Supporting Information for

Quantitative profiling of protein-derived electrophilic cofactors in bacterial cells with a hydrazine-derived probe

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Contents:
The SUPPORTING INFORMATION includes Supplemental Text and Figures S-1 to S-3. The Supplemental Text includes detailed procedures for protein extraction, biotin assay, MS analysis using DDA mode and PRM mode, respectively. Fig. S-1 contains the biotin assay to analyze the probe labeling effects with and without propyl hydrazine, followed by treatment with or without NaBH₃CN. Fig. S-2 contains the gel fluorescence scheme and results for labeling with propargyl hydrazine of different concentrations. Fig. S-3 includes the fragment ion spectra of two peptides from reactions without and with NaBH₃CN treatment, respectively.
SUPPLEMENTARY TEXT

Protein extraction from E. coli cells. E. coli cells cultured in LB were centrifuged at 4000 g for 10 min to pellet the cells. The cells were then suspended in 6 ml of lysis buffer containing PBS (pH 7.4) and 1× protease inhibitor. Then the cells were put on ice and lysed by putting a sonication probe in it. One sonication cycle included 5 s of sonication and 10 s of pause, and it totally took 10 min. The maximum temperature was set as 45 °C to prevent protein denaturation during sonication. The samples were centrifuged at 10000 g for 5 min, and the supernatant was transported to a new tube. The protein concentration was quantified by Bradford dye.

Biotin assay for analysis of probe labeling effects. For biotin assay, we had 3 protein samples and each sample has 100 μg of protein in 50 μl lysis buffer. The first one was treated with 10 mM of propyl hydrazine for 1 h, followed by 1 mM propargyl hydrazine. The second sample was only treated with 1 mM of propargyl hydrazine for 1 h. The third one was treated with 1 mM propargyl hydrazine for 1 h, followed by reduction with 20 mM NaBH₃CN for 30 min. Then click reaction was conducted in each sample to biotinylate the probe-labeled proteins. The click reaction system included 0.2 mM biotin-azide, 1% sodium dodecyl sulfate (SDS), 1 mM sodium ascorbate, 0.5 mM THPTA, and 1 mM CuSO₄. The samples were loaded into 12% SDS-PAGE gel for electrophoresis along with protein marker. The gel electrophoresis was set at 150 V, and it was run for additional 10-20 min after the blue dye moved out of the gel. The proteins were then transferred from the gel to nitrocellulose membrane, which was run at 300 mA for 90 min. The protein on membrane were stained with Ponceau S. to check the protein amount in each lane. Then the proteins on membrane were destained using PBS, followed by being blocked with 5% BSA in TBST buffer for 1 h at RT. Streptavidin-HRP in TBST containing 5% BSA was incubated with the membrane, followed by rotation overnight at 4 °C. After wash with TBST for 3 times, SuperSignal West femto kit was added onto the membrane,
incubated for 3 min at darkness. The membrane was drained, and then put on Li-cor plate, and chemiluminescence channel was selected to visualize the biotinylated proteins.

**Profiling of protein-derived electrophiles using mass spectrometry.** To identify protein-derived electrophilic cofactors, we had 3 groups of proteins with 4 replicates in each group. The 3 groups of proteins were labeled as “Blank”, “Probe”, and “Compete”, respectively. Each sample has 2 mg proteins dissolved in PBS (pH 7.4). The “Blank” group was treated with H₂O, the “Probe” group was treated with 1 mM propargyl hydrazine that was dissolved in water, while the “Compete” group of protein was treated by 10 mM propyl hydrazine for 1 h, followed by labeling with 1 mM propargyl hydrazine for 1 h at RT. Then the samples all underwent precipitation with cold acetone, washing with methanol, and re-suspension in PBS (pH 7.4). Click reaction mixture was added to the samples to ligate biotin group to the probe-labeled proteins. The click reagents were 0.5 mM THPTA, 1 mM CuSO₄, 1 mM sodium ascorbate and 0.2 mM biotin-azide. Then NAP-10 column, a type of size exclusion chromatography, was used to remove the click reagents. After loading into the column, the proteins were eluted into 1.4 ml of 1% IGEPAL, 0.1% SDS in PBS (pH 7.4). Then the proteins were mixed with 100 μl of magnetic streptavidin beads, followed by rotation at 4 °C overnight. The beads for capturing biotinylated proteins were washed with 1% IGEPAL and 0.1% SDS in PBS (twice), 6 M urea (3 times) and PBS (3 times), and then suspended in 20 mM Tris-HCl (pH 8.0) containing 2 mM CaCl₂. On-beads samples were reduced with 5 mM DTT at 56 °C for 30 min and treated with 20 mM IAA at RT for 20 min in darkness. Samples were treated with 10 mM DTT at 37 °C for 10 min, followed by incubation with 1 μg sequencing-grade trypsin at 37 °C overnight to digest the captured proteins to peptides. The peptides were collected from the supernatant and stored in 1% formic acid. The peptides were desalted by C18 StageTip and eluted with 50% acetonitrile containing 0.1% formic acid. The eluted peptides were dried and stored at -80 °C prior to dissolution in 0.1% formic acid for
nanoLC-MS/MS analysis.

