (error within 27%, N=3). The masses of quantified peptide pairs range from 2.8 kDa to 6.6 kDa. By centering the 1 *m/z* mass isolation window at the most abundant isotope predicted from the chemical formula of cross-

linked peptide pair, co-isolation of isotope clusters from light and heavy channels was avoided and accurate quantification for peptide pair as large as 6.6 kDa was achieved.

CONCLUSIONS

This work demonstrates proteome-wide *in vivo* quantitative CL-MS using isotope labeled PIR cross-linkers with the model organism *E. coli* and enables extension of qCL-MS to *in vivo* samples where SILAC may not be practical. Nearly one thousand cross-linked peptide pairs from 382 interacting proteins were quantified through comparison of peak areas of light and heavy cross-linked peptide pair precursors extracted from LC-ReACT data. Similar to other MS^1 based quantification approaches for proteome-scale data-dependent experiments, major challenges for accurate quantification of the cross-linked peptide pairs come from low abundance of many cross-linked species, co-elution of cross-linked and non cross-linked peptide pairs, and relatively slow instrument duty cycle for data sampling. We also demonstrated that targeted PRM based relative quantification using isotope labeled cross-linkers is a complementary approach for accurate and sensitive quantification of cross-linked peptides in complex samples. With PIR cross-linkers widely available from many peptide synthesis companies and PRM methods for reliable peptide quantification gaining widespread utility, we anticipate that the qCL-MS strategy described here could have broad impact for probing *in vivo* protein conformational and protein-protein interaction dynamics resultant from drug treatments, development, aging, stresses or nearly any conditional perturbations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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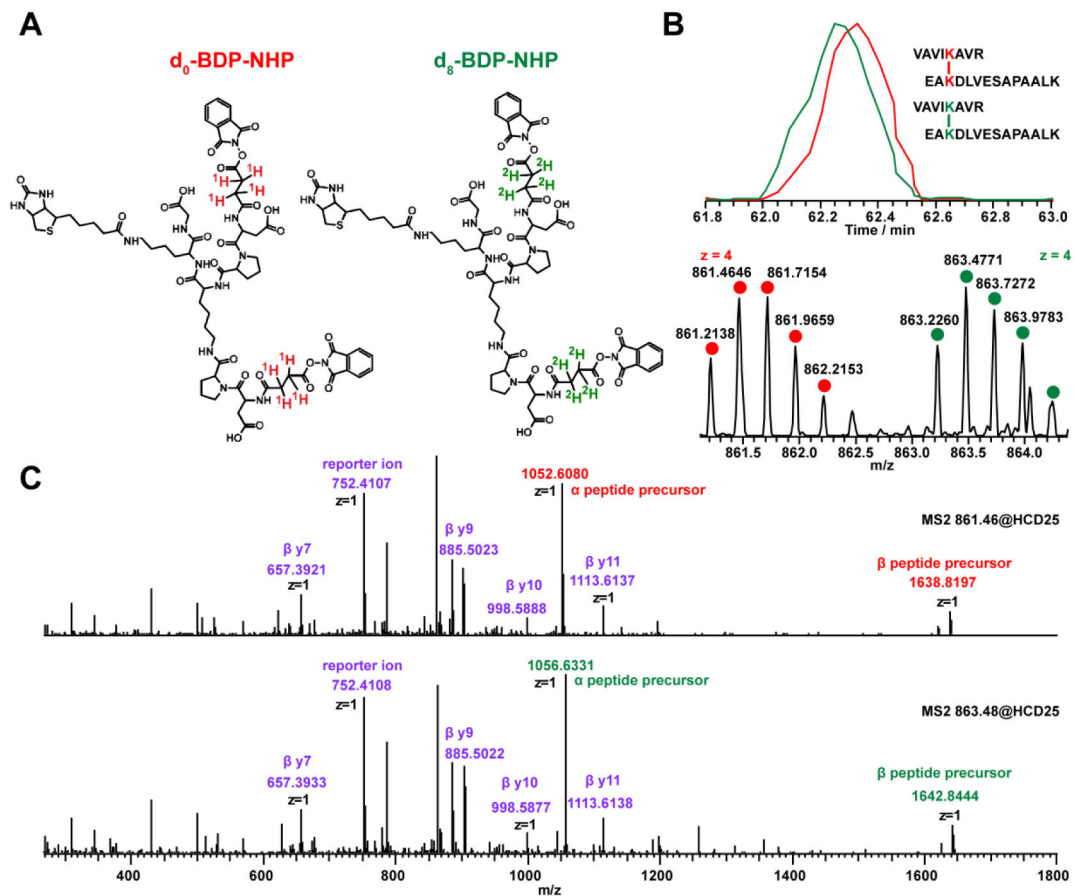
