Gold nanoparticle enrichment method for identifying *S*-nitrosylation and *S*-glutathionylation sites in proteins

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Supporting Information

I. General Information. Gold (III) chloride trihydrate, trisodium citrate dihydrate, reduced and oxidized glutathione, sodium nitrite, iodoacetamide, N-ethylmaleimide and dithiothreitol were purchased from Sigma. All commercial reagents were used without further purification. Zeba™ Desalt Spin Columns, trifluoroacetic acid, recrystalized α-cyano-4-hydroxycinnamic acid and ascorbic acid were purchased from Thermo Scientific. Siliconized micro-centrifuge tubes were purchased from Bio Plas Inc and were used for all protein processing steps. Sequencing grade Trypsin was purchased from Promega, Endoproteinase Glu-C was purchased from Roche Applied Science. Vivapure C-18 Micro spin columns were purchased from Sartorius Stedim Biotech. High purity water and acetonitrile were purchased from Honeywell Burdick and Jackson.

II. Gold Nanoparticle (AuNP) Synthesis. AuNPs were synthesized according to the method of Grabar *et al.*[1]. All glassware was washed and rinsed thoroughly with Milli-Q (18.2 MΩ) high purity water. In a 1 L double-neck round bottom flask, 197 mg gold (III) chloride trihydrate ($HAuCl_4 \bullet 3H_2O$) was dissolved in 500 mL of Milli-Q water and refluxed for 1 h in a hot-oil bath (~100 °C) with vigorous stirring. Following reflux, 571 mg trisodium citrate dihydrate ($Na_3C_5H_5O_7 \bullet 2H_2O$) was dissolved in 50 mL Milli-Q water and injected rapidly into the gold chloride solution. A distinct colour change can be seen from yellow to clear, then to dark burgundy (almost black) signifying the formation of a colloidal solution. After 10 min, the heat was removed and the solution was allowed to cool to room temperature. The resulting nanoparticles are 11 ± 1 nm in diameter, exhibit a maximum absorbance at 521 nm and are stable for months at 4 °C. The resulting concentration is ~1.3×10^{10} AuNPs / μL.

III. S-nitrosoglutathione (GSNO) Synthesis. All steps were performed in the dark due to the photosensitivity of the SNO bond. 10.3 mg sodium nitrite ($NaNO_2$) was dissolved in 4 mL of ice-cold 0.5 M HCl. 46.1 mg of reduced glutathione (GSH) was then dissolved in 1 mL of ice-cold 0.5 M HCl and added to the $NaNO_2$ solution. The mixture was incubated in the dark at 4 °C for 30 min and the pH was adjusted to 7.4 by the addition of dilute NaOH. GSNO concentration was determined from the absorption maximum of the SNO bond at 335 nm ($\varepsilon_{335} = 980\ M^{-1}cm^{-1}$). The resulting solution was stored as 1 mL aliquots at -80 °C.

IV. Protein Nitrosylation and Alkylation. Recombinant PDI and hYVH1 were purified as previously described[2,3]. Fully reduced proteins (2 μM) were nitrosylated by incubation with 1 mM GSNO in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at room

(1) Grabar, K. C.; Freeman, R. G.; Hommer, M. B.; Natan, M.J. *Anal. Chem.* **1995**, 67, 735-43.
(2) Sliskovic, I.; Raturi, A.; Mutus, B. *J. Biol. Chem.* **2005**, 280, 8733-41.
(3) Bonham, C. A.; Vacratsis, P. O. *J. Biol. Chem.* **2009**, 284, 22853-64.

temperature. Iodoacetamide (IAM) was then added to a final concentration of 10 mM and incubated for 2 h at room temperature. Excess GSNO and IAM were removed by two passes through Zeba™ Desalt Spin Columns as per the manufacturer's specifications. At this step, the buffer was exchanged to 50 mM ammonium bicarbonate (pH ~8, unadjusted).

V. Protein Nitrosylation, Glutathionylation and Differential Alkylation. Fully reduced recombinant hYVH1 (30 μM) was nitrosylated or glutathionylated by incubation with 1 mM GSNO or oxidized glutathione (GSSG) in 25 mM Tris-HCl 50 mM NaCl (pH 7.5) in the dark, shaking for 1 h at room temperature. Iodoacetamide was then added to a final concentration of 20 mM and incubated in the dark, shaking for 1 h at room temperature. Both samples were desalted as above, except nitrosylated hYVH1 was exchanged into 25 mM Tris-HCl 50 mM NaCl (pH 7.5) buffer. Ascorbate, N-ethylmaleimide, and copper (II) sulfate were then added to final concentrations of 30 mM, 20 mM, and 300 nM respectively. The sample was incubated in the dark, shaking for 3 h at room temperature, then desalted as above for subsequent proteolysis.

VI. Proteolytic Digestion. A 10:1 protein:protease (by mass) ratio was maintained for all digests. PDI was digested for 18 h with Endoproteinase Glu-C shaking at room temperature. hYVH1 was digested for 12 h with Trypsin shaking at 37 °C. For solution digest spectra, the samples were quenched by a final concentration of 1 % formic acid.

