Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

A Prochelator Peptide Designed to use Heterometallic Cooperativity to Enhance Metal Ion Affinity

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Materials & Methods

Reagents used:

HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), PAR (4-(2-pyridylazo)resorcinol), H_2PO_4, TCEP (tris(2-carboxyethyl)phosphine hydrochloride), DTT (dithiothreitol), and $ZnSO_4$ were purchased from Sigma Aldrich and $Tb(III)Cl_3.6H_2O$ from Acros. All stock solutions were made in Milli-Q water. Concentrations of metal stock solutions were verified by titration with xylenol orange. TCEP stock solutions were adjusted to neutral pH using NaOH and used immediately. The pH of prepared peptide samples was checked before and after experiments by using an InLab micro pH electrode from Mettler Toledo; no pH drift was detected over the course of the experiments..

Peptide synthesis, purification and concentration determination:

Peptides were synthesized by standard solid-phase synthesis on a Protein Technologies PS3 automated peptide synthesizer on Wang resin (Chem-Impex). All peptide sequences reported in this paper contain a native-like amine N-terminus and carboxylic acid C-terminus. Names and amino acid sequences for all peptides are listed in Figure 1 of the main text, with the exception of A_4CysHisPCP, which has the sequence AAAACIDTNNDGWYEGDEHAAAA. Standard FMOC (9-fluorenylmethoxy-carbonyl)-protected amino acids (Chem-Impex) were coupled with HBTU (O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate; Novabiochem) in the presence of N-methylmorpholine in DMF (N-N'-dimethylformamide) in 20 min cycles. FMOC deprotection was performed with 20% piperidine in DMF. Side-chain deprotection and cleavage from the resin was performed with a 5-mL cocktail of TFA (triflouroacetic acid: EDT (ethane dithiol): TIS (triisopropyl silane) (95:2.5:2.5%) for approximately 3 h under N_2. A second addition of 75 uL EDT and 65 uL TMSBr (bromotrimethylsilane) was added during the final 30 min to minimize cysteine oxidation. TFA was evaporated under N_2, and the crude peptides were washed three times with cold diethyl ether and allowed to air dry. Purification was achieved by analytical reversed-phase chromatography on a Waters 600 Controller HPLC equipped with a Hichrom C18 Ultrasphere 5 ODS column (250 × 4.6mm) with a variety of isocratic gradients ranging from 21–15% acetonitrile in water, optimized for each peptide. HPLC fractions of peptides were lyophilized and the peptide powder was dissolved in Milli-Q water.

Concentrations of peptide stock solutions were determined by diluting aliquots into 8M urea and measuring the absorbance at 280 nm (extinction coefficients of 5650 $L.mol^{-1}$ for tryptophan and 1300 $L.mol^{-1}$ for each tyrosine)[1]. Samples were aliquot, frozen in liquid nitrogen and stored at -20 °C until use. Peptides purities were checked by liquid chromatography-mass spectrometry (LC-MS) collected on an Agilent 1100 Series HPLC equipped with a 3-μm C18 Luna column in line with an electrospray mass spectrometer (see figure S9). Purity based on the UV trace was found to be ≥ 93% for all the peptides, with the exception of slightly lower estimates for ΔCys_{PCP} (≥ 88%) and $CysHis_{PCP}$ (≥ 85%). Peptide samples incubated in typical buffer conditions (5 mM HEPES buffer pH 7.4 with 50 μM TCEP) were found by HPLC analysis to remain in their cysteine-reduced forms for at least 90 min (see figure S7). All experiments of the presented article were carried in less than 90 min.

Spectrophotometry

Absorbance measurements were performed on a Cary 50 UV-Vis Spectrophotometer in quartz cuvettes, path length 1 cm.

Luminescence experiments were done on a PerkinElmer Victor 3V Multilabel Plate Reader or a PerkinElmer LS50B Luminescence Spectrophotometer, in phosphorescence mode. Samples were loaded in 1-cm quartz cuvettes for measurements on the spectrophotometer with instrument settings as follows: delay 0.1 ms, gate 8 ms, excitation: 280 nm, range: 500-600 nm, scan speed: 400 nm/min). Equivalent settings (delay 0.1 ms, gate 8 ms, excitation 280 nm; emission 545 nm) were used on the plate reader, with samples loaded into the wells of Non-Binding Surface coated polystyrene plates (Corning).

Luminescence lifetimes were measured on a PerkinElmer Victor 3V Multilabel Plate Reader. For all experiments except the H_2O/D_2O experiment, a standard terbium lifetime measurement was used (gate: 0.5 ms, delay: 0.1, 0.5, 1.0, 1.5, 2.0, 3.0 ms). The plate was shaken before each measurement was performed. A long lifetime measurement was used for H_2O/D_2O, (gate: 0.5 ms, delay: 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0 ms), under the same conditions.

Fittings and calculations

Determination of Tb-peptide concentration: The concentration of Tb-peptide complex in solution was determined by comparison to a standard curve of Tb luminescence intensity vs peptide concentration under conditions of excess Tb to ensure that every peptide is loaded with Tb. The contribution of emission intensity from uncomplexed Tb excited at 280 nm is negligible, therefore all the intensity originates from the Tb-peptide complex.

Lifetime and q: The lifetime and decay rate of Tb luminescence were obtained from luminescence intensity data recorded after several delay times and calculated according to Eqs. 1 and 2 (where I_t is the intensity of luminescence at time t, I_0 is the intensity of luminescence at time t= 0, τ is the lifetime and *k* is the decay rate).

$$I_t = I_0 e^{-kt} \quad \text{Eq. 1}$$

$$k = 1/\tau \quad \text{Eq. 2}$$

The number of water molecules (q) bound to Tb was calculated by using Eq. 3,[2] where A is an empirical constant related to the amount of quenching per OH oscillator (A for Tb = 4.2 ms) and *k* is the luminescence decay rate in either H_2O or D_2O.

$$q = A(k_{H_2O} - k_{D_2O}) \quad \text{Eq. 3}$$

Zn affinity: Competition with PAR. The apparent association constants (log β_2) of $Zn(Peptide)_2$ reported in Table 1 were determined from spectrophotometric titrations with PAR as a competitive ligand. Global fit analysis of the titration data was performed with the software SPECFIT; apparent association constants of $Zn(PAR_2)$ and ZnPAR were incorporated in the fitting model (log β_2 = 12.34 and log K_1 = 6.6, respectively).[3,4]

