Supplemental Information for

Elucidation of a copper binding site in proinsulin C-peptide and its implications on metal-modulated activity

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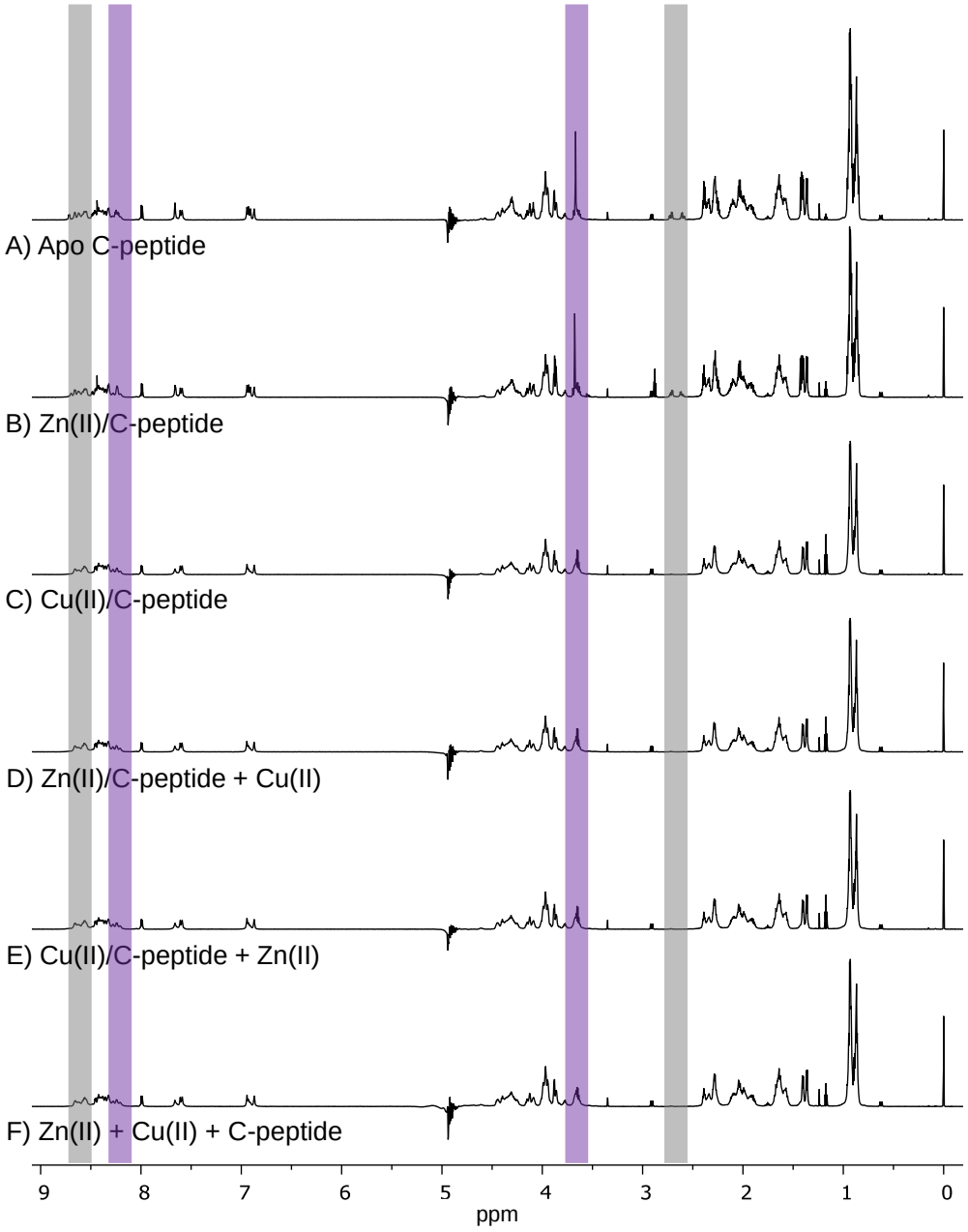