VII. AuNP-Peptide Binding and Elution. AuNPs (500 μL) were pelleted by centrifugation (16,000 x g for 15 min). The supernatant was discarded and 40 μL of unquenched protein digests were added to the AuNP pellet and incubated 30 min at 37 °C. The AuNP-peptide complex was centrifuged, the supernatant was aspirated and the AuNP-peptide pellet was washed in 200 μL of 10 mM ammonium bicarbonate (pH ~7.6, unadjusted) three times. After the wash-spin steps the AuNP-bound peptides were then eluted in 20 μL of 100 mM dithiothreitol (DTT). Following addition of DTT, the AuNPs were sonicated for 5 min in a water bath to disperse any AuNP aggregates. This was followed by a 2 h incubation after which the AuNPs were again pelleted and the supernatant harvested. To ensure the complete elution of peptides from the AuNP surface, a second addition of 20 μL of 100 mM DTT was incubated with the AuNPs for 8 h with gentle agitation. The AuNPs were centrifuged and the resulting supernatant was combined with that from the first DTT elution step. Samples were acidified by the addition of 100 μL 0.1 % trifluoroacetic acid, then desalted and concentrated using Vivapure C-18 Micro spin columns as per the manufacturer's protocol. All digests were mixed 1:1 with 10 mg/mL matrix solution (recrystalized α-cyano-4-hydroxycinnamic acid in 60 % acetonitrile, 0.1 % trifluoroacetic acid) on the target plate by dried droplet method and analyzed by MALDI-TOF MS using an Applied Biosystems Voyager DE-Pro Mass Spectrometer. Peptide mass fingerprints and tandem mass spectrometry (MS/MS) using post source decay (PSD) was performed on selected parent ions as previously described[3] and compared to *in silico* fragmentation using the Protein Prospector software (http://prospector.ucsf.edu/).

Residue	Sequence	Monoisotopic (*m/z*)	IAM (+ 57 Da)
325 – 331	F**C**HRFLE	951.5	1008.5
31 – 45	FYAPW**C**GH**C**KALAPE	1692.7	1749.7/1806.7
287 – 306	FFGLKKEE**C**PAVRLITLEEE	2351.2	2408.2
374 – 394	FYAPW**C**GH**C**KQLAPIWDKLGE	2462.2	2519.2/2576.2

Table S1. Protein disulfide isomerase thiol-containing peptides. Mass fingerprint reference table of target thiol-containing peptides from recombinant protein digest using Endoproteinase Glu-C.

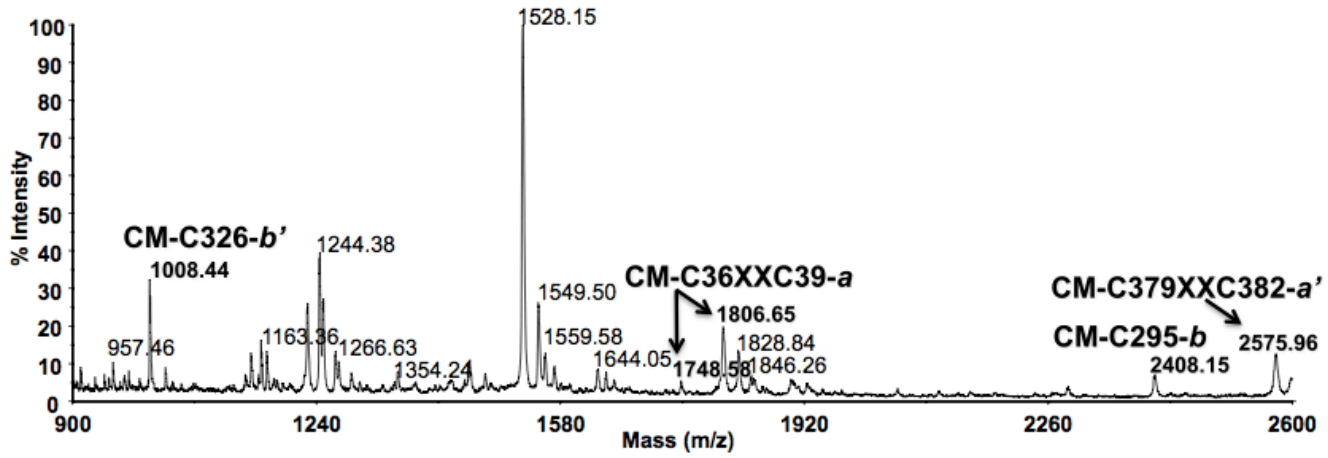

Figure S1. Mass fingerprint of reduced, CM-labeled, Endoproteinase Glu-C digested PDI (no AuNPs). Modified thiol-containing peptides are highlighted, their peptide masses in bold.

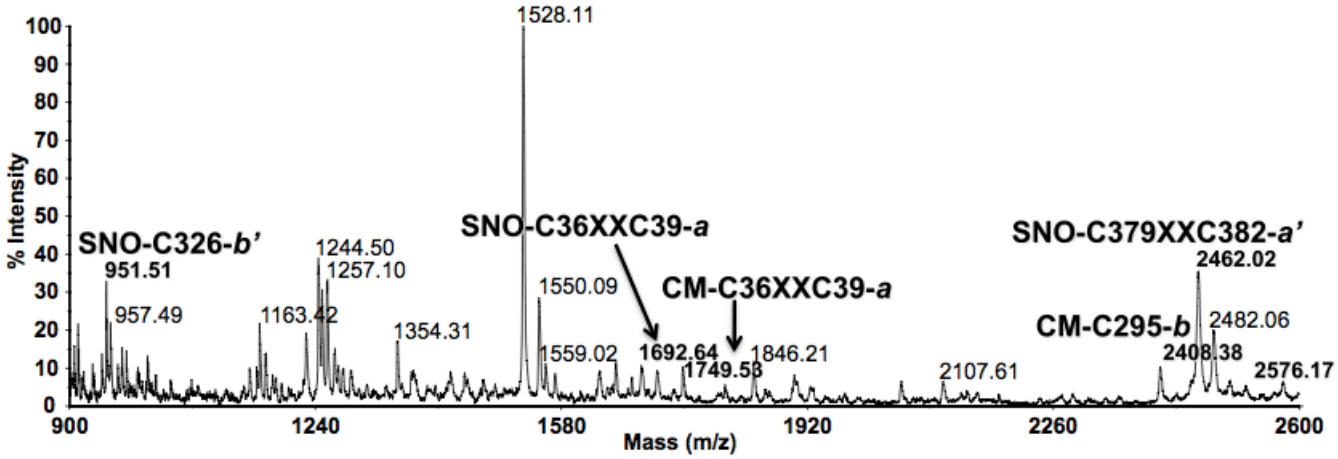

Figure S2. Mass fingerprint of reduced, *S*-nitrosylated, CM-labeled, Endoproteinase Glu-C digested PDI (no AuNPs). Modified thiol-containing peptides are highlighted, their peptide masses in bold.

