Supporting Information

Chemical Methods for the Detection of Protein N-Homocysteinylation via Selective Reactions with Aldehydes

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Materials and General Methods. All aqueous solutions were prepared in water purified by a Milli-Q purification system (Millipore, Billerica, MA). The pH measurements were made on an Accumet Basic meter, model AB15 (Thermo Fisher Scientific, Pittsburgh, PA), using a Semi-micro PerpHecT ROSS combination pH probe, model 8203BN (Thermo Fisher Scientific). The pH for solutions less than 1 mL was estimated using pH indicator strips with a pH 2.5-4.5 or 1-6 range with 0.5 pH unit accuracy (EMD Chemicals Inc., Gibbstown, NJ). Coomassie G-250, 10 X Tris/Glycine/SDS buffer, and ready Tris-HCl gels were from Bio-Rad (Hercules, CA). EZ-Run protein marker was from Thermo Fisher Scientific (Pittsburgh, PA). SDS-PAGE (2X) protein loading buffer was from National Diagnostic (Atlanta, Georgia). Peptide stock solutions were prepared in water, and the concentrations were determined by UV absorption at 280 nm using the extinction coefficient 1,490 M^{-1} cm^{-1} for tyrosine and 5,500 M^{-1} cm^{-1} for tryptophan, estimated by ProtParam on ExPASy (www.expasy.org/tools/protparam.html).[1, 2]

LCQ Analysis. LC-MS data for protein analysis was obtained from a Surveyor HPLC system (Thermo Fisher, Waltham, MA) coupled to an LCQ Ion Trap mass spectrometer (Thermo Fisher, Waltham, MA) equipped with a PicoView ESI sources (New Objective, Woburn, MA). Approximately 2 pmol of sample was loaded on a self-packed reversed phase column (75 μm i.d. × 10 cm, Magic C4 resin, 5 μm particle size, 300 Å pore size, Michrom Bioresources, Auburn, CA) and eluted at 200 nL/min. A gradient of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) was performed by starting at 1% mobile phase B and increasing to 30% mobile phase B over 5 min, then increasing to 55% mobile phase B over 25 min, and finally increasing to 90% B in 5 min. Before the next sample was injected, the column was equilibrated and desalted at 1% mobile phase B for 20 min. The

ion transfer tube of the linear ion trap was held at 250 °C and the ion spray voltage was set at 2.3 kV. The mass spectrometer altered between a full MS scan (m/z 400-2000).

MALDI Mass Spectrometry. Peptide Samples were desalted prior to MALDI analysis using C18 standard bed Zip Tips (Millipore, Billerica, MA) unless otherwise noted. Peptide samples (0.5 μL) were spotted on a polished standard 192-well stainless steel MALDI sample plate followed by the addition of α-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution (0.4 μL, 10 mg/mL in a mixture of acetonitrile/water/TFA, v:v:v, 50:50:0.05). The resulting mixtures were air-dried and analyzed using an AB 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Framingham, MA). MS and MS/MS spectra were acquired in reflector positive mode. Human angiotensin I ($[M + H]^+$) 1296.67), was used for the external calibration and the data were analyzed using Data Explorer software 4.6.

1. Preparation of N-Homocysteinylated Neurotensin

Neurotensin (Neu, 150 μM) was modified with D,L-Hcy TL hydrochloride (50 mM, Thermo Fisher Scientific) in 0.2 M ammonium bicarbonate, pH 8, at 37 ºC for 12-16 hr. After incubation, the concentration of modified Neu was measured at 280 nm using the extinction coefficient of 2,980 M^{-1} cm^{-1}. Modified Neu was diluted to 10 μM by a mixture of 50 % (v/v) acetonitrile and 0.05% (v/v) TFA, and analyzed by MALDI-TOF/TOF MS.

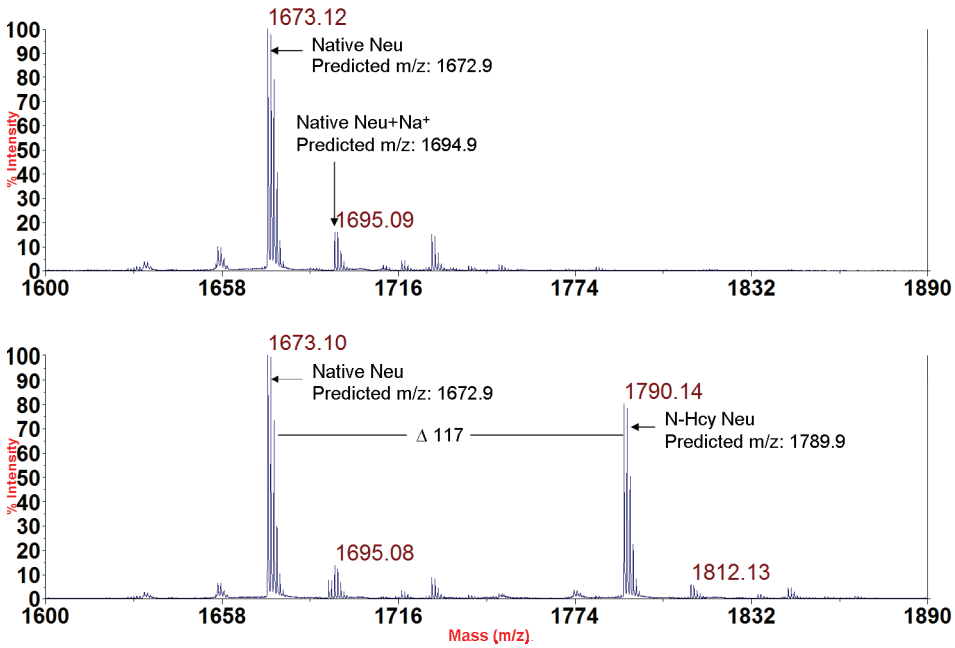


Figure S1.1. MALDI MS spectra of native neurotensin (top) and neurotensin treated with homocysteine thiolactone (bottom) showing the treated sample contained about 50% N-homocysteinylated (N-Hcy) neurotensin.