Figure S1. ^{1}H NMR spectra of A) apo C-peptide, B) Zn(II)/C-peptide, C) Cu(II)/C-peptide, D) equimolar Cu(II) added to Zn(II)/C-peptide, E) equimolar Zn(II) added to Cu(II)/C-peptide, and F) Cu(II) and Zn(II) added to apo C-peptide simultaneously. Grey highlighted regions of spectra are enlarged and shown in Figure 3 and are due to CHβ protons on D4 (2.7 ppm) and backbone amide protons from E3 (8.7 ppm). Purple highlighted regions of the spectra are due to glycine protons (3.7 ppm) and the backbone amide proton from L5 (8.2 ppm). All spectra were collected at 800 MHz in 95:5 H_2O:D_2O and 10 mM Tris-d_{11}, pH 7.4 at 10 °C. Addition of Zn(II) induces shifts in proton resonances while Cu(II) obliterates proton resonances within these regions. The spectra of Cu(II) competition with Zn(II) (D, E, and F) result in spectra that resemble Cu(II)/C-peptide and not Zn(II)/C-peptide indicating that Cu(II) displaces Zn(II) for binding C-peptide.

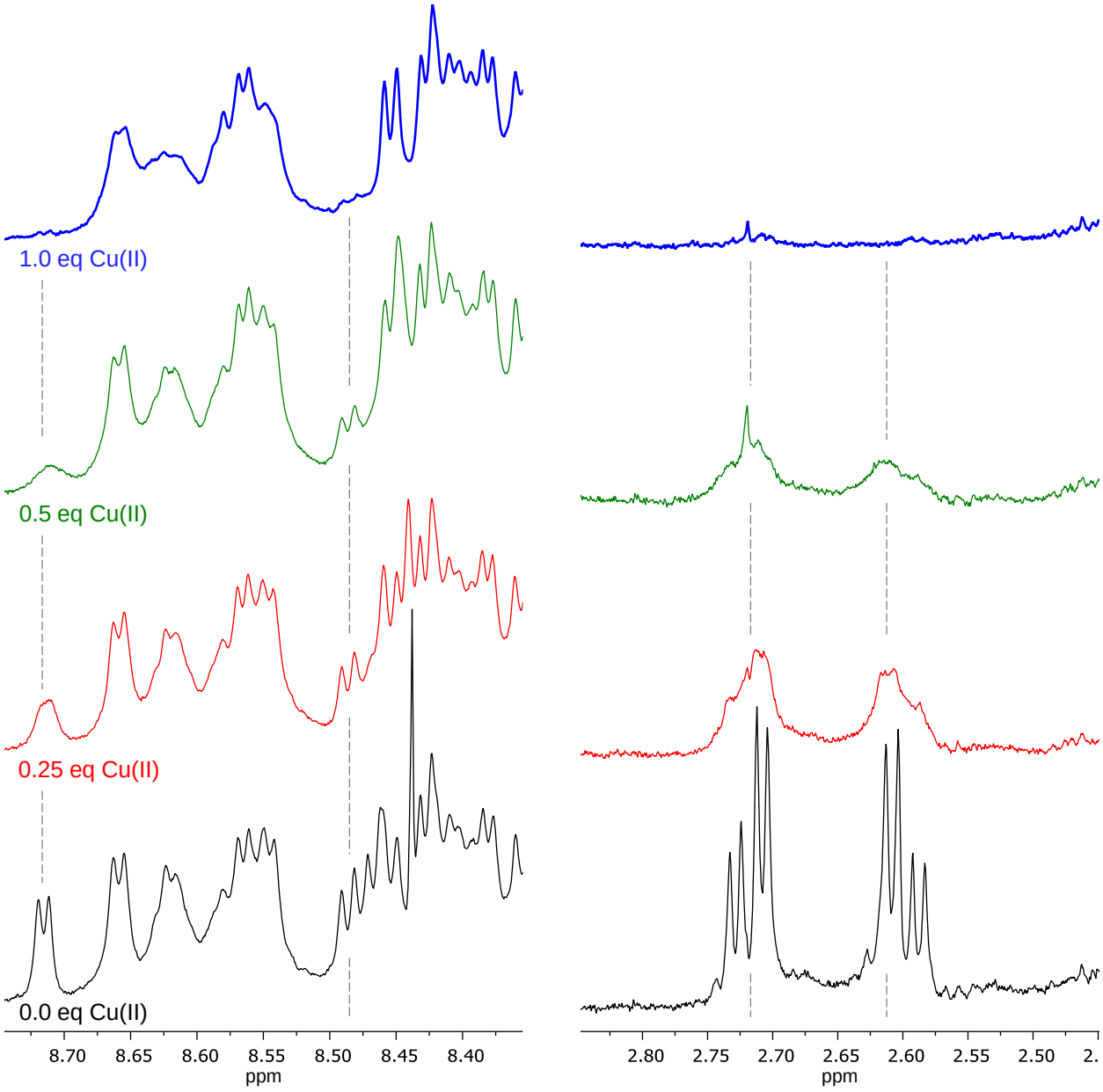

Figure S2. NMR spectra of 250 µM apo C-peptide (black) and 250 µM C-peptide with 62.5 µM (red), 125 µM (green), and 250 µM (blue) Cu(II). Cu(II) obliterates the proton resonances of CHβ protons on D4 (2.6 ppm) and backbone amide protons from D4 (8.45 ppm) and E3 (8.7 ppm) (dashed lines). Solutions were prepared in 95:5 (v/v) H2O:D2O with 10 mM Tris-d11 at pH 7.4 and spectra were collected at 800 MHz and 10 °C.