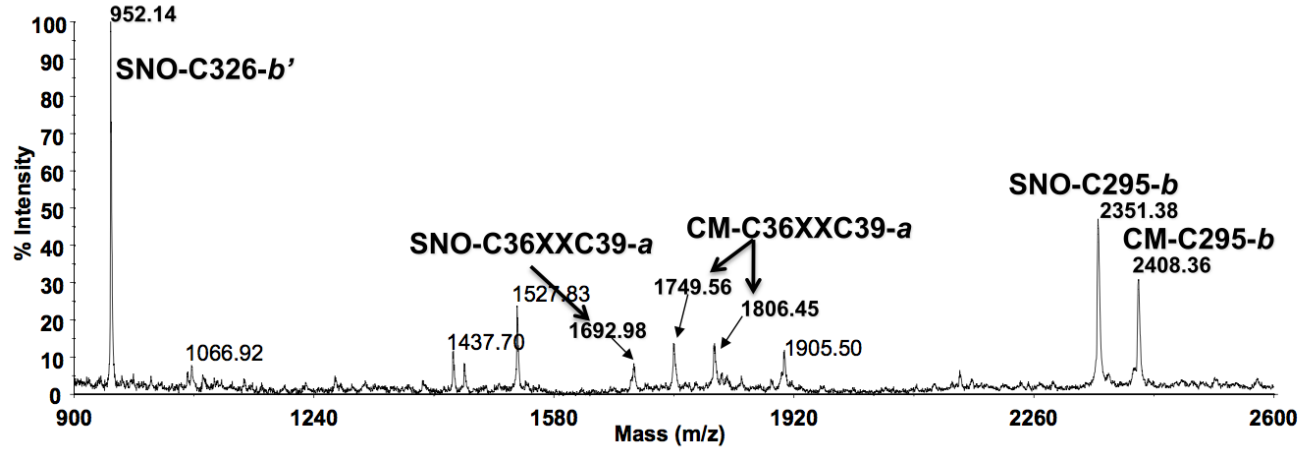

Figure S3. Mass fingerprint of reduced, *S*-nitrosylated, CM-labeled, Endoproteinase Glu-C digested, AuNP-bound PDI DTT elution. Modified thiol-containing peptides are highlighted, their peptide masses in bold.

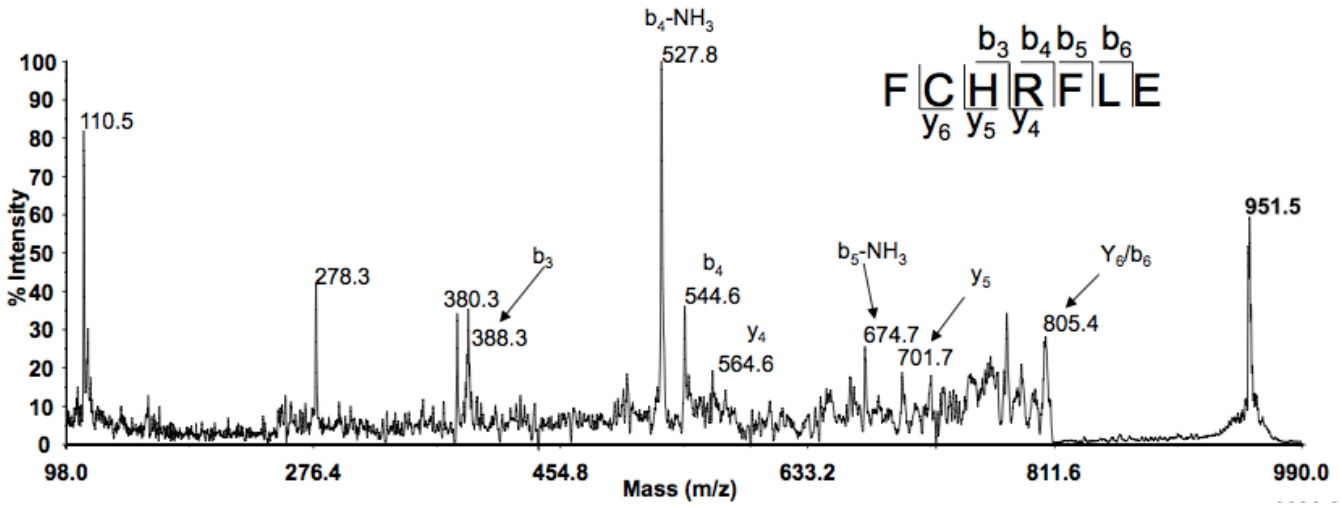

Figure S4. MS/MS analysis of PDI ***b'*** domain parent ion peak *m/z* 951.5 from reduced, *S*-nitrosylated, CM-labeled, Endoproteinase Glu-C digested, AuNP-bound DTT elution sample.