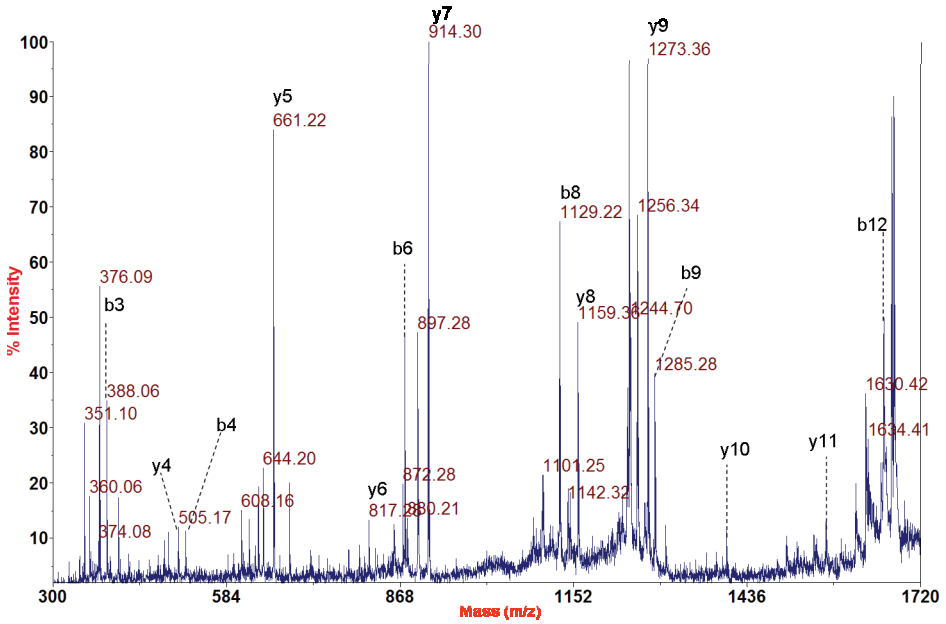


Figure S1.2. MALDI-TOF/TOF-MS/MS spectrum of 1790.14 (Predicted: 1789.9) $[M+H]^+$ precursor ion corresponding to N-Hcy neurotensin. The observation of b6 and y8 confirms the modified position at the lysine residue.

Table S1.1. Observed b and y ions of N-Hcy Neu sequence.

b ion	Predicted	Observed		y ion	Predicted	Observed
b1	---	---	Pyro E	y13	---	---
b2	225.12	---	L	y12	1678.91	
b3	388.19	388.06	Y	y11	1565.83	1565.4
b4	517.23	517.08	E	y10	1402.76	1402.35
b5	631.27	---	N	y9	1273.72	1273.36
b6	876.39	876.14	K*	y8	1159.68	1159.37
b7	973.44	---	P	y7	914.56	914.3
b8	1129.55	1129.22	R	y6	817.5	817.28
b9	1285.65	1285.28	R	y5	661.4	661.22
b10	1382.7	---	P	y4	505.3	505.17
b11	1545.76	---	Y	y3	408.25	---
b12	1658.85	1659.41	I	y2	245.19	---
b13	---	---	L	y1	132.1	---

1. K*: modified lysine.

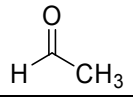
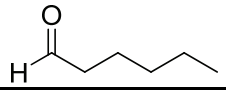
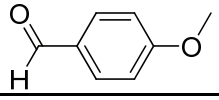
2. ---: not detectable.

2. Thiazine Adduct Formation of N-Homocysteinylated Neurotensin

Hcy TL modified neurotensin (10 μM or 100 μM, total peptide concentration) without desalting was treated with 10 equivalents of aldehydes (100 μM or 1 mM) in 100 mM ammonium bicarbonate pH 8, at 25 ºC for 15 -19 hr. Thiazine adducts were analyzed by MALDI-TOF/TOF MS.

Three aldehydes, two aliphatic and one aromatic in Table S2.1 were chosen to modify the N-homocysteinylated neurotensin. After treatment, the corresponding thiazine adducts were analyzed by mass spectrometry. Mass spectrometric analysis of the three thiazine adducts showed that thiazines were formed with the N-homocystamide group.

Table S2.1. Aldehydes for thiazine adduct formation.

Name	Molecular Formula	MW (Da)	CAS Number	Structure
Acetaldehyde	C_2H_4O	44.05	75-07-0	
Hexanal	$C_6H_{12}O$	100.16	66-25-1	
p-Anisaldehyde	$C_8H_8O_2$	136.15	123-11-5	

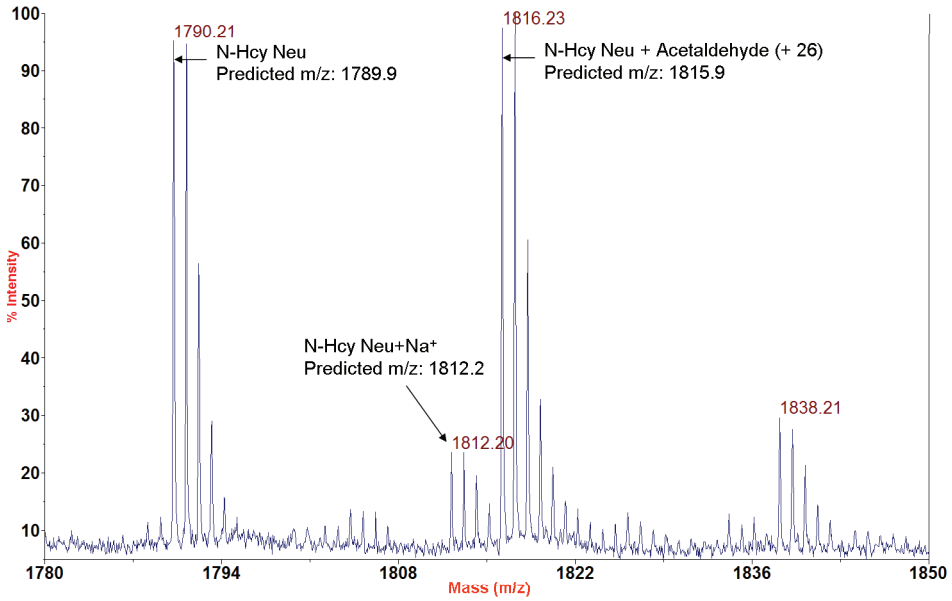


Figure S2.1. MALDI MS spectrum of acetaldehyde treated N-homocysteinylated Neu.

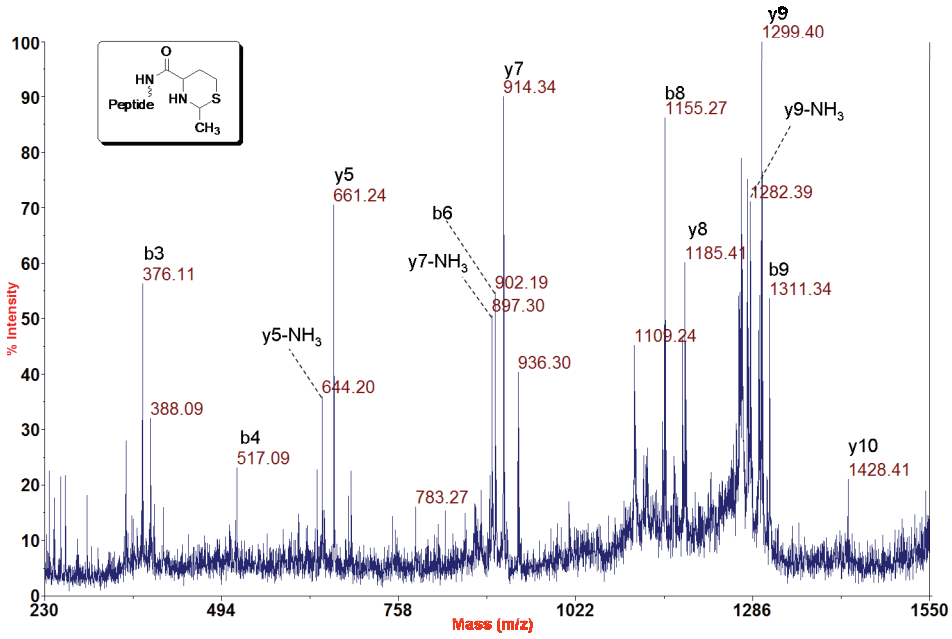


Figure S2.2. MALDI-TOF/TOF MS/MS spectrum of thiazine adduct from acetaldehyde treated N-Hcy Neu. The precursor ion was chosen 1816.2 $[M+H]^+$ corresponding to the thiazine adduct. The observation of b6 and y8 confirm the modified position at the N-homocysteine group. The structure of thiazine adduct is shown in the insert.