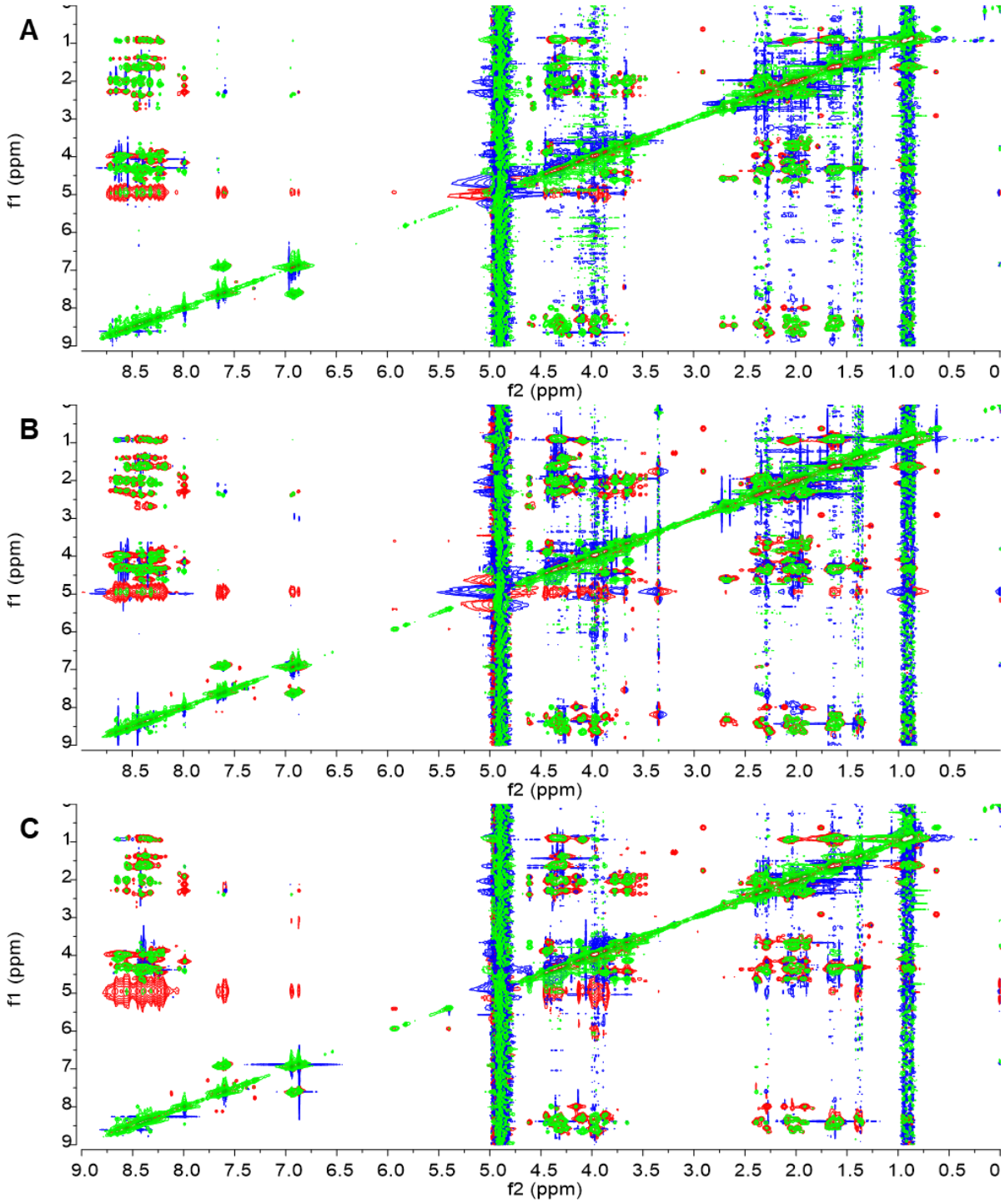

Figure S3. Overlay of full ^{1}H-^{1}H TOCSY (red-blue) and ^{1}H-^{1}H NOESY (green-blue) spectra for 1.5 mM A) apo C-peptide, B) Zn(II)/C-peptide, and C) Cu(II)/C-peptide. All spectra were collected at 800 MHz in 95:5 H_2O:D_2O and 10 mM Tris-d_{11}, pH 7.4 at 10 °C.