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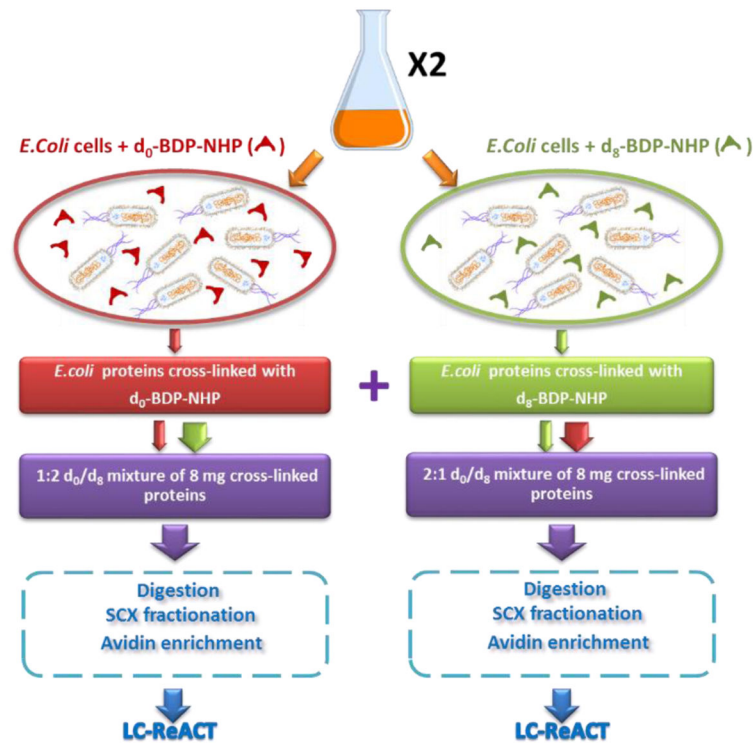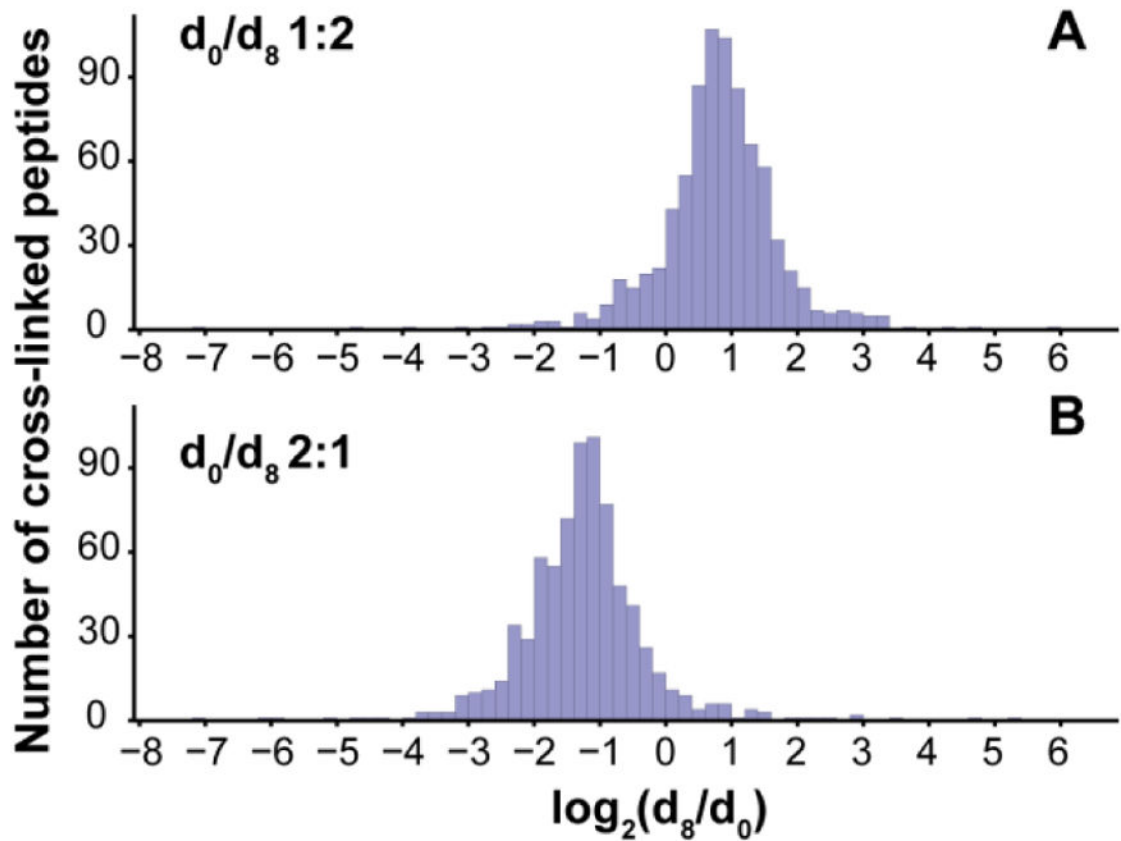
Figure 1.
(A) Chemical structure of d_0-BDP-NHP and d_8-BDP-NHP. (B) An example of EICs (top) and high-resolution MS^1 spectra (bottom) of d_0 BDP-NHP and d_8 BDP-NHP cross-linked *E. coli* peptide pairs VAVIKAVR-EAKDLVESAPAALK (intra cross-links from 50S ribosomal protein) mixed in 1:1 ratio (monoisotopic *m/z* 861.2138, 863.2260, +4). (C) High-resolution MS/MS spectra of the d_0-BDP-NHP (top) and d_8-BDP-NHP (bottom) cross-linked peptide pairs. Upon HCD, the reporter ion (m/z 752.4107), α peptide precursor VAVIKAVR (m/z 1052.6080 light, m/z 1056.6331 heavy), β peptide precursor (m/z 1638.8197 light, m/z 1642.84444 heavy) are released from BDP-NHP cross-linked peptide pairs.
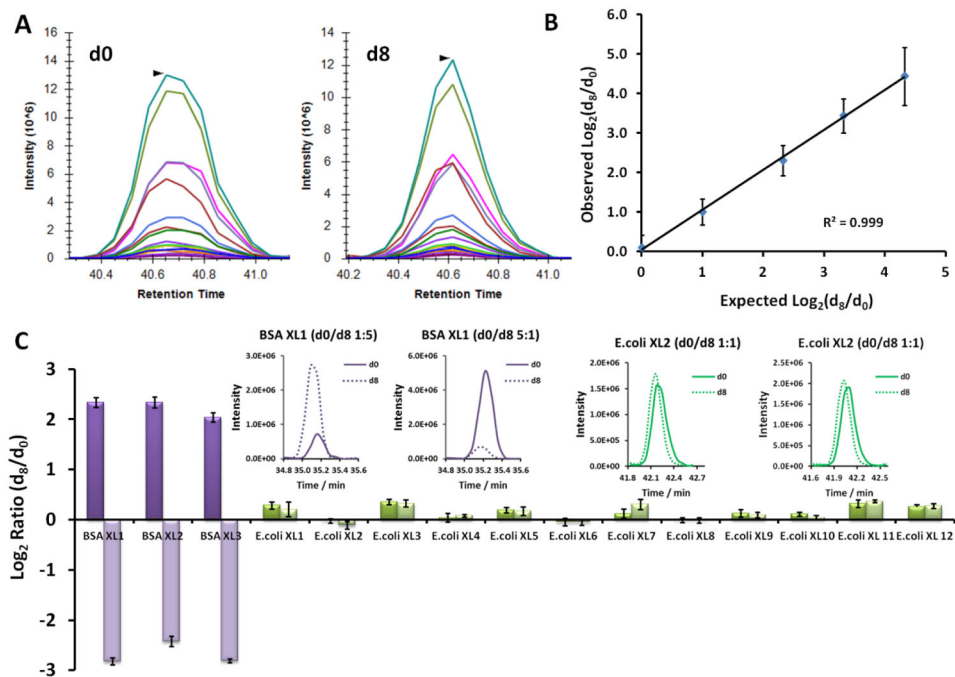
Figure 2.