Table S2.2. Observed b and y ions of N-Hcy Neu thiazine adduct from acetaldehyde.

b ion	Predicted	Observed		y ion	Predicted	Observed
b1	---	---	Pyro E	y13	---	---
b2	225.12	---	L	y12	1704.93	---
b3	388.19	388.09	Y	y11	1591.84	---
b4	517.23	517.09	E	y10	1428.78	1428.41
b5	631.27	---	N	y9	1299.74	1299.4
b6	902.41	902.19	K*	y8	1185.69	1185.41
b7	999.46	---	P	y7	914.56	914.34
b8	1155.56	1155.27	R	y6	817.5	---
b9	1311.66	---	R	y5	661.4	661.24
b10	1408.72	---	P	y4	505.3	---
b11	1571.78	---	Y	y3	408.25	---
b12	1684.86	---	I	y2	245.19	---
b13	---	---	L	y1	132.1	---

1. K*: thiazine adduct formed on lysine residue.

2. ---: not detectable.

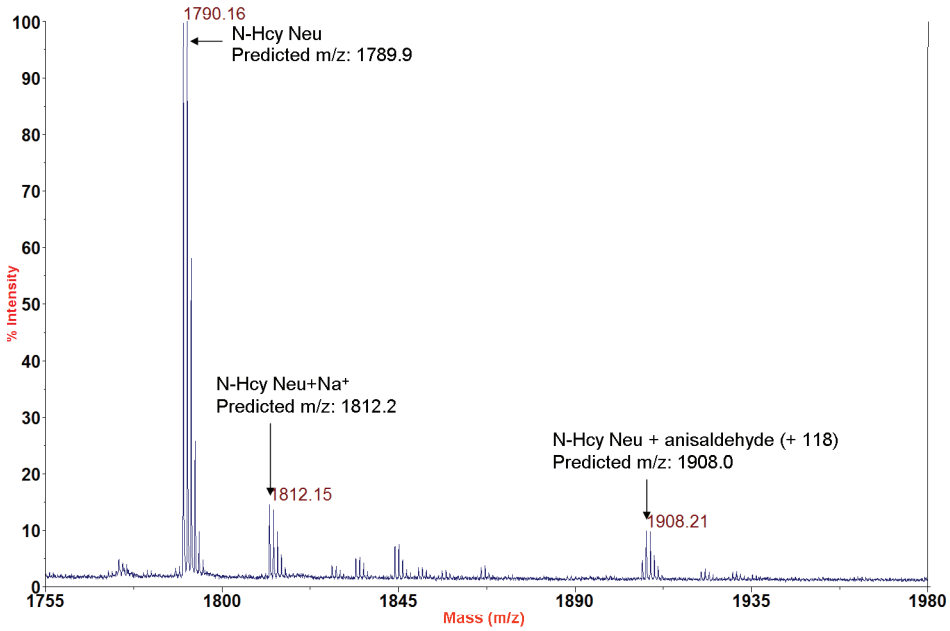


Figure S2.3. MALDI MS spectrum of anisaldehyde treated N-homocysteinylated Neu.

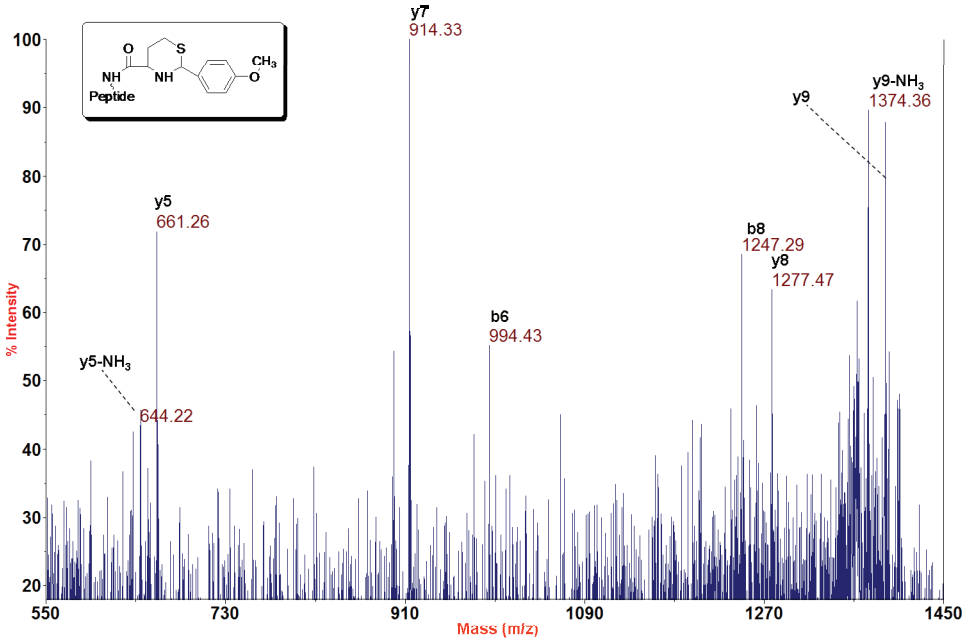


Figure S2.4. MALDI-TOF/TOF MS/MS spectrum of thiazine adduct from anisaldehyde treated N-Hcy Neu. The precursor ion was chosen 1913.2 (predicted: 1908.0) $[M+H]^+$ corresponding to the thiazine adduct. The observation of b6 and y8 confirms the modified position at the N-homocysteine group. The structure of thiazine adduct is shown in the insert.

Table S2.3. Observed b and y ions of N-Hcy Neu thiazine adduct from anisaldehyde treatment.

b ion	Predicted	Observed		y ion	Predicted	Observed
b1	---	---	Pyro E	y13	---	---
b2	225.12	---	L	y12	1796.95	---
b3	388.19	---	Y	y11	1683.87	---
b4	517.23	---	E	y10	1520.80	---
b5	631.27	---	N	y9	1391.76	1391.43
b6	994.43	994.43	K*	y8	1277.72	1277.47
b7	1091.49	---	P	y7	914.56	914.33
b8	1247.59	1247.29	R	y6	817.50	---
b9	1403.69	---	R	y5	661.40	661.26
b10	1500.74	---	P	y4	505.30	---
b11	1663.81	---	Y	y3	408.25	---
b12	1776.89	---	I	y2	245.19	---
b13	---	---	L	y1	132.10	---

1. K*: thiazine adduct formed on lysine residue.

2. ---: not detectable.