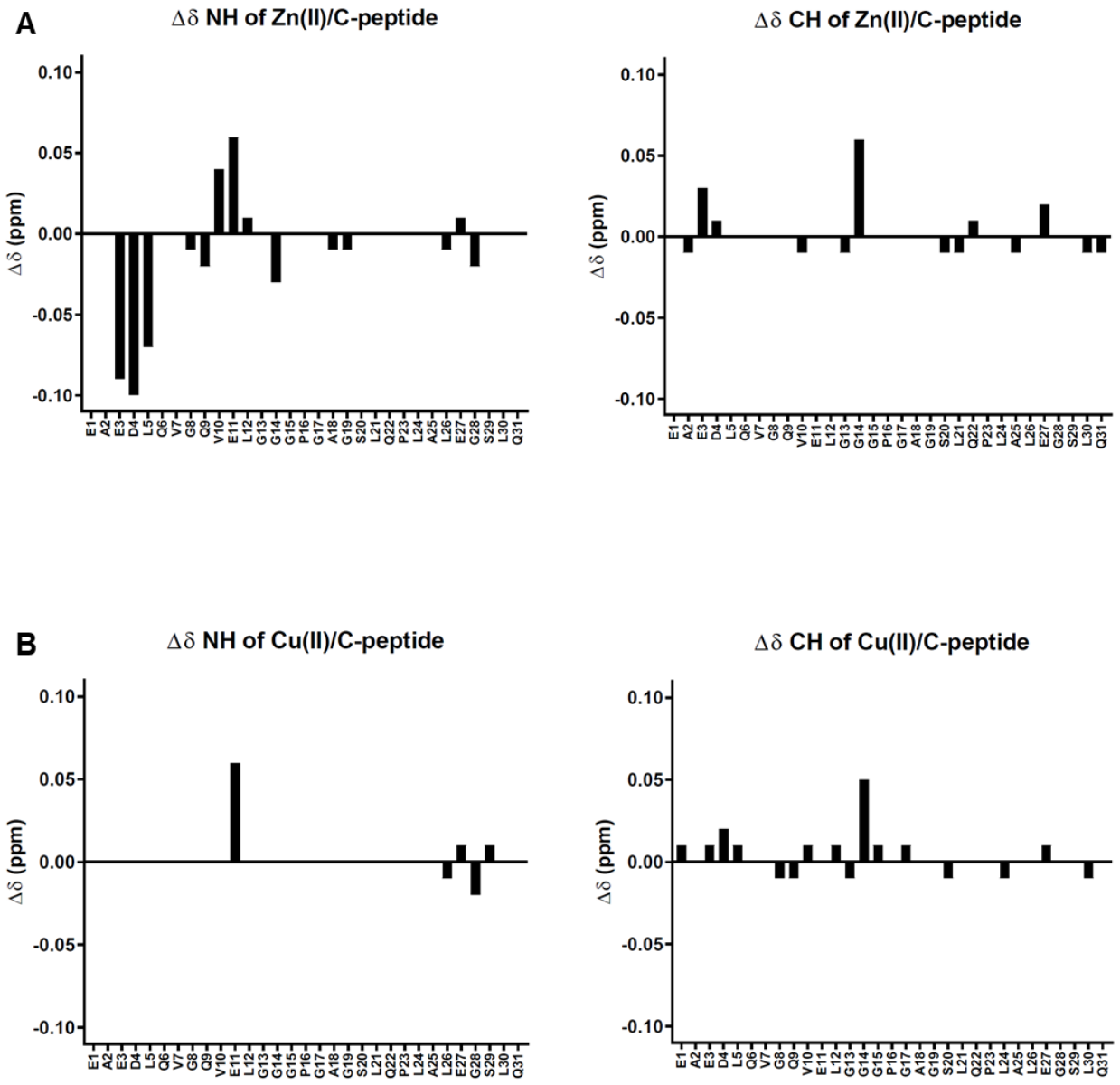

Figure S4. The Δδ (δ(M^{2+}/C-peptide) − δ(apo C-peptide)) of ^{1}H chemical shifts (ppm) of the backbone amide (NH) and CHα protons plotted against C-peptide sequence for A) Zn(II)/C-peptide and B) Cu(II)/C-peptide. Δδ plots for the backbone side chain protons are shown in Figure S5. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. The Δδ of NH for E3, D4, L5, V10, and E11 show significant effects on the ^{1}H resonances compared with other NH protons and indicate a possible location of metal binding. ^{1}H chemical shift assignments are tabulated in Table S1-S5.