The MS data was acquired with an UltiMate 3000 RSLCnano system coupled with an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Scientific). C18-packed nanoLC column was used to separate peptides in 90-min LC gradient (mobile phase A was 0.1% formic acid and 2% acetonitrile in water, and mobile phase B was 0.1% formic acid and 2% water in acetonitrile). Data-dependent acquisition mode (DDA) was implemented to collect tandem MS data: precursor ions of peptides were fragmented by HCD within a cycle time of 3 s if they ranked high in MS1 scanning with charge state between +2 and +7. Orbitrap mass spectrometer was used to detect both precursor and fragment ions.

The MS data were analyzed with MaxQuant (version 1.6.10) against an E.coli protein database (UniProt 2019.03.23). The data processing parameters were as follows: mass tolerance of 20 ppm for both precursor and fragment ions, cysteine carbamidomethyl as fixed modification, protein N-end acetylation and methionine oxidation as variable modifications, and two missing tryptic cleavages. To match between runs, retention time window was set as ±5 min. The quantified protein information underwent median-centered normalization.\(^1\) Then fold changes for each protein were calculated as follows: Probe group divided by Blank group as Enrichment ratio, and Probe group divided by Compete group as Competition ratio. The proteins meeting the criteria of Enrichment ratio>10 and Competition ratio>2 were considered as candidates that can derived electrophilic cofactors.

**Verification of protein electrophiles using PRM mode.** Before PRM data analysis, the acquired DDA data were analyzed by Proteome Discover 1.4 to obtain files with msf format. The detailed Proteome Discoverer search parameters were: E.coli protein database was used (UniProt 2019.03.23); maximum missing cleavage site was set as 2; mass tolerance was 20 ppm for precursor ion and 0.02 Da for fragment ions; spectrum matching only focused on b ions and y ions; dynamic modification
included methionine oxidation; static modification included cysteine carboxymethyl. The msf files were then imported to Skyline 19.0 for library establishment. The proteins of interest were listed, and only their unique peptides were selected for PRM analysis. The corresponding peptide information was obtained from the DDA data, including retention time, m/z value and ion charge. The precursor ion information was exported to an excel file and used as parameters for PRM analysis.

PRM samples were treated in the same way as the DDA samples, and each group had 4 replicates. During PRM acquisition, the peptides of interest were selectively fragmented, and both of their precursor and fragment ions were detected. Retention time window of ±10 min was utilized to ensure detection of peptides, and less than 30 peptides were simultaneously analyzed to maintain a reasonable cycle time. The acquired PRM data were directly imported into Skyline to quantify the precursor ions with their 3 isotopic peaks and fragment ions (b ions and y ions) that ranked top 5. The related peptide information was then exported. The peak areas of the fragment ions were summed up as the peptide signal intensity. The selected peptides were then integrated as protein levels.
Supporting Figures

**Figure S-1** Biotin assay to analyze the protein labeling efficiency by 1 mM propargyl hydrazine, with and without competition by 10 mM propyl hydrazine, with and without NaBH₃CN treatment.
Figure S-2 Gel fluorescence profiling of protein-derived electrophiles in E.coli cells. (A) The scheme of gel fluorescence profiling. (B) Gel fluorescence analysis of proteins labeled by propargyl hydrazine of different concentrations (0, 0.02, 0.1, 0.5, and 2 mM, respectively).
Figure S-3 (A) The MS spectra extracted from the probe labeling group without NaBH$_3$CN treatment showed the fragment ions of peptide ($m/z=630.5390$), with marked b ions and y ions. (B) The extracted MS spectra from reaction with NaBH$_3$CN treatment showed the fragment ions of peptide ($m/z=618.0323$), with identified b ions and y ions.
References