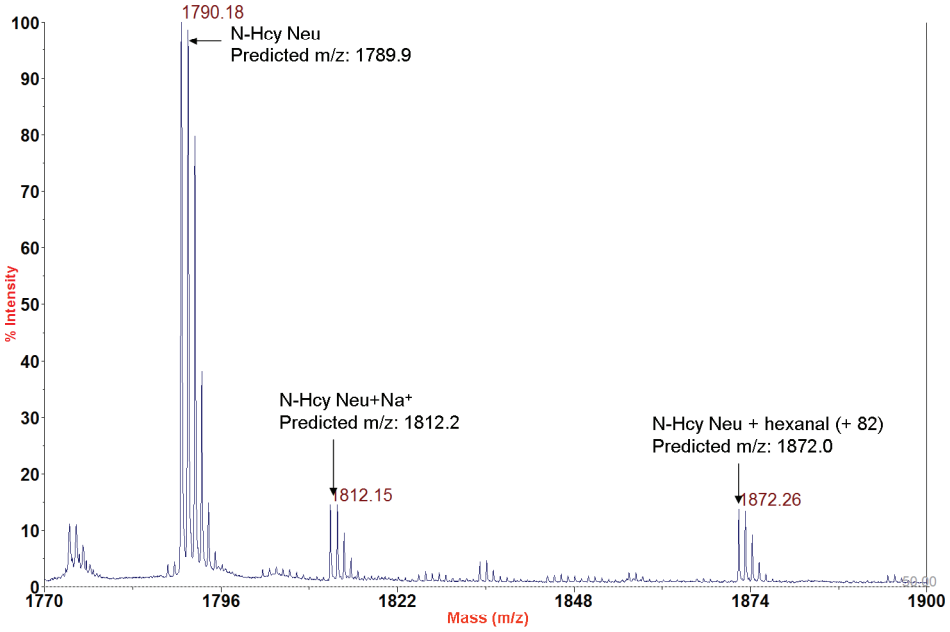


Figure S2.5. MALDI MS spectrum of hexanal treated N-homocysteinylated Neu.

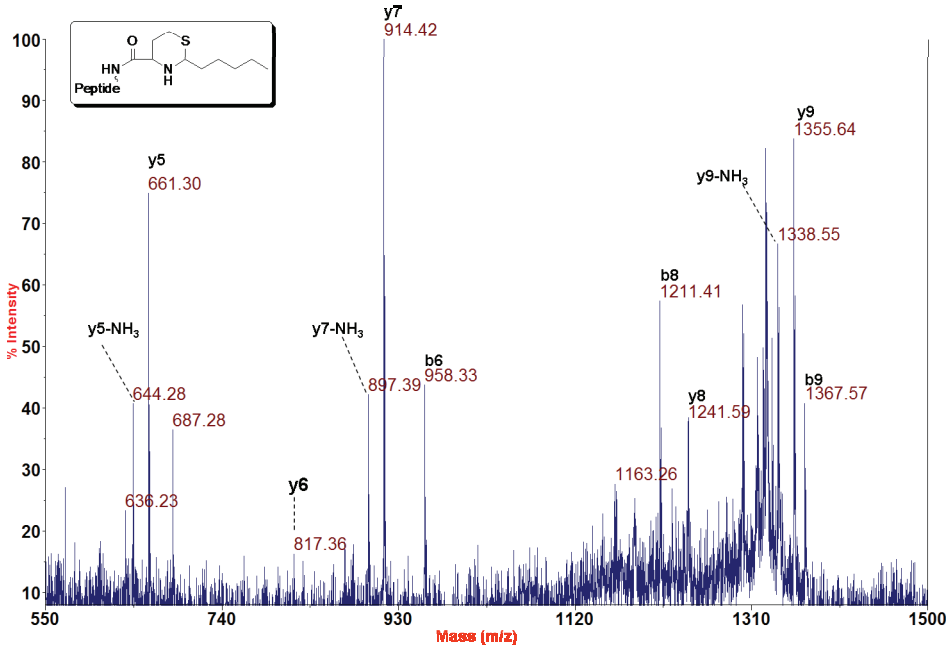


Figure S2.6. MALDI-TOF/TOF MS/MS spectrum of thiazine adduct from anisaldehyde treated N-Hcy Neu. Precursor ion was chosen 1872.2 (predicted: 1872.0) $[M+H]^+$ corresponding to the thiazine adduct. The observation of b6 and y8 confirm the modified position at the N-homocysteine group. The structure of thiazine adduct is shown in the insert.

Table S2.3. Observed b and y ions of N-Hcy Neu thiazine adduct from hexanal.

b ion	Predicted	Observed		y ion	Predicted	Observed
b1	---	---	Pyro E	y13	---	---
b2	225.12	---	L	y12	1760.99	---
b3	388.19	---	Y	y11	1647.90	---
b4	517.23	---	E	y10	1484.84	---
b5	631.27	---	N	y9	1355.80	1355.64
b6	958.47	958.33	K*	y8	1241.76	1241.59
b7	1055.52	---	P	y7	914.56	914.42
b8	1211.62	1211.41	R	y6	817.50	817.36
b9	1367.73	1367.57	R	y5	661.40	661.30
b10	1464.78	---	P	y4	505.30	---
b11	1627.84	---	Y	y3	408.25	---
b12	1740.93	---	I	y2	245.19	---
b13	---	---	L	y1	132.10	---

1. K*: thiazine adduct formed on lysine residue.

2. ---: not detectable.

3. Analysis of N-Homocysteinylated Myoglobin.

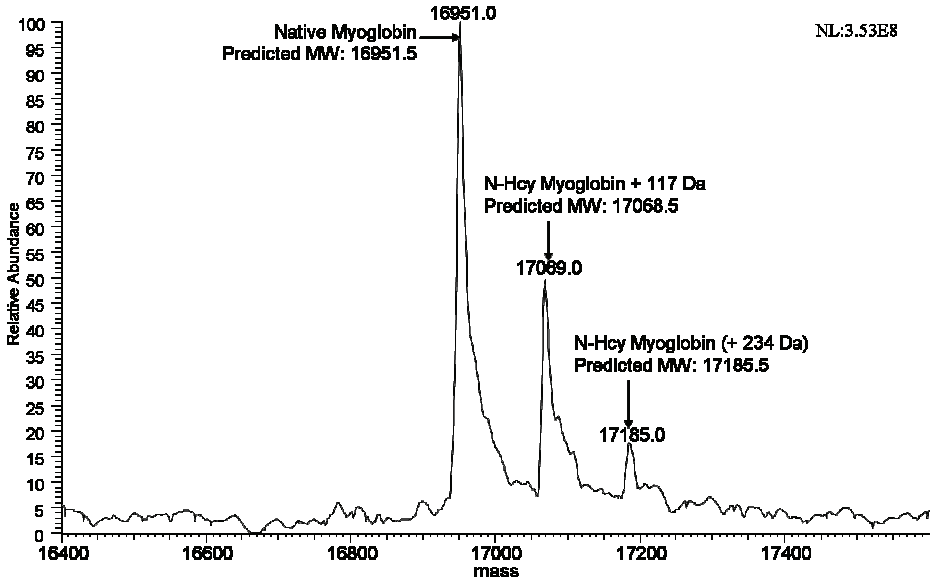


Figure S3.1. Mass spectrum of Hcy TL modified equine skeletal myoglobin.