Illustration of sample preparation work flow for large-scale relative quantification of differentially cross-linked *E. coli* cells. Two batches of cells were cultured for biological replicates. Cultured cells from one batch were split into two equal halves and cross-linked with d_0-BDP-NHP and d_8-BDP-NHP respectively. After cell lysis, 8 mg of cross-linked *E. coli* lysates were pooled in d_0/d_8 1:2 and 2:1 ratios based on total protein mass. The lysates were digested, fractioned by SCX, enriched with avidin beads, and then analyzed by LC-ReACT in triplicates for MS^1 peak intensity based quantification.

Figure 3.
Histograms showing distribution of measured $\log_2(d_8/d_0)$ values of cross-linked *E. coli* peptide pairs from the (A) 1:2 d_0/d_8 mixing sample (n=825, median 0.81, IQR=0.87) and (B) 2:1 d_0/d_8 mixing sample (n=771, median −1.24, IQR=0.94).

Figure 4.
Targeted PRM based relative quantification of d_0/d_8 BDP-NHP cross-linked peptide pairs.
(A) An example of PRM transitions of 1: 1 mixed d_0-BDP-NHP (left) and d_8-BDP-NHP
(right) cross-linked BSA peptide pair SLGKVGTR-LSQKFPK. Only unique transitions
with 4 Da mass difference between the fragment ions from light and heavy channels are
shown. (B) Calibration curve showing PRM based relative quantification of 13 pairs of d_0-
and d_8- BDP-NHP cross-linked BSA peptides mixed with ratios of 1:1, 1:2, 1:5, 1:10 and
1:20. Standard deviation of the measured $Log_2(d_8/d_0)$ value was calculated based on
duplicate PRM measurements of 13 d_0/d_8 -BDP-NHP cross-linked peptide pairs (n=26). (C)
PRM based relative quantification of trace amount of differentially cross-linked BSA
peptides (d_0/d_8 1:5 deep colored, and 5:1 light colored) spiked into cross-linked *E. coli* cell
lysate digests (d_0/d_8, 1:1). The *E. coli* cell digests were mixed with cross-linked BSA digest
at a total *E. coli* protein /BSA mass ratio of 190:1. Three d_0/d_8 BDP-NHP cross-linked BSA
peptide pairs and twelve d_0/d_8 BDP-NHP cross-linked *E. coli* peptide pairs were measured
by PRM. Standard deviation of $Log_2(d_8/d_0)$ value was calculated based on triplicate PRM
measurements. Insets show examples of summed EICs of differentially cross-linked peptide
pair SLGKVGTR-LCVLHEKTPVSEK (purple, d_0/d_8 1:5 and 5:1) from BSA, and
QENQIKAVR-GITLKIADGQQK (green, d_0/d_8 1:1) from the *E. coli* ABC transporter
periplasmic-binding protein, in two separate PRM experiments for the two *E. coli* samples
spiked with different BSA d_0/d_8 cross-linking ratio.