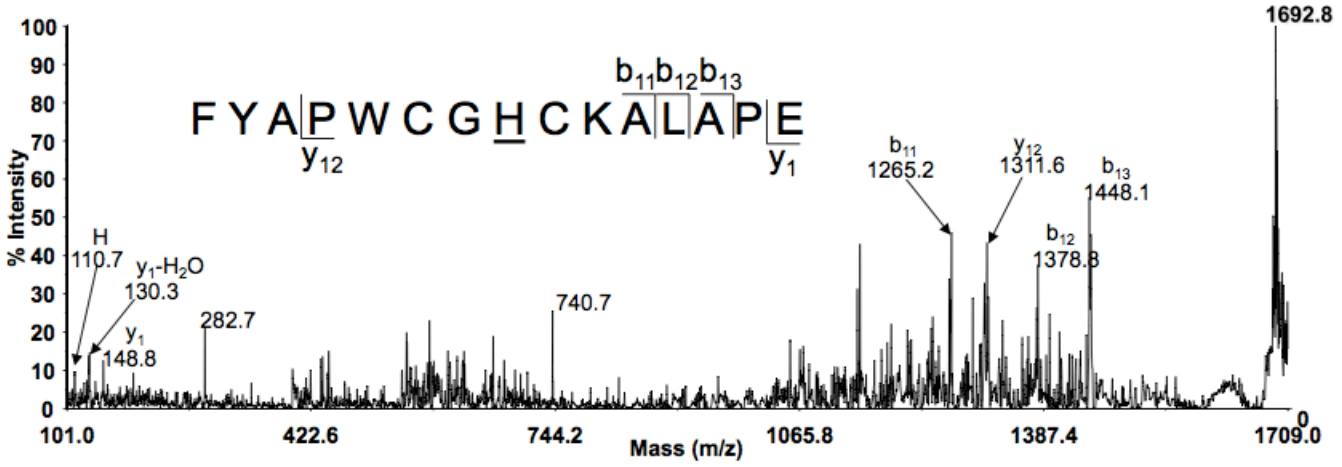

Figure S5. MS/MS analysis of PDI ***a*** domain parent ion peak *m/z* 1692.8 from reduced, *S*-nitrosylated, CM-labeled, Endoproteinase Glu-C digested, AuNP-bound DTT elution sample.

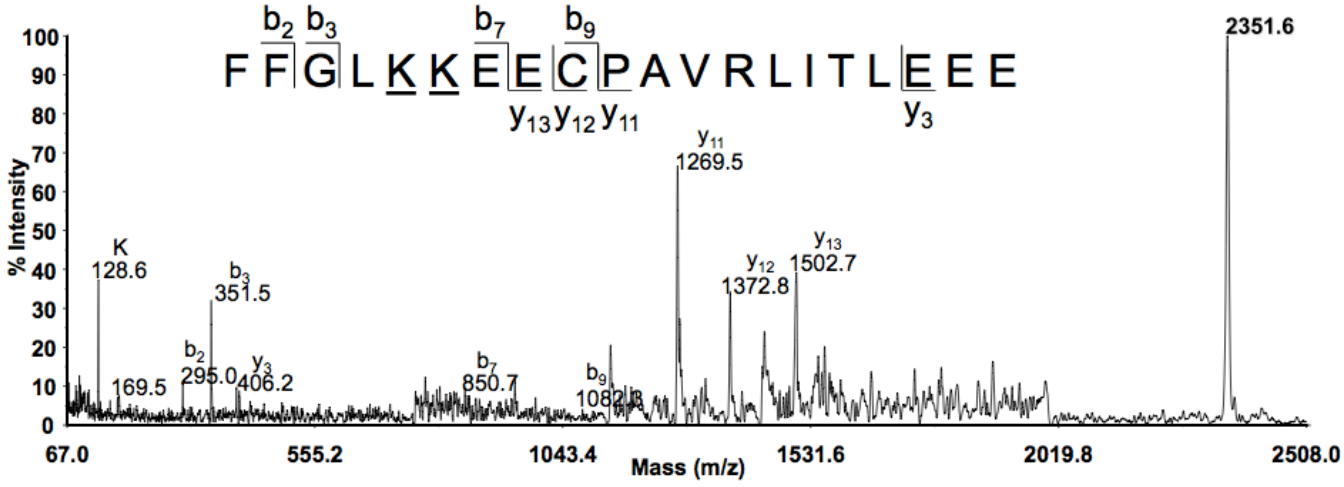

Figure S6. MS/MS analysis of PDI ***b*** domain parent ion peak *m/z* 2351.6 from reduced, *S*-nitrosylated, CM-labeled, Endoproteinase Glu-C digested, AuNP-bound DTT elution sample.

Residue	Sequence	Monoisotopic (*m/z*)	IAM (+ 57 Da)	NEM (+ 125 Da)	GSSG (+ 305 Da)
102 – 110	**C**VAFIGQAR	964.5	1021.5	1089.6	1269.5
115 – 126	AVLVH**C**HAGVSR	1248.7	1305.7	1373.7	1553.7
302 – 316	LGSFNWYGEQ**C**S**C**GR	1706.7	1763.7/1820.7	1831.8/1956.8	2011.7/2316.7
1 – 25	GSPEFMLEAPGPSDG**C**ELSNPSASR	2535.1	2592.1	2660.2	2840.1
26 – 54	VS**C**AGQMLEVQPGLYFGGAAAVAEPDHLR	2986.5	3043.5	3111.5	3291.5

Table S2. Human YVH1 thiol-containing peptides. Mass fingerprint reference table of target thiol-containing peptides from recombinant protein digest using Trypsin. Monoisotopic peptide *m/z* 1706.7 is capable of a mixture of modifications dependent on the experimental set-up.