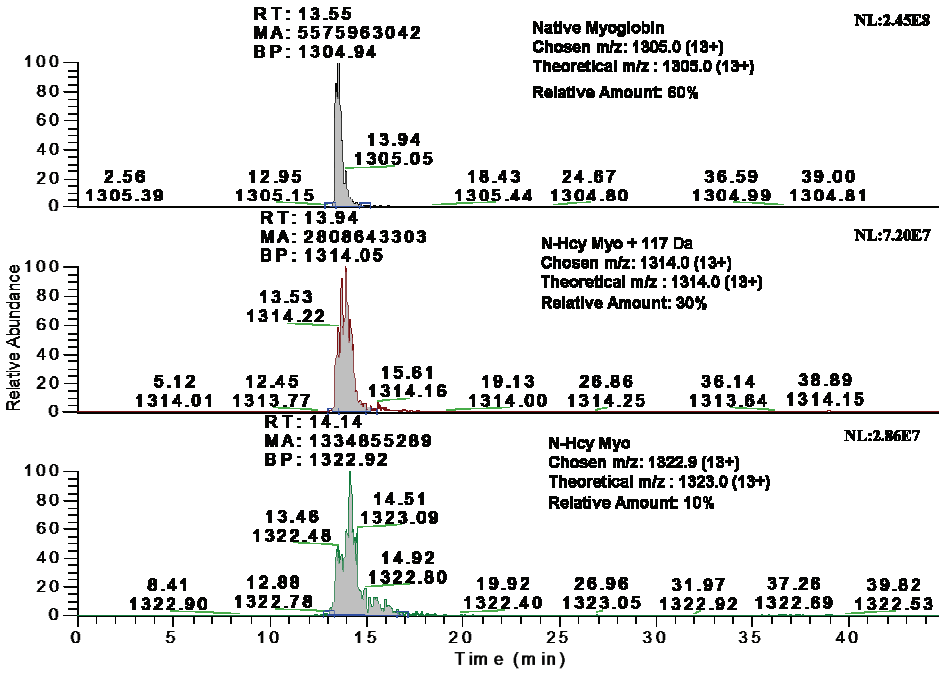


Figure S3.2. Extracted ion chromatograms (XICs) of native myoglobin and N-Hcy myoglobin. The relative amount of N-homocysteinylated myoglobin was about 40% after calculating the peak areas of different protein forms. RT: retention time; MA: peak area by manual integration; BP: base peak. NL: normalized intensity.

4. Quantification and Detection Limits of Rhodamine Labeling

The initial concentration of 25 μM (total protein concentration) Hcy TL modified myoglobin and its 2-fold series of diluted samples were incubated with 200 μM Rhodamine-aldehyde respectively, in 50 mM, pH 3.0 citric acid with 500 μM TCEP in dark room, at 25 ºC for 3 hr. In order to keep the same concentrations of myoglobin in the incubated solutions, the native myoglobin solution was added into the diluted samples to give the final concentration of 25 μM myoglobin and 70 μM LuxS was also incubated together as the negative control. After incubation, samples was separated by SDS-PAGE and analyzed by fluorescence following the procedure described in the Experimental Section. The calibration curve was made according to the fluorescence intensity versus the amount of N-Hcy myoglobin.

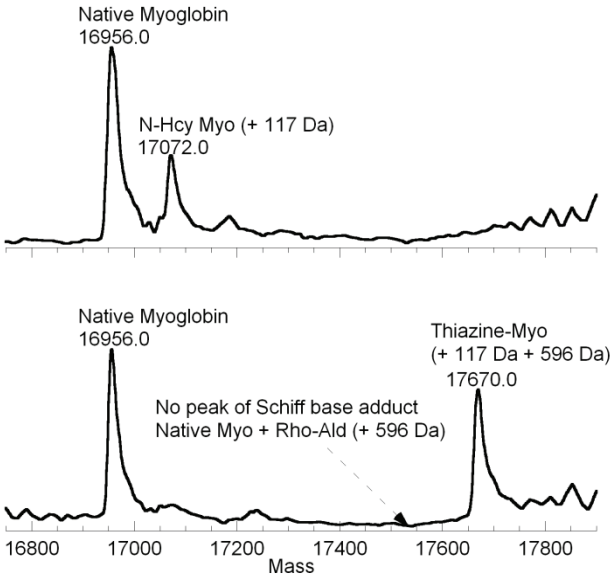


Figure S4.1. Mass spectra of native myoglobin (predicted MW: 16951.5) and N-Hcy myoglobin (predicted MW: 17069.7 (20 μM, total concentration) before (top) and after (bottom) treatment with Rhodamine-aldehyde (400 μM) at pH 3. The predicted MW for the thiazine adduct of N-Hcy myoglobin is 17665.4 and for Schiff base of the native form is 17548.2 (not observed).

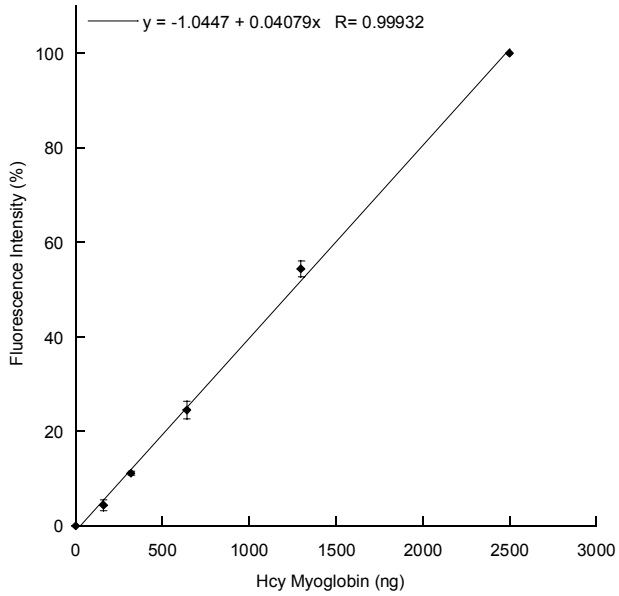


Figure S4.2. Linear correlation between the amount of N-Hcy myoglobin and fluorescence intensity. Shown are mean ± SE for triplicates at five concentrations (160 ng, 320 ng, 640 ng, 1.3 µg and 2.5 µg) and the line of best fit determined by linear regression (r^2=0.999, relative standard deviation (RSD): 3%-25%).

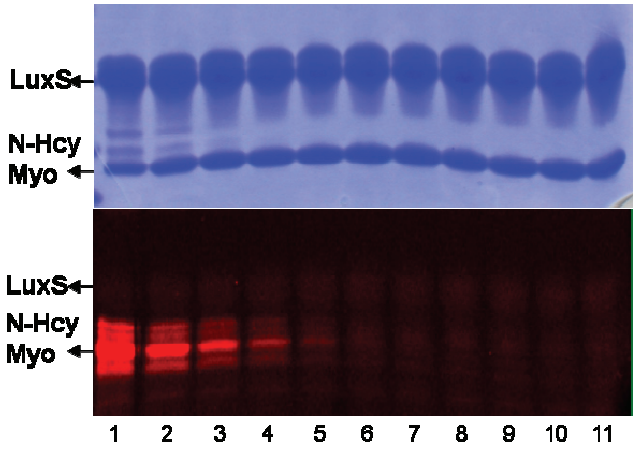


Figure S4.3. SDS-PAGE Coomassie staining image (top) and fluorescent image (bottom) for LuxS, N-Hcy and native myoglobin. The experiment was repeated three times. Amount of Hcy myoglobin in lane 1: 2.5 µg, lane 2: 1.3 µg, lane 3: 640 ng, lane 4: 320 ng, lane 5: 160 ng, lane 6: 80 ng, lane 7: 40 ng, lane 8: 20 ng, lane 9: 10 ng, lane 11: 5 ng.