Tb affinity: The 1:1 conditional affinity constants of Tb for peptides were obtained either by direct Tb titration or by competitive titration with ΔY_{LBT}. Direct titration data were fit using Equation 7, which is derived from Eqs 4–6, where K_d is the dissociation constant, [P] is the concentration of "free" peptide, $[P]_{tot}$ is the total concentration of peptide, [P.Tb] is the concentration of Tb peptide complex, [Tb] is the concentration of "free" terbium, $[Tb]_{tot}$ is the total concentration of terbium.

$$Kd = \frac{[P][Tb]}{[P.Tb]} \quad \text{Eq. 4}$$

$$[Tb] = [Tb]_{tot} - [P.Tb] \quad \text{Eq. 5}$$

$$[P] = [P]_{tot} - [P.Tb] \quad \text{Eq. 6}$$

$$[P.Tb] = \frac{[P]_{tot}+[Tb]_{tot}+Kd-\sqrt{(-[P]_{tot}-[Tb]_{tot}-Kd)^2-4[P]_{tot}[Tb]_{tot}}}{2} \quad \text{Eq. 7}$$

For competitive titrations, the ΔY_{LBT} peptide was titrated into solutions of Tb + Peptide and both Tb luminescence intensity and lifetime were recorded and the luminescence data were fit to Eq. 8, where Kd is the apparent dissociation constant of ΔY_{LBT}, $[\Delta Y_{LBT}]_{tot}$ is the total concentration of ΔY_{LBT}, $[P.Tb]_{ini}$ is the initial concentration of Tb peptide complex, [P.Tb] is the concentration of Tb peptide complex at that point in the titration. The small contribution of Tb-ΔY_{LBT} to the overall intensity was corrected using both Tb lifetime and luminescence measurements.

$$[P.Tb] = [P.Tb]_{ini} - \frac{[P.Tb]_{ini}+[\mathrm{Y1\Delta/W9F}]_{tot}+Kd-\sqrt{(-[P.Tb]_{ini}-[\mathrm{Y1\Delta/W9F}]_{tot}-Kd)^2-4[P.Tb]_{ini}[\mathrm{Y1\Delta/W9F}]_{tot}}}{2} \quad \text{Eq. 8}$$

pMetal (pM). For a 1:1 metal:ligand complex, as is the case for Tb:Peptide, the concentration of uncomplexed (free) metal is calculated from Eqs. 9 and 10, where $[P]_{tot}$ = 2 μM, $[Tb]_{tot}$ = 0.5 μM, K_d is $1/(10^{\log K})$, and log K is the Tb association constant from Table 1 (either the value obtained without Zn or with Zn). The plots in Figure 6 of the main text were obtained from calculations where $[Tb]_{tot}$ was varied from 10 nM to 1 μM.

$$\mathrm{pM} = -\log[M]_{free} \quad \text{Eq. 9}$$

$$[Tb]_{free} = \frac{-[P]_{tot}+[Tb]_{tot}-Kd+\sqrt{([P]_{tot}-[Tb]_{tot}+Kd)^2+4\,Kd\,[Tb]_{tot}}}{2} \quad \text{Eq. 10}$$

For a 1:2 metal:ligand complex, like $Zn(Peptide)_2$, the concentration of free metal is calculated from Eq. 11, where x is the concentration of free Zn, β_2 is the association constant of peptide for Zn, $[P]_{tot}$ is the total concentration of the peptide (2 μM for these calculations), $[Zn]_{tot}$ is the total

concentration of Zn (0.5 μM for the values in Table 1, or ranging from 10 nM – 1 μM for Figure 6 of the main text). The equation can be solved with basic math solver software if the concentration and the affinity constant are specified. The Zn-peptide β_2 values are those listed in Table 1.

Eq. 11

$$0 = 4x^3 + x^2(4[P]_{tot} - 8[Zn]_{tot}) + x\left(-4[P]_{tot}[Zn]_{tot} + [P]_{tot}^2 + 4[Zn]_{tot}^2 + \frac{1}{\beta_2}\right) - [Zn]_{tot}\frac{1}{\beta_2}$$