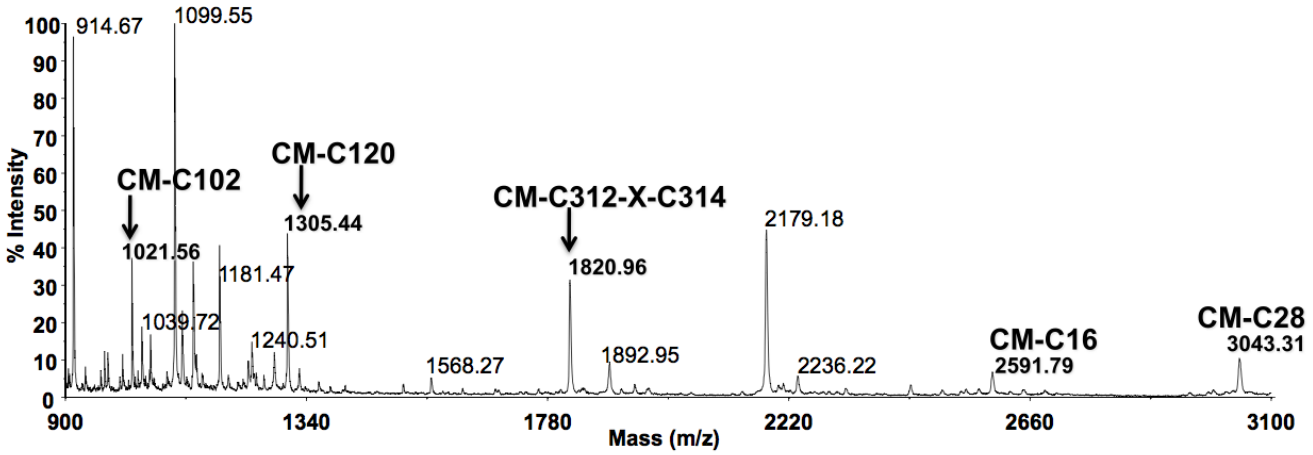

Figure S7. Mass fingerprint of reduced, CM-labeled, Trypsin digested hYVH1 (no AuNPs). Modified thiol-containing peptides are highlighted, their peptide masses in bold.

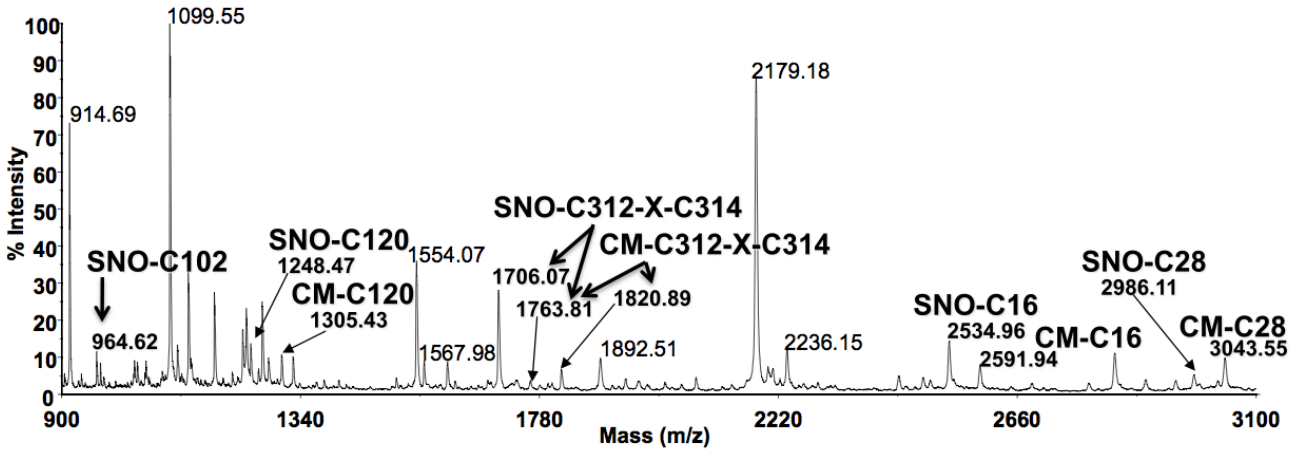

Figure S8. Mass fingerprint of reduced, *S*-nitrosylated, CM-labeled, Trypsin digested hYVH1 (no AuNPs). Modified thiol-containing peptides are highlighted, their peptide masses in bold.