5. Kinetics of 1,3-Thiazine Formation

N-Hcy myoglobin (50 μM total protein concentration, including 40% Hcy myoglobin) was incubated with 25 μM, 50 μM and100 μM, Rhodamine-aldehyde in 50 mM, pH 3.0 citric acid with 500 μM TCEP in the dark, at 25 ºC respectively. Aliquots of 5 μL were taken after 1 hr, 3 hrs and 8 hrs incubation and analyzed by LC/MS. The relative amount of Rhodamine labeling N-Hcy myoglobin versus incubation time (hr) was analyzed.

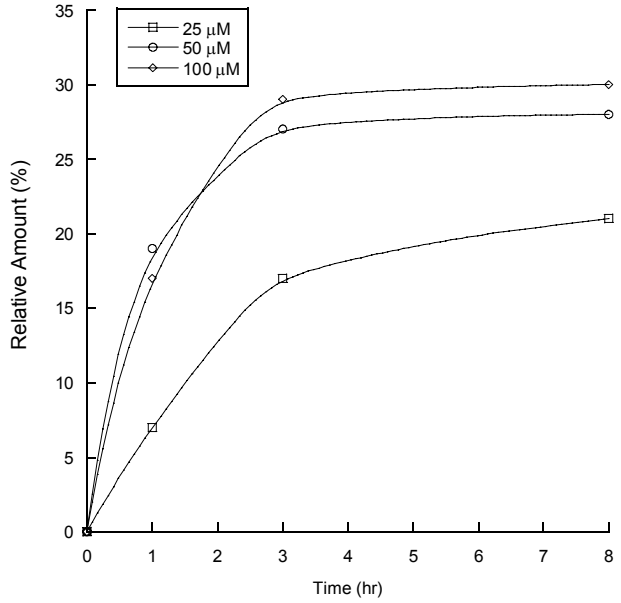


Figure S5.1. Time course plots of the Rhodamine-aldehyde labeling reaction at different concentrations of aldehyde. The relative amount was calculated by the percentage of N-homocysteinylated myoglobin in the sum of peak area of all proteins forms from the extracted ion chromatogram (XIC).

6. Detection of N-Homocysteinylated Proteins in Human Plasma

Plasma proteins (8.4 mg/mL) and Hcy TL modified myoglobin at different concentrations were incubated with 250 μM Rhodamine-aldehyde in 200 mM citric acid, 2 mM TCEP, pH 3 in the dark, at 25 ºC for 3 hr. Native myoglobin was also added into each sample to keep a constant final concentration of myoglobin. After SDS-PAGE, gels were analyzed by fluorescence as described in the Experimental Section. Precast gel (12% or 4-15%) was used for SDS-PAGE.

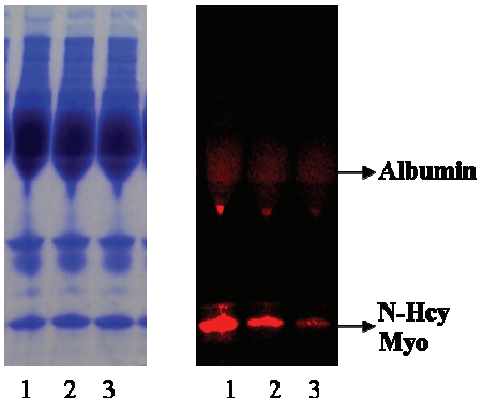


Figure S6.1. SDS-PAGE staining image (left) and fluorescence (right) image of Rhodamine-aldehyde labeling human plasma. Total plasma protein in each lane was 84 μg. N-Hcy myoglobin in lanes 1, 2 and 3 were 881, 427 and 210 ng, respectively.

7. Detection by Western Blotting and Chemiluminescence

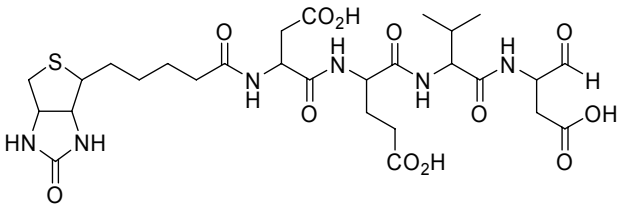
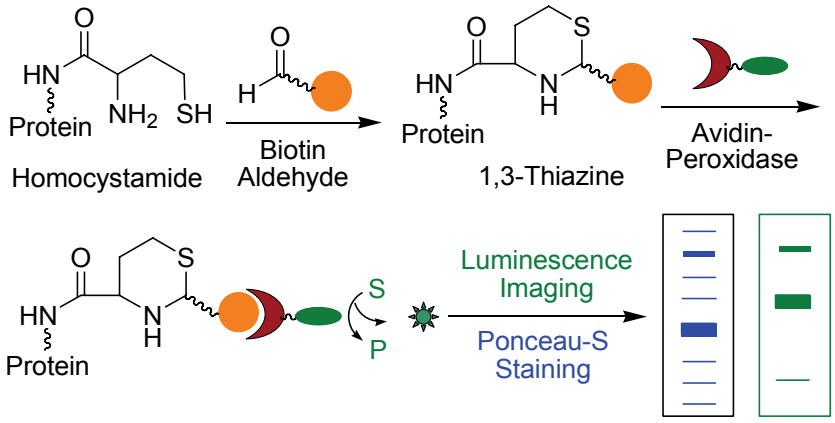


Figure S7.1. Structure of biotin aldehyde (CAS number: 178603-73-1).

Scheme S7.1. Western-blotting using biotin aldehyde and chemiluminescence assay for N-homocysteinylated proteins.



Detection Limit Assay of N-Hcy Myoglobin. Hcy TL modified myoglobin (32.5 µM. total concentration) was incubated with 200 µM biotin aldehyde (Figure S7.1) in 50 mM citric acid, 500 µM TCEP, pH 3 in the dark, at 25 ºC, for 14-16 hr. Then, 5-fold series of diluted samples were made and native myoglobin was added into every sample to keep total myoglobin at 32.5 µM. Then, 10 µL aliquot samples were mixed with 2 x SDS loading buffer (10 µL) and incubated in boiling water for 3 min. Heat treated samples were loaded into precast gel (12%) for SDS-PAGE separation. Western blotting and chemiluminescence protocols were performed as described in the Experimental Section. Interestingly, minor bands appeared with an apparent molecular weight of a myoglobin dimer.