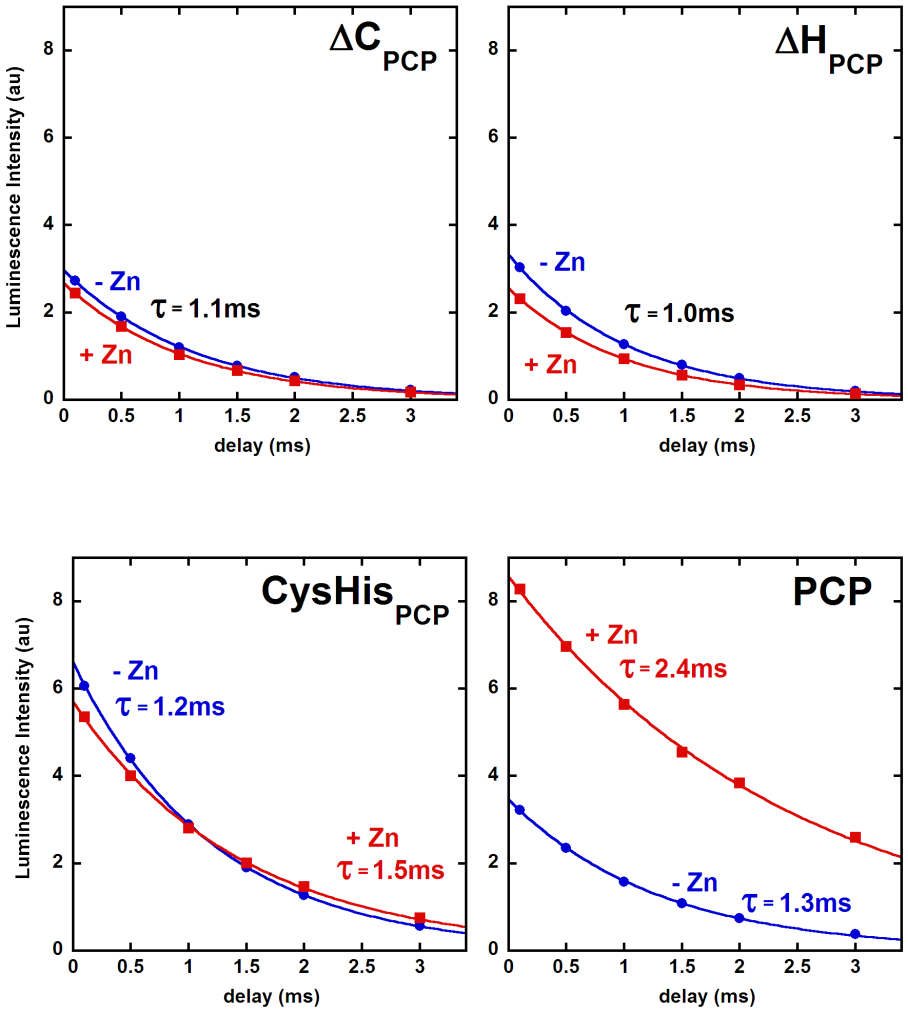

Figure S1: Tb luminescence lifetime measurements of different peptides in the absence (blue circles) or presence (red square) of Zn. Lines are the fitting by the simple exponential decay in Eq 1. Condition: [Peptide]=[Tb]=2 μM; [Zn]=0 or 2 μM [HEPES]=5 mM pH 7.4; [TCEP]=50 μM

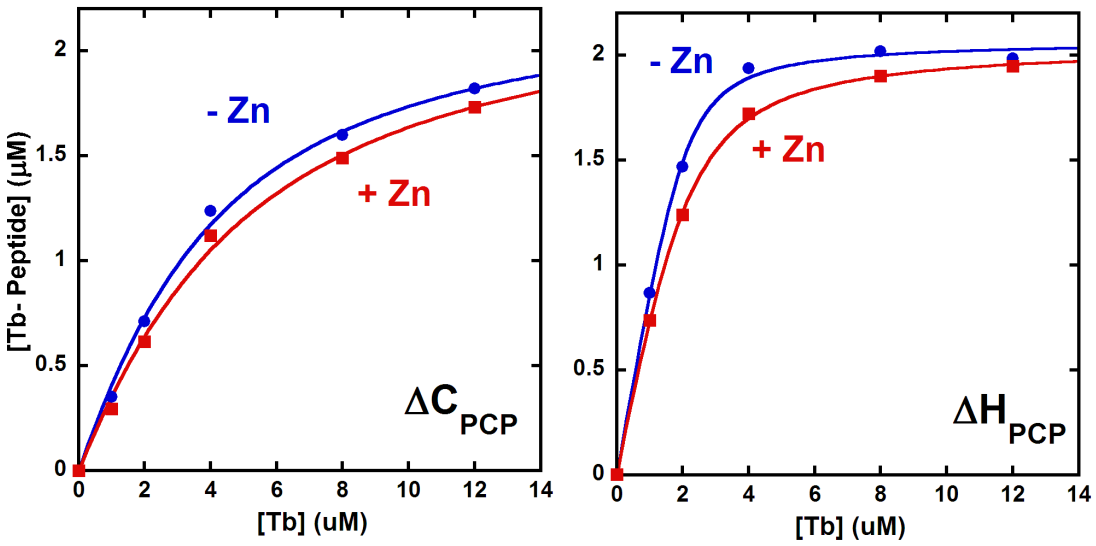

Figure S2: Tb titration of ΔC_{PCP} and ΔH_{PCP} in the absence (blue circles) or presence (red square) of Zn. Lines are the fit to Eq. 7. Condition: [Peptide]=2 μM; [Tb]=0-12 μM; [Zn]=0 or 2 μM; [HEPES]=5 mM pH 7.4; [TCEP]=50 μM

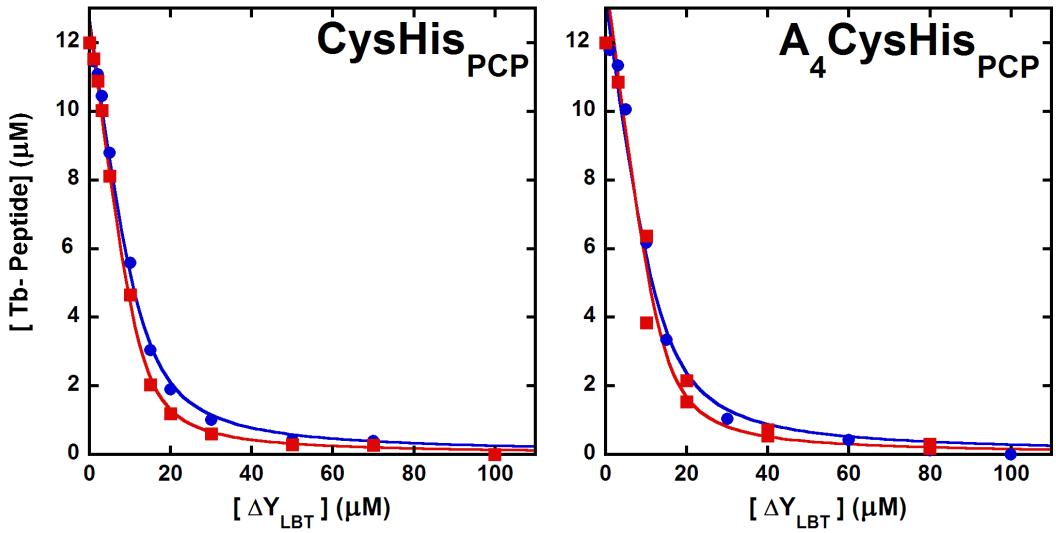

Figure S3: Tb competition using the ΔY_{LBT} as a competitor on $CysHis_{PCP}$ (left panel) or $A_4CysHis_{PCP}$ (right panel) in presence (red) or in absence of Zn (blue). Concentration of Tb-peptide complexes was determined by using both luminescence intensity and lifetime measurements. Lines represent the fit to Eq. 8 from which the log K values for Tb:peptide complexes were calculated to be 5.9 ± 0.1 and 6.2 ± 0.1 for $CysHis_{PCP}$ in the presence and absence of Zn, respectively. Very similar values were obtained for $A_4CysHis_{PCP}$ (5.8 ± 0.2 and 6.0 ± 0.1), providing evidence that truncating the alanine tails in the case of $CysHis_{PCP}$ does not affect the Tb-binding properties. Condition: [$CysHis_{PCP}$]=[$A_4CysHis_{PCP}$]=15 μM; [Tb]=12 μM; [Zn]=0 or 15 μM; [HEPES]=5 mM pH 7.4; [TCEP]=100 μM