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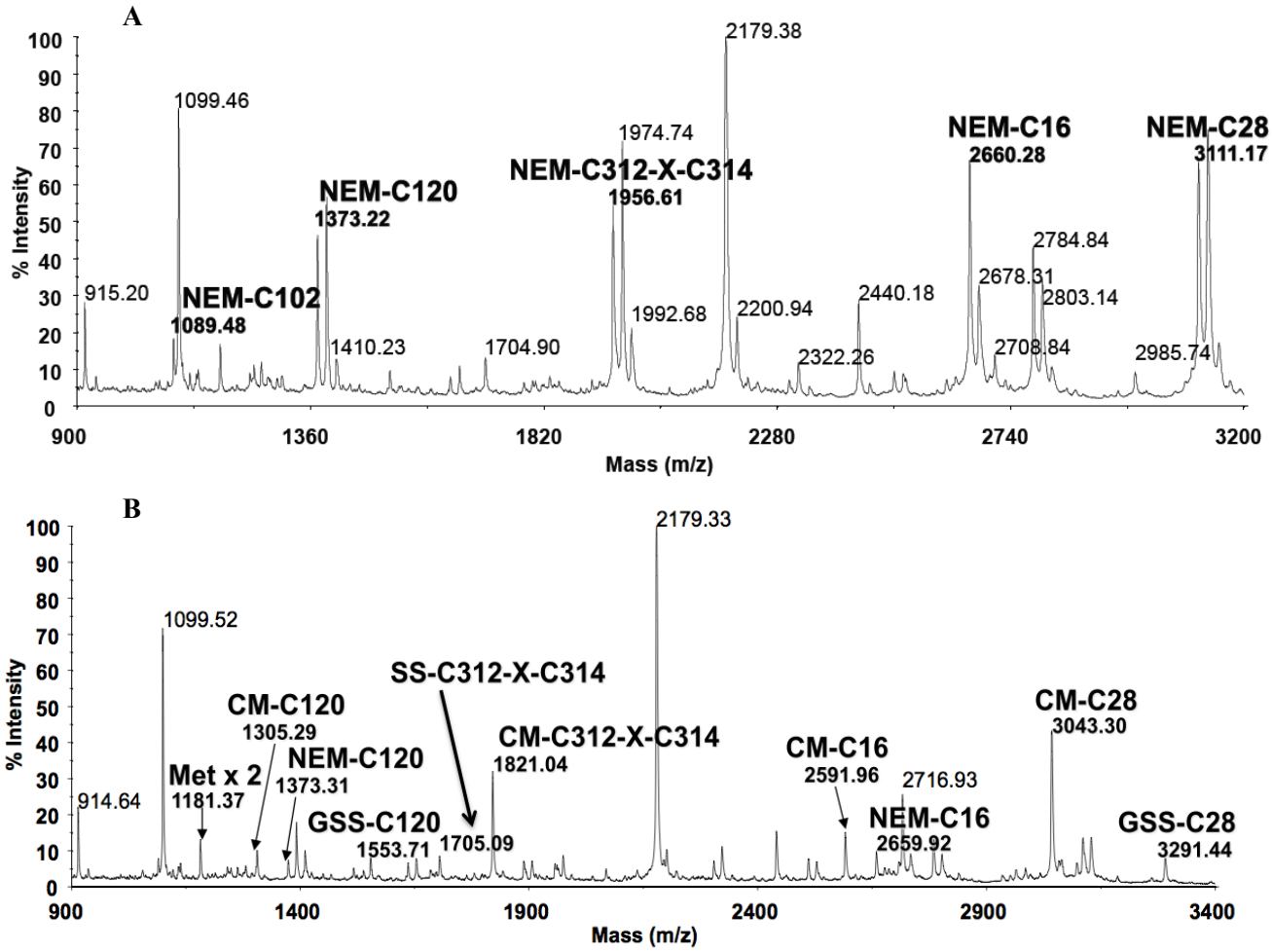

Figure S9. (***A***) Mass fingerprint of reduced, NEM-labeled, Trypsin digested hYVH1 (no AuNPs) (***B***) Mass fingerprint of reduced, *S*-nitrosylated, CM-labeled, denitrosylated, NEM-labeled, Trypsin digested hYVH1 (no AuNPs). Modified thiol-containing peptides are highlighted, their peptide masses in bold.

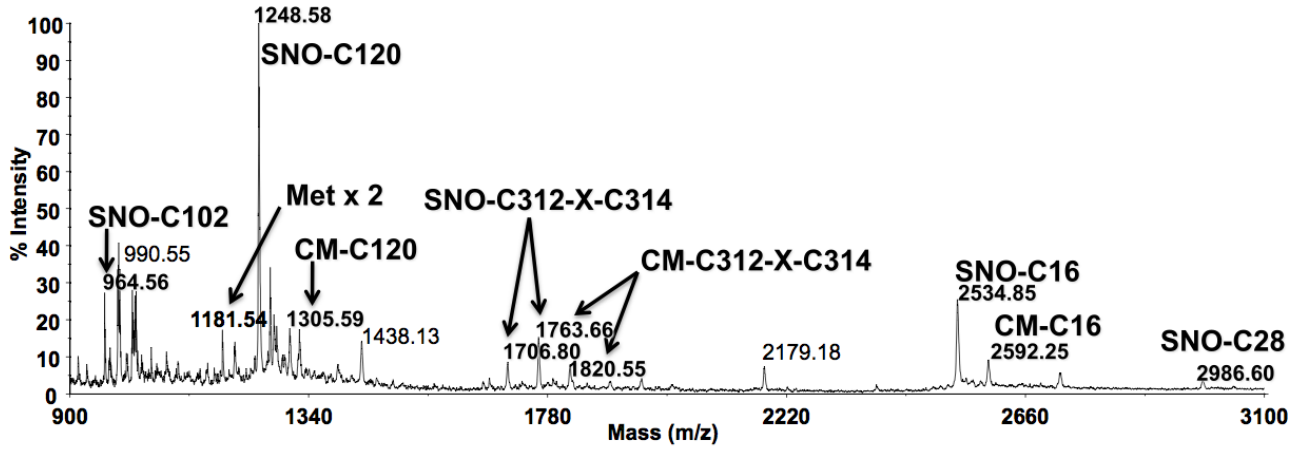

Figure S10. Mass fingerprint of reduced, *S*-nitrosylated, CM-labeled, Trypsin digested, AuNP-bound hYVH1 DTT elution. Modified thiol-containing peptides are highlighted, their peptide masses in bold.