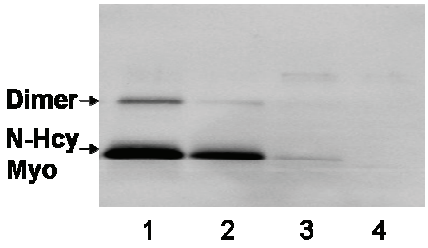


Figure S7.2. Chemiluminescent gel image of Hcy TL modified myoglobin samples. Amounts of N-Hcy myoglobin in lane 1, 2, 3 and 4 were: 80, 40, 20 and 10 ng, respectively.

Detection of N-Hcy proteins in Mice Plasma. Mice plasma was kindly provided by Joseph Loscalzo and Diane Handy at Brigham and Women's Hospital (Boston, MA). Mice plasma proteins (4 mg/mL) are incubated with 250 μM biotin aldehyde in 200 mM citric acid, 2 mM TCEP, pH 3 in the dark, at 25 ºC, for 5 hr. Then, aliquots of 20 μL were mixed with 2 x SDS loading buffer (20 μL) and incubated in boiling water for 5 min. Heat treated samples were loaded into precast gel (4-15%) for SDS-PAGE. Western blotting and chemiluminescence protocols were performed as described in the Experimental Section.

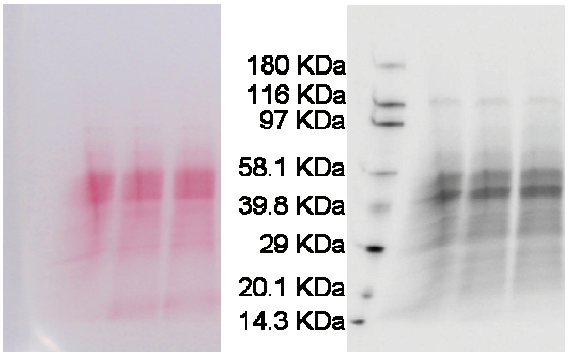


Figure S7.3. Ponceau-S staining (left) and chemiluminescent (right) images of mice plasma treated with biotin aldehyde. "M" denotes biotinylated protein markers. In image to the right, chemiluminescence intensity indicated the amount of N-Hcy proteins; in the image to the left, the color density from Ponceau-S staining indicated the total amount of protein. Mice plasma (80 μg) was loaded into each lane.

8. Affinity Enrichment of N-Homocysteinylated Peptides and Proteins

Enrichment of N-Hcy Neurotensin

Hcy TL modified neurotensin (24 μM) was incubated with 5 mg CPG aldehyde beads (Millipore, Billerica, MA) in 50 μL binding solution (100 mM citric acid, 2 mM TCEP, pH 3) at room temperature with gentle rotation for 4 hr. After binding, the washing and elution steps were followed as described in the Experimental Section.

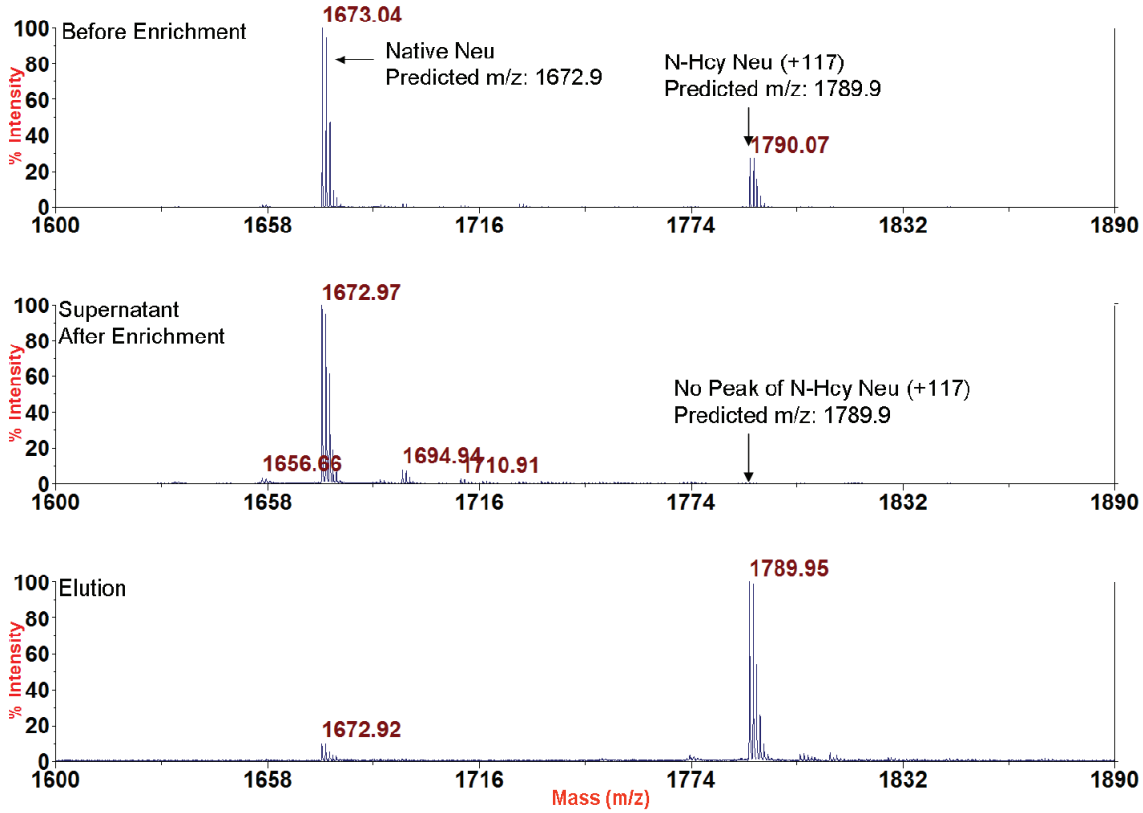


Figure S8.1. MALDI MS spectra of N-Hcy Neu at different steps of enrichment. Top: before enrichment; middle: supernatant after binding; bottom spectrum: eluted peptide solution.

Enrichment of N-Hcy Myoglobin

Hcy TL modified myoglobin (120 μM) was incubated with 107 mg POROS AL 20 resin (vendor and catalog number) in 50 mM potassium phosphate, 1 mM TCEP, pH 8.0 at room temperature for 3 hr with gentle agitation. After that, the slurry was centrifuged at 500 x g for 2 min. The supernatant was removed and the binding efficiency was determined by LC/MS.