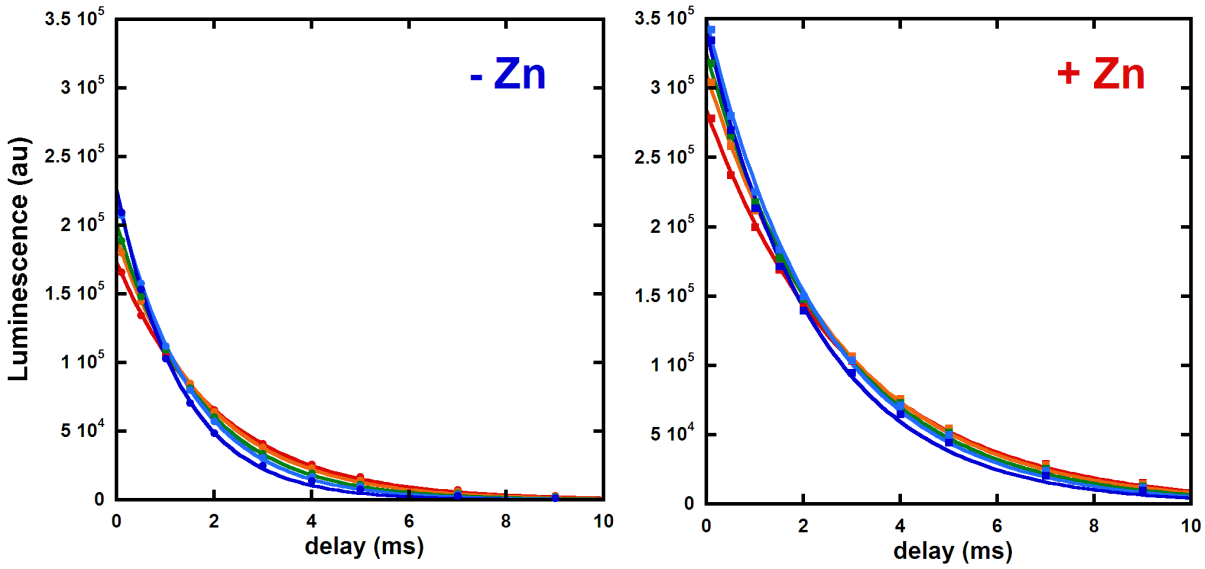

Figure S4: Lifetime measurement of Tb-PCP at 1 (dark blue), 0.8 (light blue), 0.6 (green), 0.4 (orange), 0.2 (red) ratios of H_2O/D_2O in absence (left panel) or in presence (right panel) of Zn. Lines are the fit to Eq. 1. Condition: [Peptide]=2µM; [Tb]=2 µM; [Zn]=0 or 2 µM [HEPES]=5 mM pH 7.4; [TCEP]=50 µM