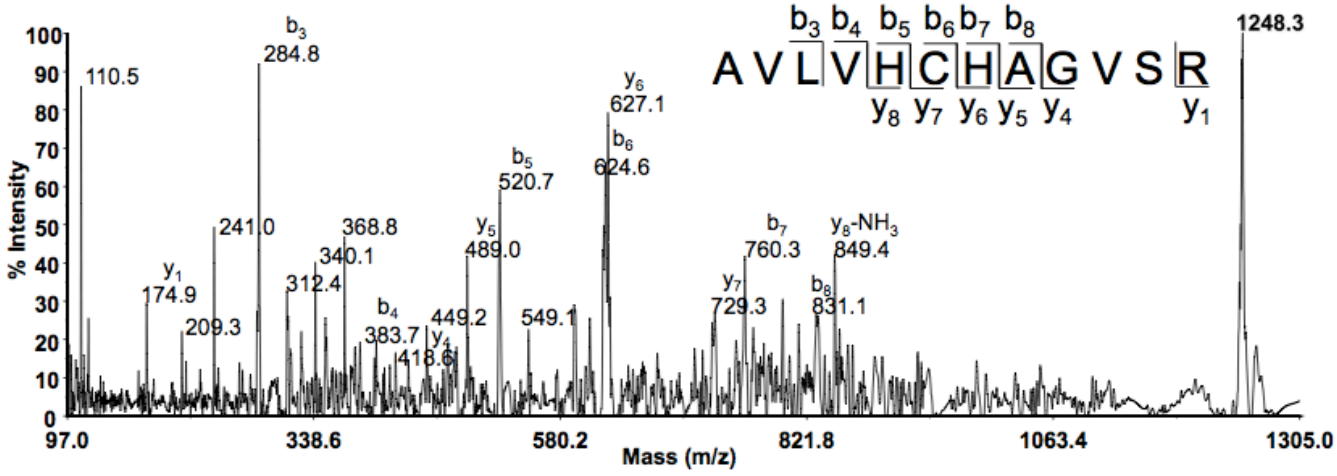

Figure S11. MS/MS analysis of hYVH1 **active site** parent ion peak *m/z* 1248.3 from reduced, *S*-nitrosylated, CM-labeled, Trypsin digested, AuNP-bound DTT elution sample.

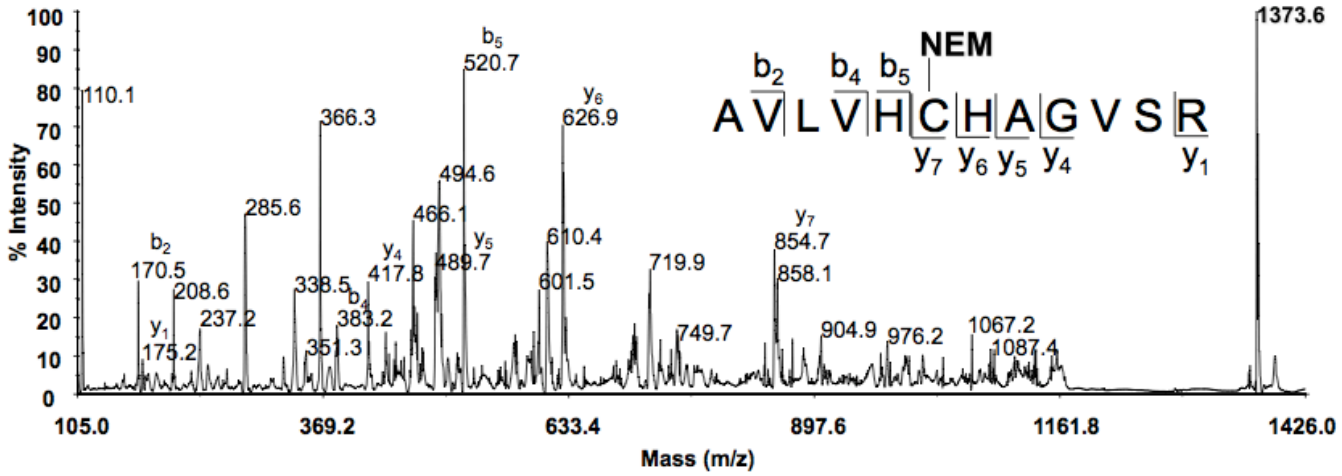

Figure S12. MS/MS analysis of hYVH1 **active site** parent ion peak *m/z* 1373.6 from reduced, *S*-nitrosylated, CM-labeled, denitrosylated, NEM-labeled, Trypsin digested, AuNP-bound DTT elution sample.