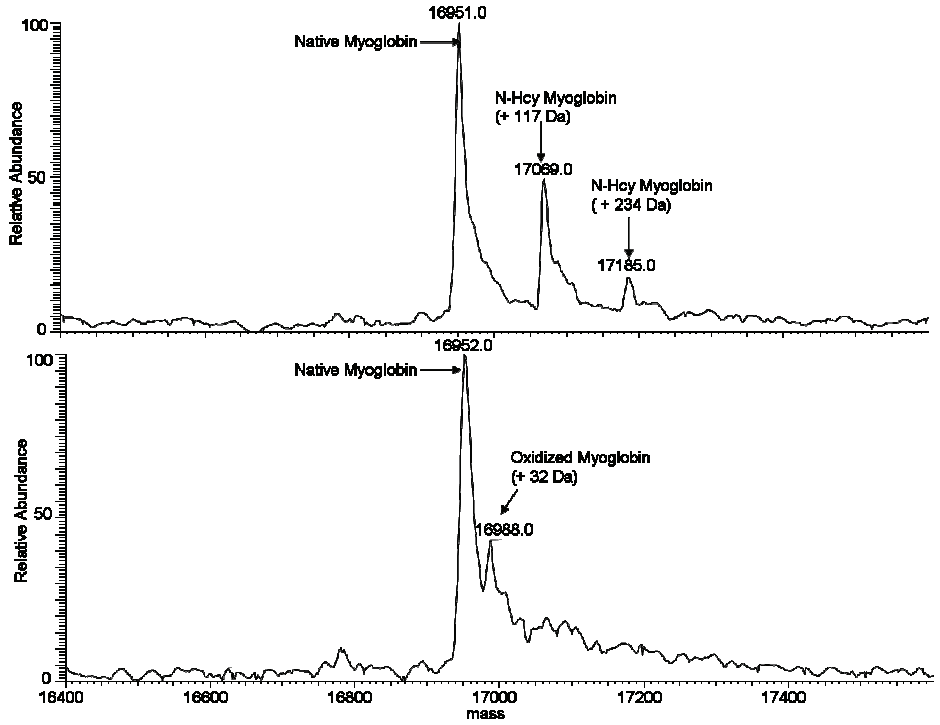


Figure S8.2. Mass spectra of native myoglobin (predicted MW: 16951.5) and N-Hcy myoglobins (predicted MW: 17068.5 and 17185.5) before enrichment (top) and supernatant after enrichment (bottom).

Enrichment of N-Hcy Peptides from Digested Modified Human Hemoglobin

Table S8.1. Modified lysine residues and the corresponding solvent accessibility.

α-chain of Human Hemoglobin		
Modification Site	Sequence	Relative Accessibility (%)
K 7 & K 11	VLSPADK*TNVK*AAWGK	19.4 (K 7)
K 11	TNVK*AAWGK	36.7
K 11 & K 16	TNVK*AAWGK*VGAHAGEYGAEALER	
K 16	AAWGK*VGAHAGEYGAEALER	42
K 56	TYFPHFDLSHGSAQVK*GHGK	42.7
K 60	---	37
K 61	K*VADALTNAVAHVDDMPNALSALSDLHAHK	31.7
K 90	VADALTNAVAHVDDMPNALSALSDLHAHK*LR	43.3
K 99	VDPVNFK*LLSHCLLVTLAAHLPAEFTPAVHASLDK	42.7
K 139	FLASVSTVLTSK*YR	26.9
K 127	---	7.7
β-chain of Human Hemoglobin		
K 8	VHLTPEEK*SAVTALWGK	38.7
K 8 & K 17	VHLTPEEK*SAVTALWGK*VNVDEVGGEALGR	
K 17	SAVTALWGK*VNVDEVGGEALGR	33.9
K 59	FFESFGDLSTPDAVMGNPK*VK	26.8
K 59 & K61	FFESFGDLSTPDAVMGNPK*VK*AHGK	37.6 (K 61)
K 65	AHGK*KVLGAFSDGLAHLDNLK	33
K 66	K*VLGAFSDGLAHLDNLK	35
K 66 & K 82	K*VLGAFSDGLAHLDNLK*GTFATLSELHCDK	
K 82	VLGAFSDGLAHLDNLK*GTFATLSELHCDK	41.4
K 95	GTFATLSELHCDK*LHVDPENFR	50.6
K 120	LLGNVLVCVLAHHFGK*EFTPPVQAAYQK	64.8
K 132	---	26.2
K 144	VVAGVANALAHK*YH	28.8

Note: 1. ---: Not detected.

2. K*: modified lysine residue.

3. Relative accessibility of lysine residues was acquired from the script language command "access" of Swiss-Pdb Viewer 3.7. The range is between 100% (maximum surface exposure) and 0% (completely buried amino acid). PDB number of human hemoglobin is 1xz2.

α-chain:

VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGK

KVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPA

VHASLDKFLASVSTVLTSKYR

β-chain:

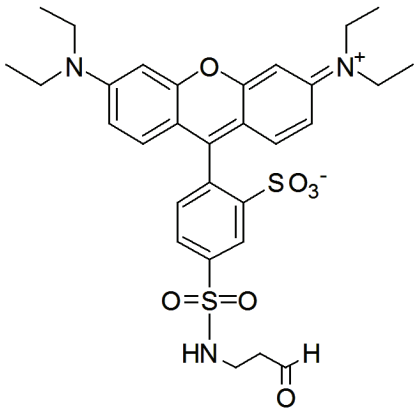
VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKV

KAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGK

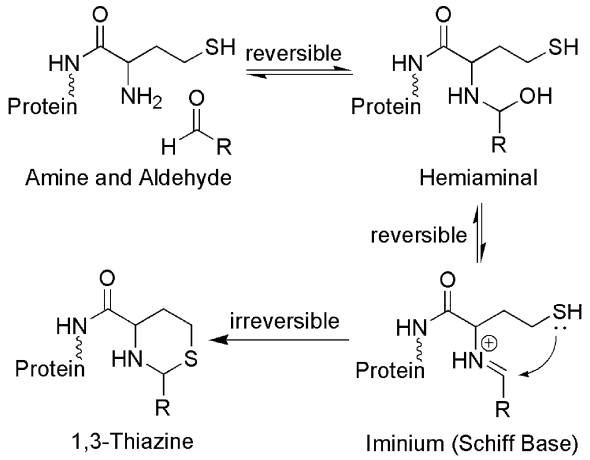
EFTPPVQAAYQKVVAGVANALAHKYH

Figure S8.3. Sequences of α-chain (UniProtKB/Swiss-Prot number: P69905) and β-chain (UniProtKB/Swiss-Prot number: P68871) of human hemoglobin.

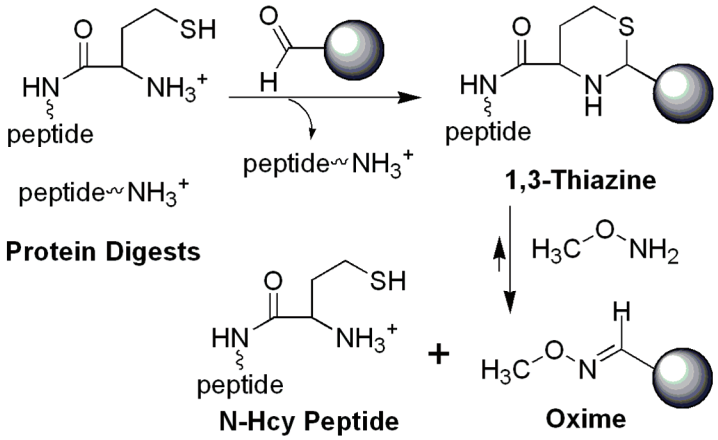
9. Structure of Rhodamine Aldehyde



10. Chemistry of 1,3-Thiazine Formation from Homocystamide and Aldehyde



11. Strategy for Affinity Enrichment of N-homocysteinylated Peptides



References:

(1) Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182*, 319-326.

(2) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. *Protein. Sci.* **1995**, *4*, 2411-2423.