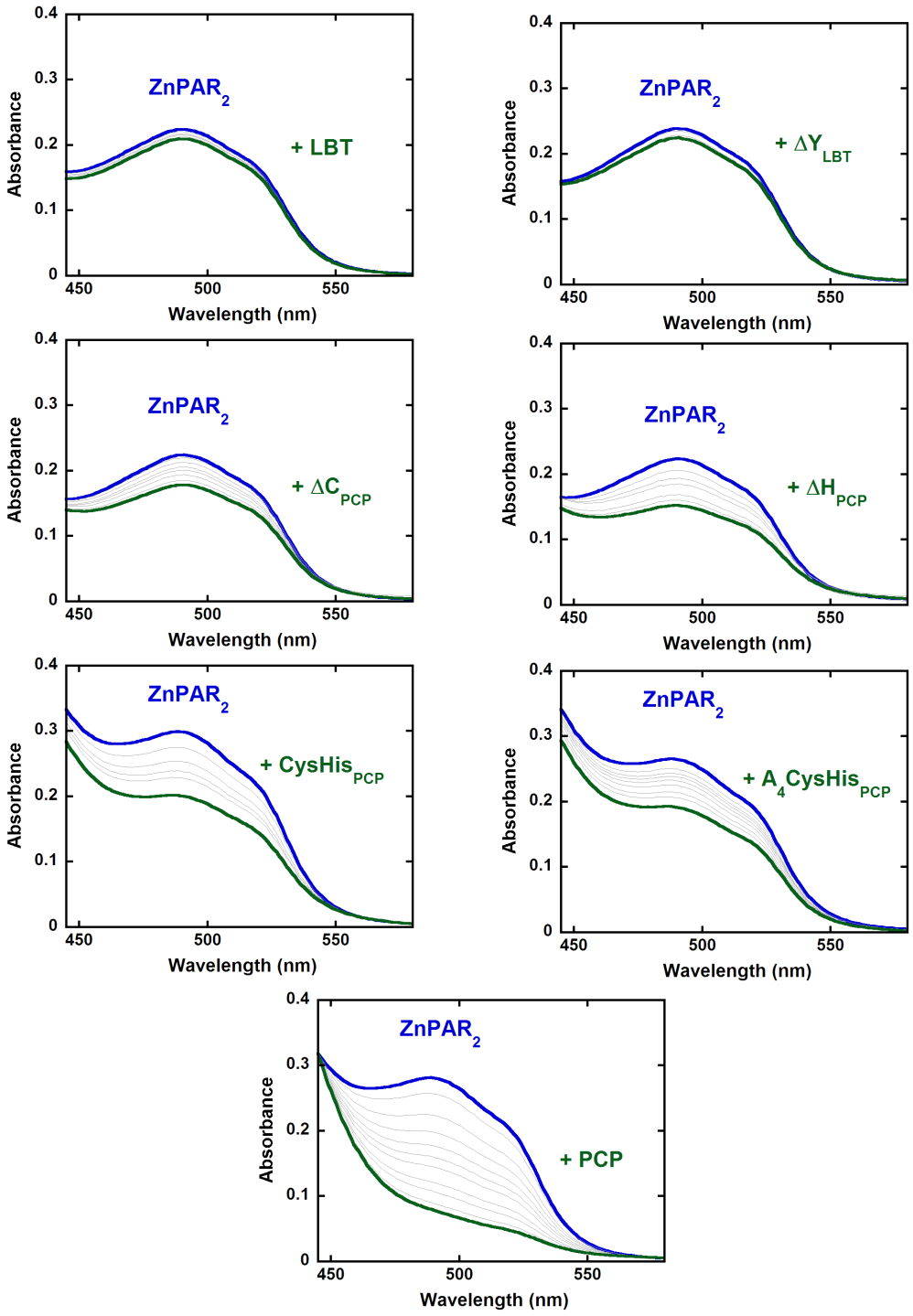

Figure S5: Zn competition between peptides and PAR. To $Zn(PAR)_2$ (blue line), sequential amounts of peptide were added (gray lines). These spectra were analyzed to determine the apparent association constants (log β_2) of $Zn(Peptide)_2$ that are reported in Table 1 of the main text, with the exception of $A_4CysHis_{PCP}$, for which log β_2 was found to be 11.3 ± 0.2, regardless of Tb status. This value matches that of $CysHis_{PCP}$, providing evidence that truncating the alanine tails in the case of $CysHis_{PCP}$ does not affect the Zn-binding properties. Green lines correspond to [LBT]=20 μM; [ΔY_{LBT}]=18 μM; [ΔC_{PCP}]=30 μM; [ΔH_{PCP}]=14 μM; [$CysHis_{PCP}$]=30 μM; [$A_4CysHis_{PCP}$]=30 μM; [PCP]=20 μM. Conditions: [HEPES]=5 mM pH 7.4; [TCEP]=100 μM; [Zn]=3 μM; [PAR]=10 μM (for LBT, $\Delta Y_{LBT,}$ $\Delta C_{PCP,}$ ΔH_{PCP}); [PAR]=20 μM (for $CysHis_{PCP}$, $A_4CysHis_{PCP,}$ PCP).

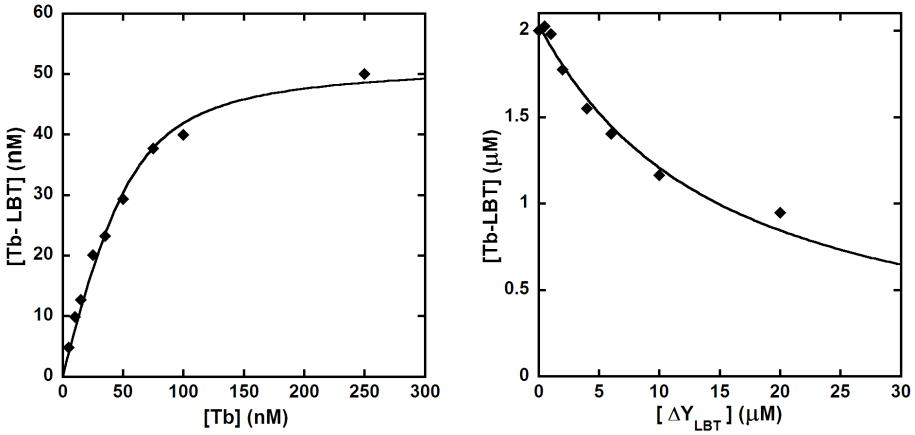

Figure S6: Direct titration of LBT with Tb (left panel) and its competition for Tb with ΔY_{LBT} (right panel). Curve fitting gives conditional K_D = 16 ± 5 nM and K_D = 120 ± 12 nM for LBT and ΔY_{LBT} respectively (see fitting section for details). Note that the 16 nM K_D reported here for LBT differs from the 57 nM value reported by Imperiali and coworkers;[5,6] however, notable variations in the solution conditions and the peptide itself explain these differences. Our measurements were done at pH 7.4 with no added salt, whereas the original work was at pH 7.0 with 100 mM NaCl. Furthermore, our LBT has a free C-terminal carboxylic acid whereas the parent LBT terminated with an amide. Variations in the conditional affinity constants are therefore to be expected. Condition: [HEPES]=5 mM pH 7.4; *left panel* [LBT]=50 nM; [Tb]=[0-250 nM]; *right panel* [LBT]=[Tb]=2 μM; [ΔY_{LBT}]=0–20 μM