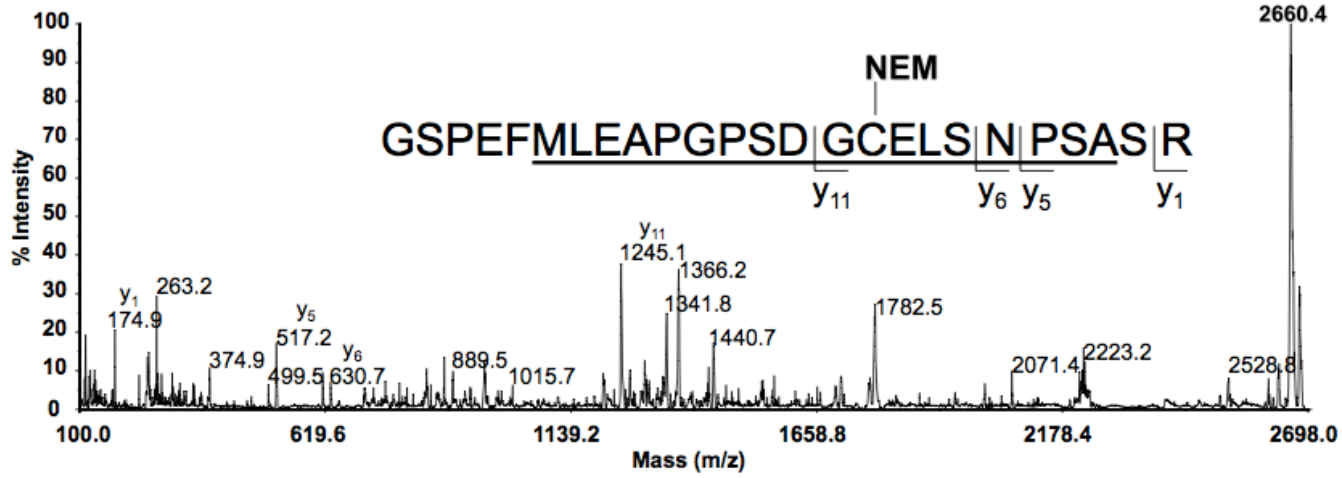

Figure S13. MS/MS analysis of hYVH1 parent ion peak *m/z* 2660.4 from reduced, *S*-nitrosylated, CM-labeled, denitrosylated, NEM-labeled, Trypsin digested, AuNP-bound DTT elution sample.

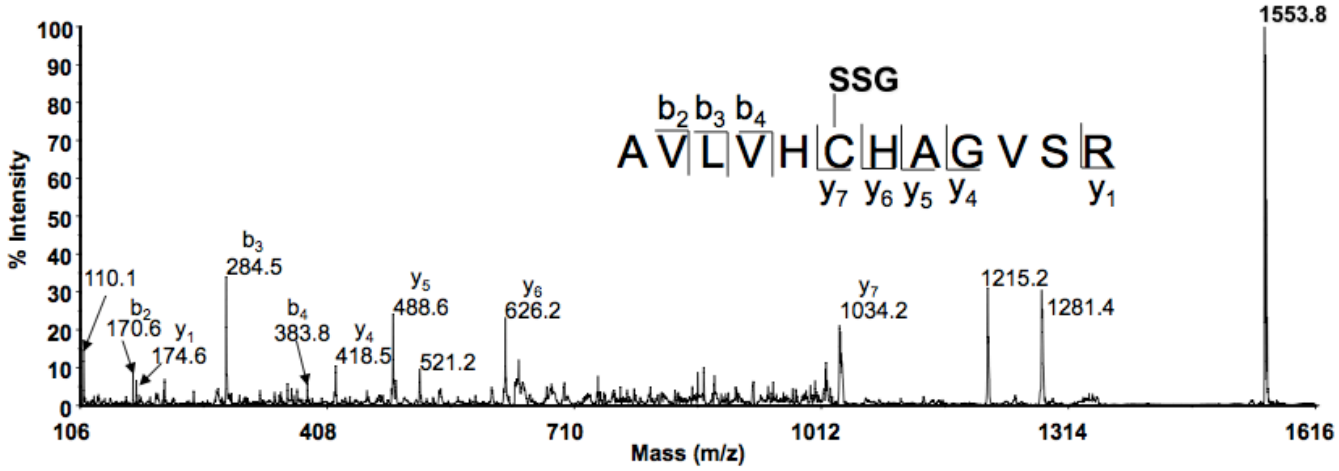

Figure S14. MS/MS analysis of hYVH1 **active site** parent ion peak *m/z* 1553.8 from reduced, *S*-glutathionylated, CM-labeled, Trypsin digested, pre-AuNP-bound sample.

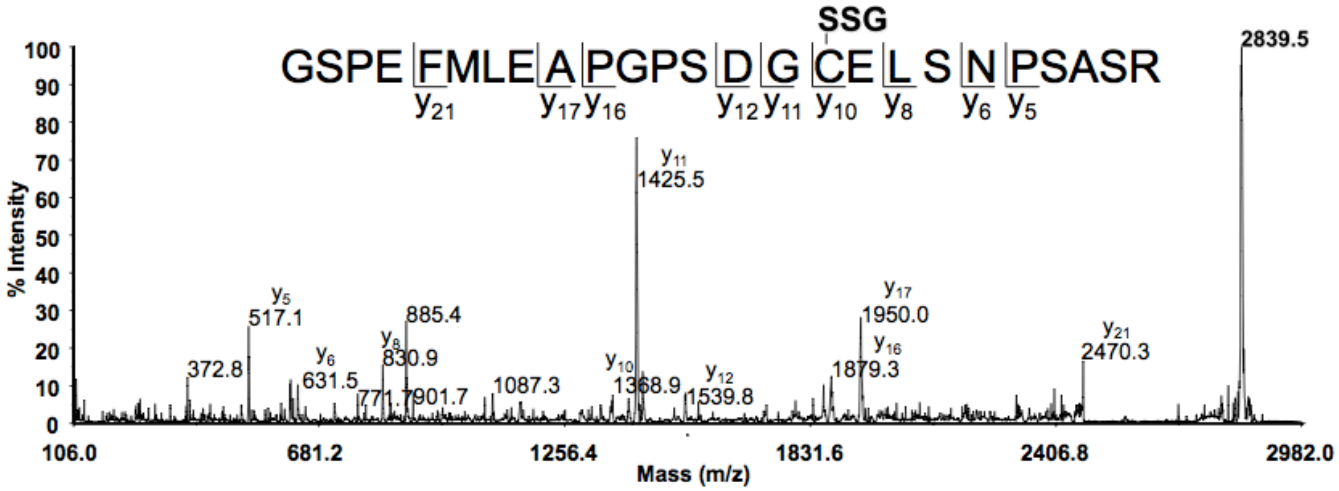

Figure S15. MS/MS analysis of hYVH1 parent ion peak *m/z* 2839.5 from reduced, *S*-glutathionylated, CM-labeled, Trypsin digested, pre-AuNP-bound sample.