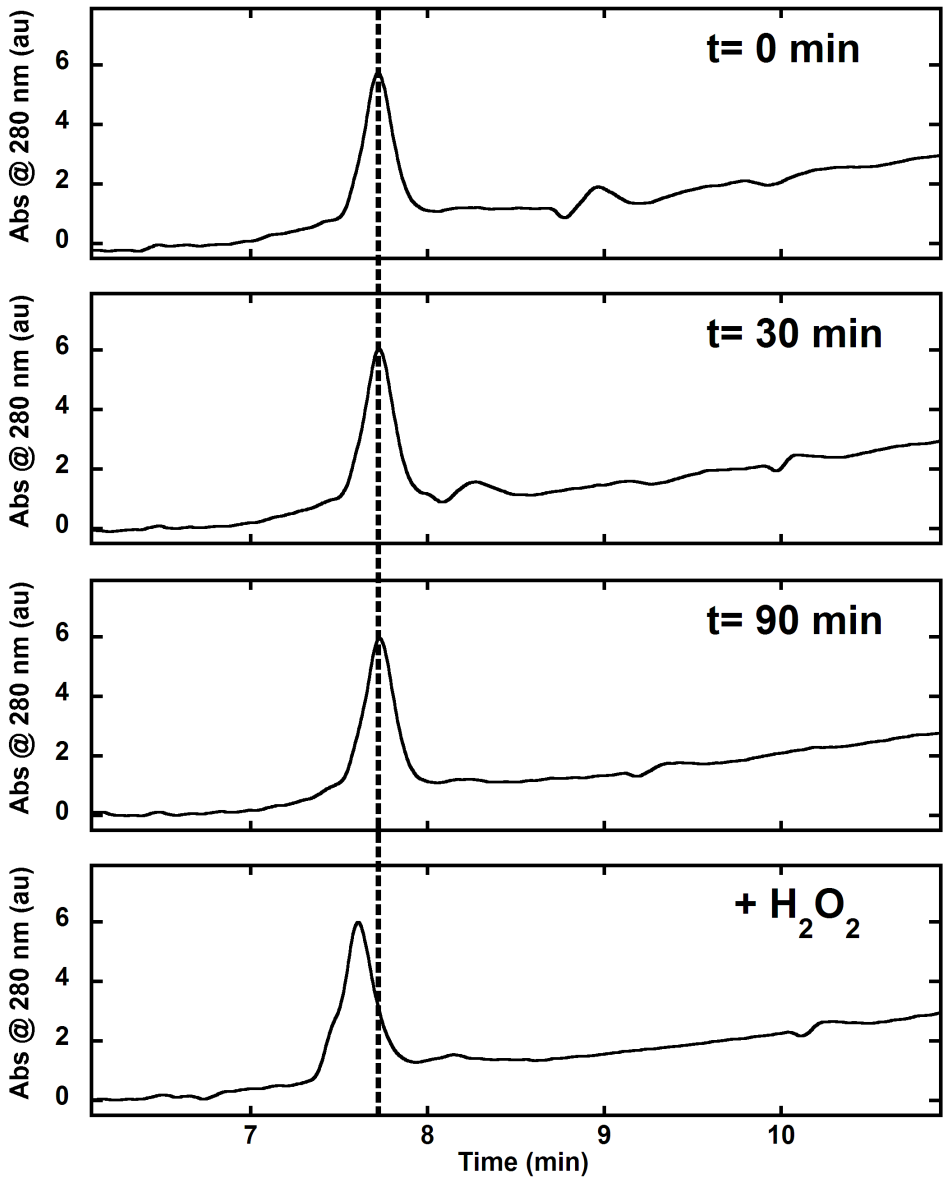

Figure S7a: LC trace of 10 μM samples of PCP incubated in HEPES buffer pH 7.4 with 50 μM TCEP for 0, 30 and 90 min (top 3 traces) and after addition of H_2O_2 (500 μM) (bottom trace). The dashed line emphasizes the slight change in the peptide elution time upon H_2O_2 addition, consistent with cysteine oxidation (see Figure S7b). These results provide evidence that PCP remains in the reduced form under these buffer conditions for at least 90 min. Elution: H_20/MeCN/HCOOH (95/5/0.1) to (50/50/0.1) over 15 min.

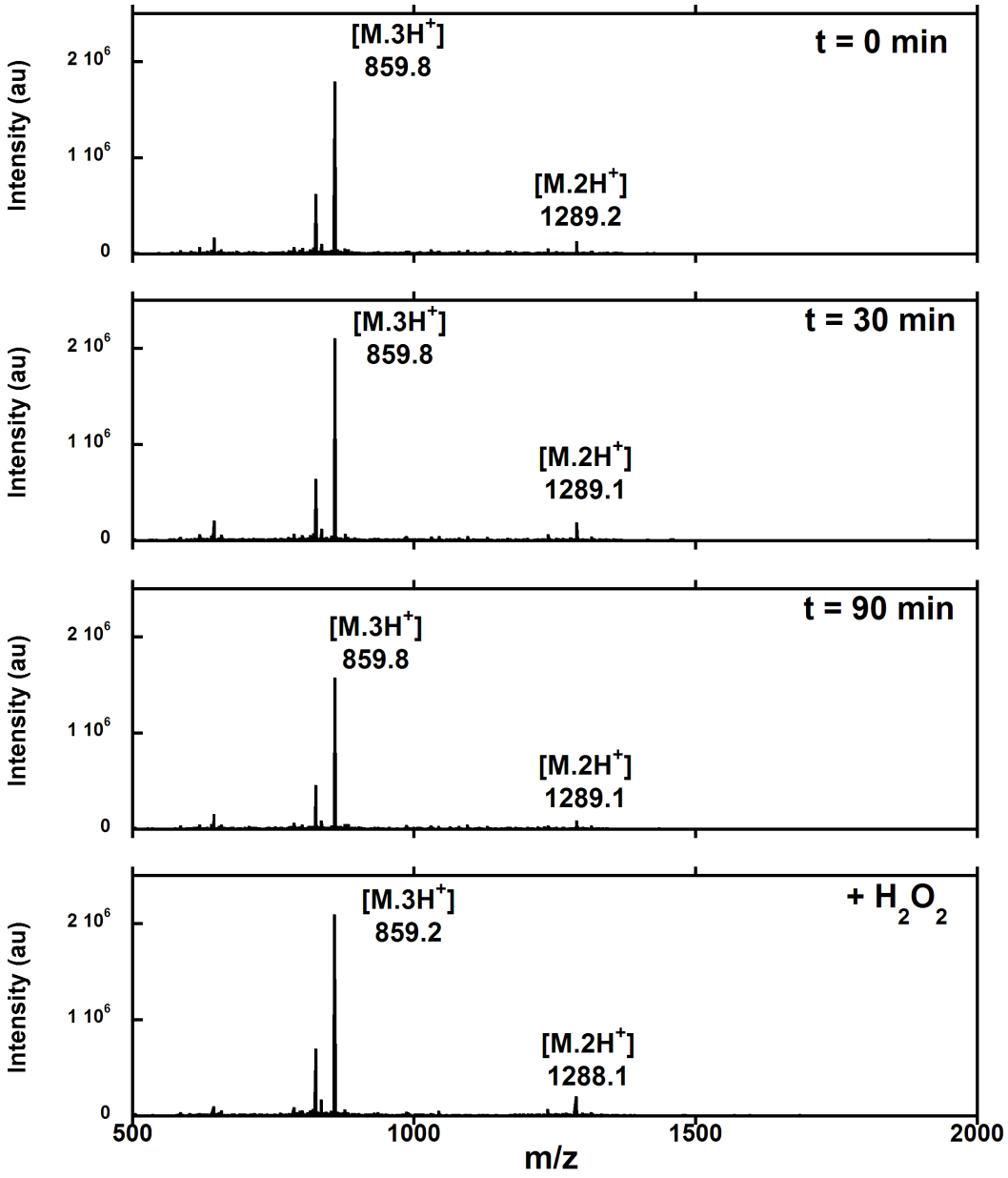

Figure S7b: Extracted ESI mass spectra corresponding to the LC peaks shown in FigureS7a. The top 3 spectra are consistent with the fully reduced PCP peptide, whereas the H_2O_2-treated sample matches the expected mass for disulfide formation.

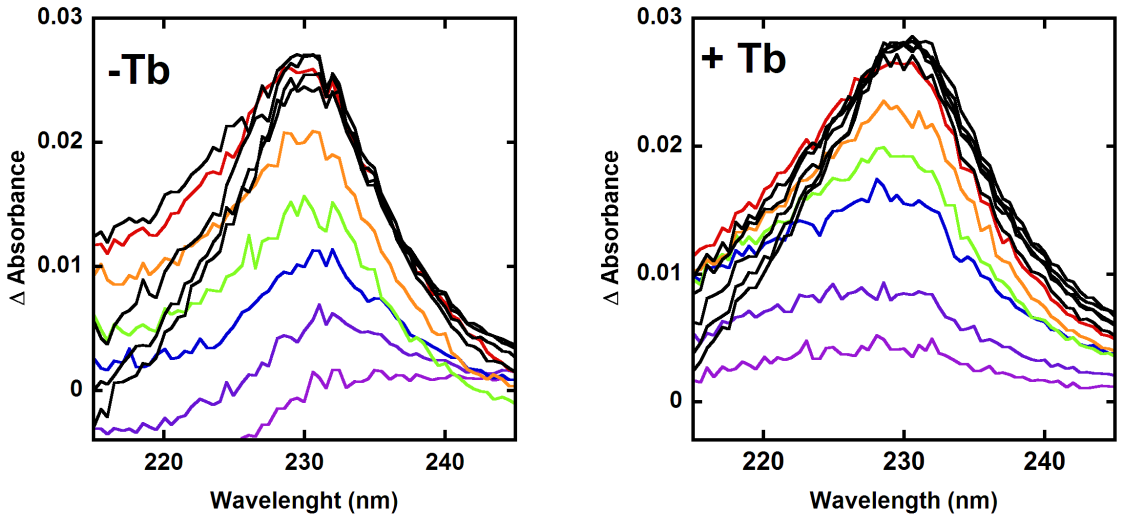

Figure S8a: Difference UV spectra between apo-PCP and subsequent addition of Zn corresponding to 0.05 (purple), 0.1 (indigo), 0.2 (blue), 0.3 (green), 0.4 (orange), 0.5 (red), 0.6-1 (black) equivalents in the absence (left panel) or presence of Tb. Condition: [PCP]=10 μM; [Zn]=0–10 μM; [Tb]=0–10 μM; [TCEP]=50 μM; 10 mM phosphate buffer, pH 7.4

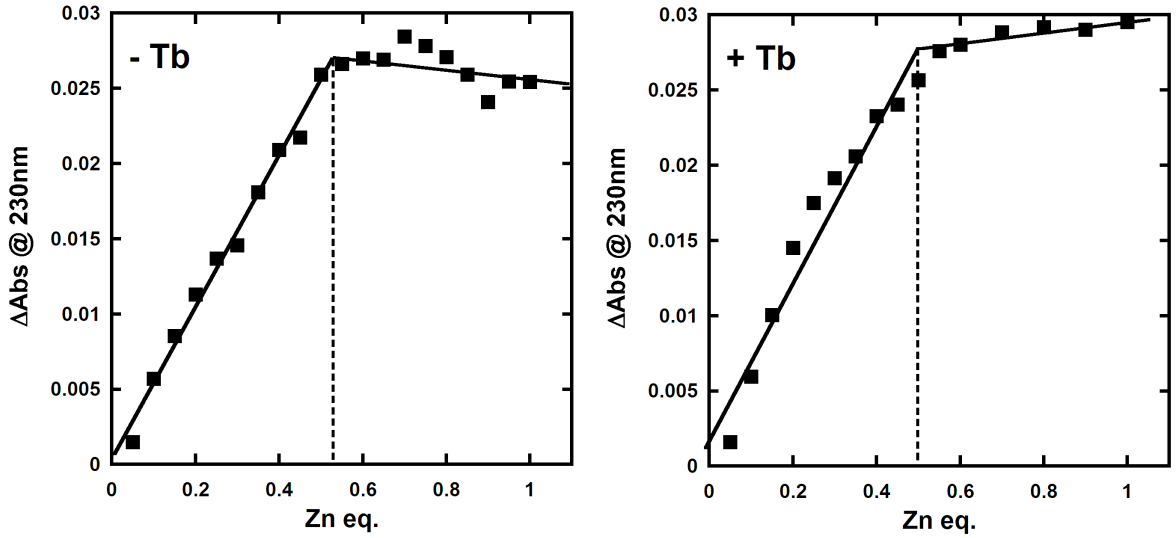

Figure S8b: Corresponding plot of difference UV at 230 nm as a function of Zn equivalent in the absence (left panel) or presence (right panel) of Tb. Black solid lines are linear regression of data points between 0.05–0.5 and between 0.55–1 Zn equivalent. The dashed line emphasizes the $Zn(PCP)_2$ stoichiometry suggested by the data. Condition: [PCP]=10 μM; [Zn]=0–10 μM; [Tb]=0–10 μM; [TCEP]=50 μM; 10 mM phosphate buffer, pH 7.4

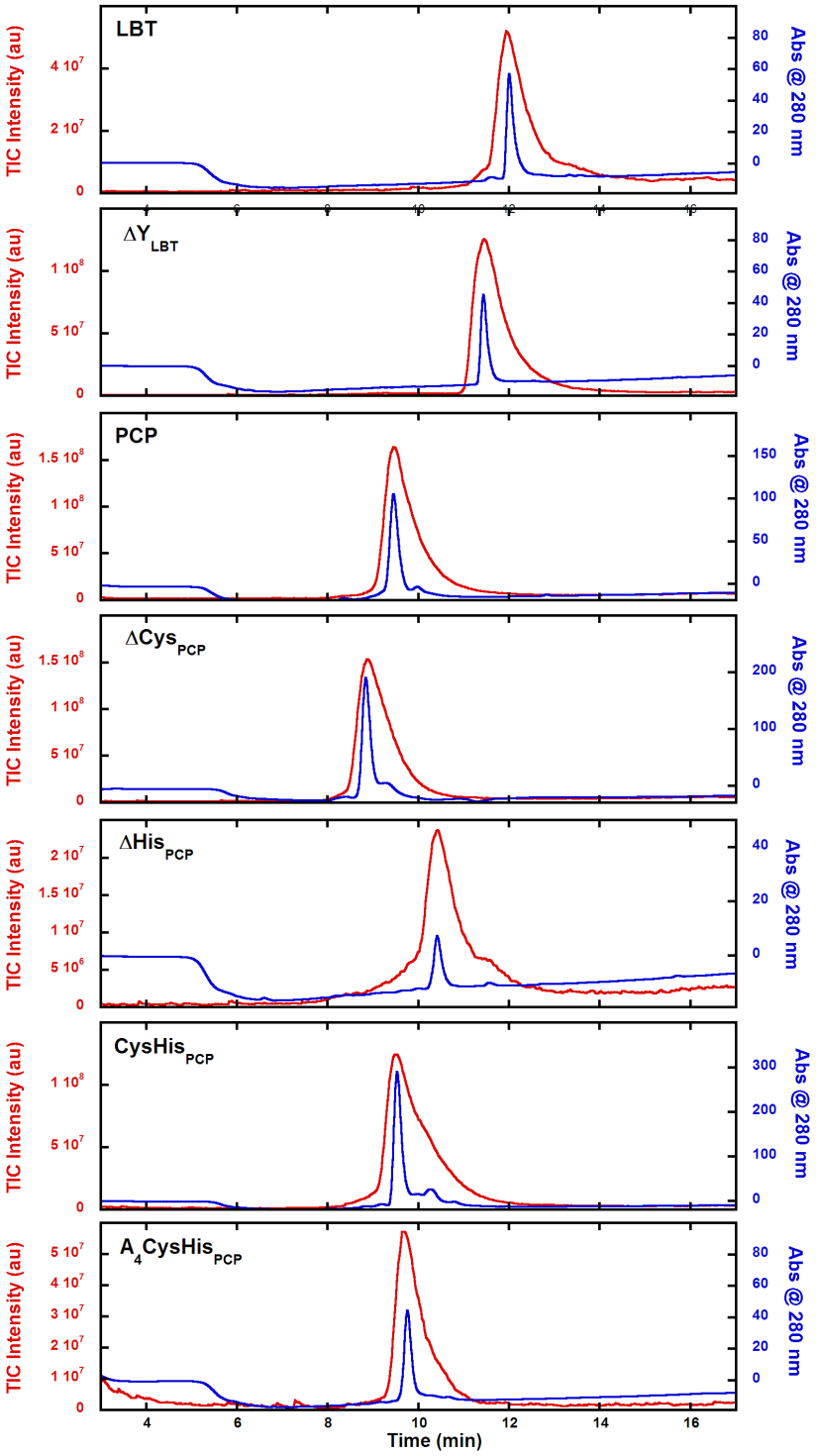

Figure S9a: LC-MS traces of peptides. Detection via Total Ionic Courant (red) and Absorbance at 280 nm (blue). Elution: H_20/MeCN/HCOOH (100/0/0.1) to (50/50/0.1) over 20 min. Condition: 2-µL injection of peptide samples at various concentrations. [LBT]=100 µM; [ΔY_{LBT}]=300 µM; [PCP]=300 µM [ΔC_{PCP}]=300 µM; [ΔH_{PCP}]=50 µM; [$CysHis_{PCP}$]=300 µM; [$A_4CysHis_{PCP}$]=100 µM.

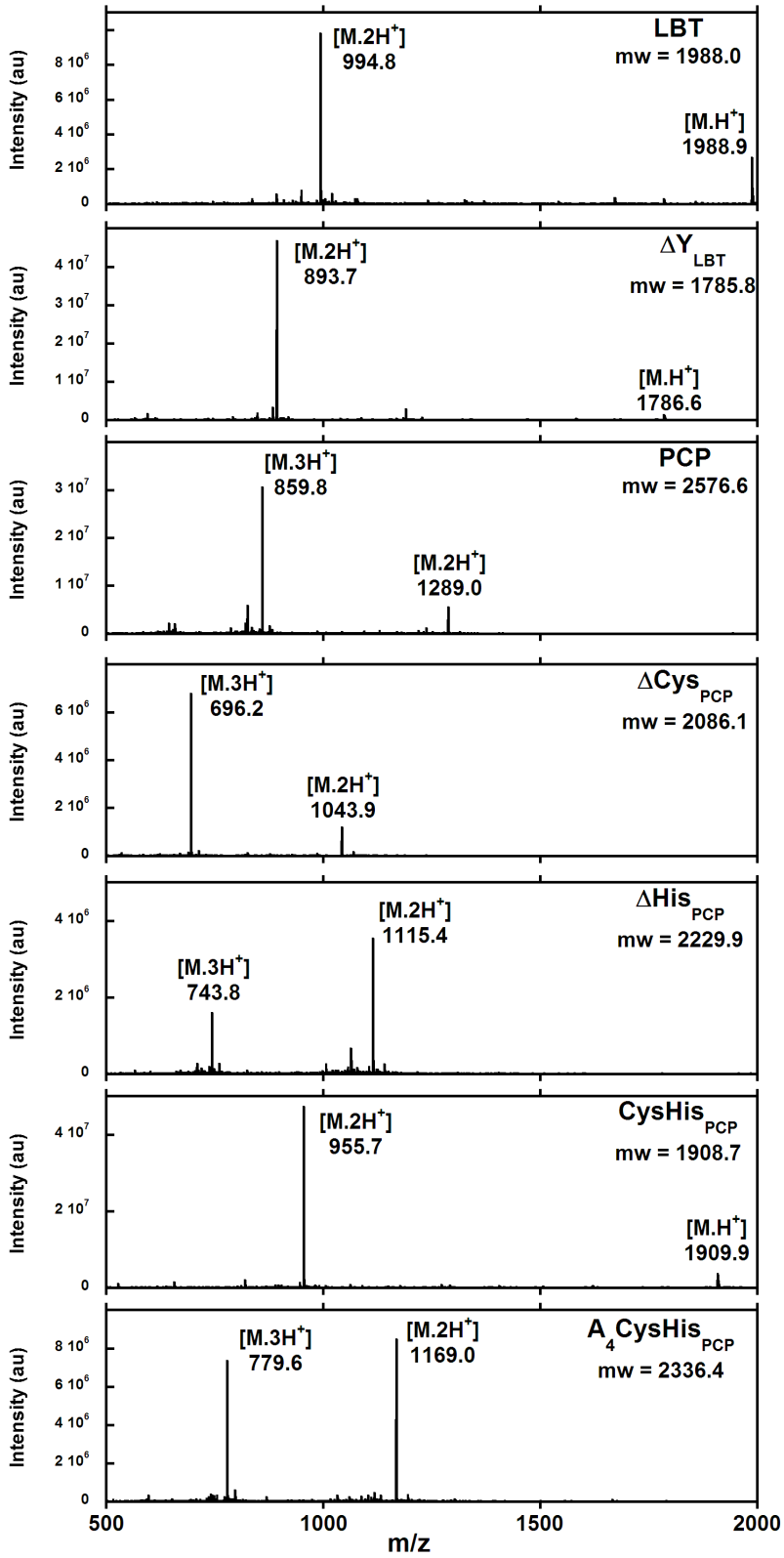

Figure S9b: ESI mass spectra extracted from the major TIC peaks shown in FigureS9a for each peptide.

References

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