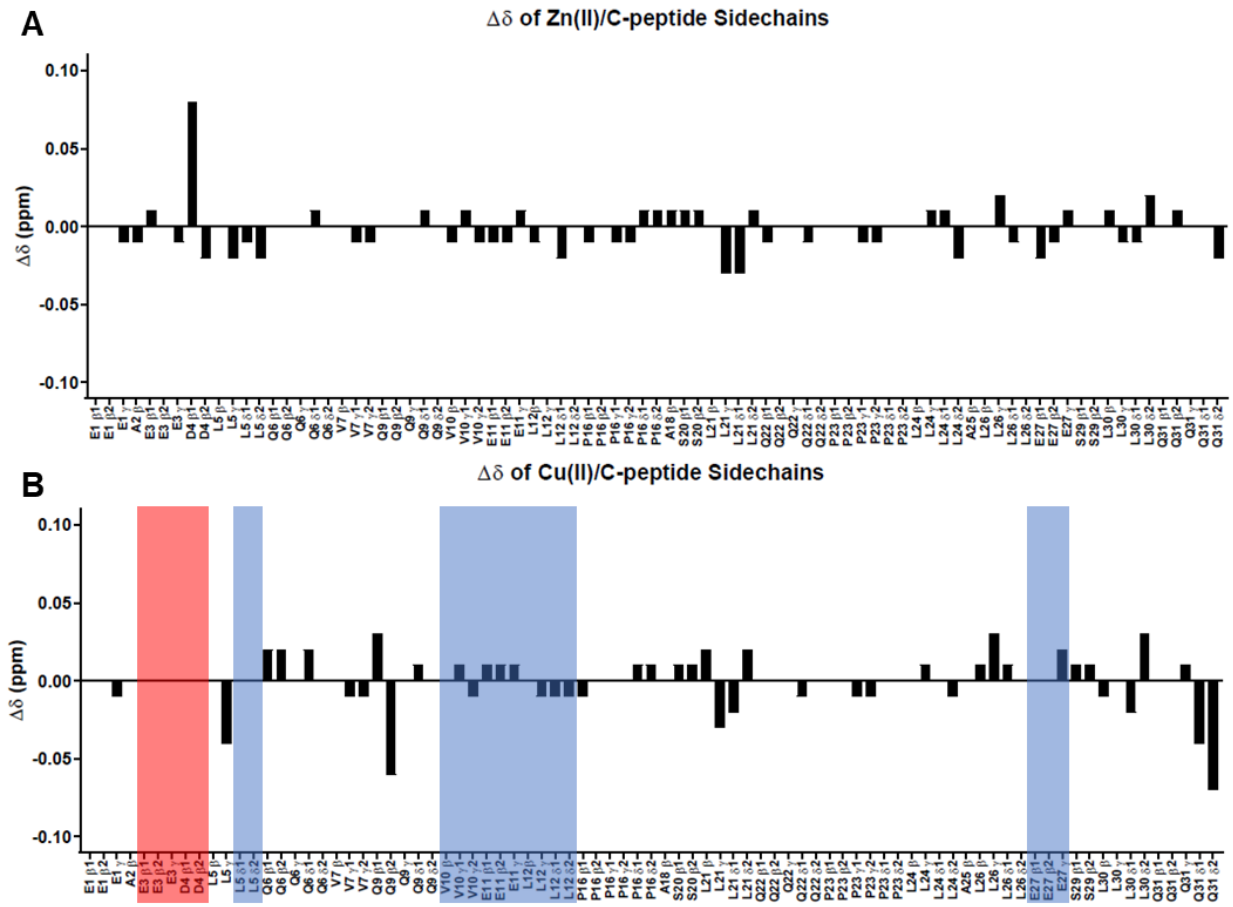

Figure S5. The Δδ (δ(M^{2+}/C-peptide) − δ(apo WT C-peptide)) of ^{1}H chemical shifts (ppm) of amino acid side chains plotted against WT C-peptide sequence for A) Zn(II)/C-peptide and B) Cu(II)/C-peptide. Δδ plots for the backbone amide and CHα protons are shown in Figure S4. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. The proton resonances with shading indicate reduced (blue) or obliterated (red) intensities. Coordination by Zn(II) on D4 and by Cu(II) on E3 and D4 show significant effects on the ^{1}H resonances compared with other amino acid protons and indicate location of metal binding. ^{1}H chemical shift assignments are tabulated in Tables S1-S5.

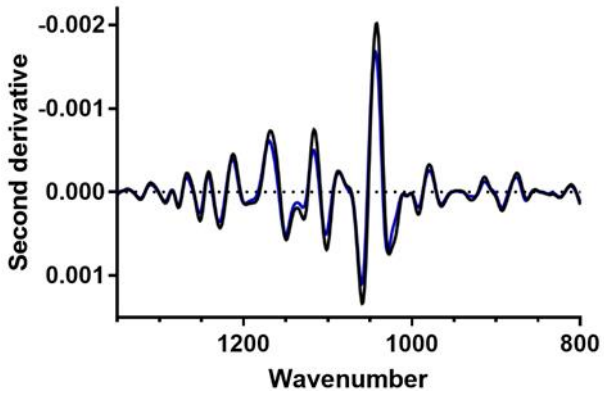

Figure S6. Second derivative FTIR spectra of WT C-peptide (black) and Cu(II)/C-peptide (blue) at low wavenumbers. The second derivative patterns between apo and Cu(II)-bound peptides are similar in this region indicating that the stretching and bending frequencies of serine are not affected by Cu(II).

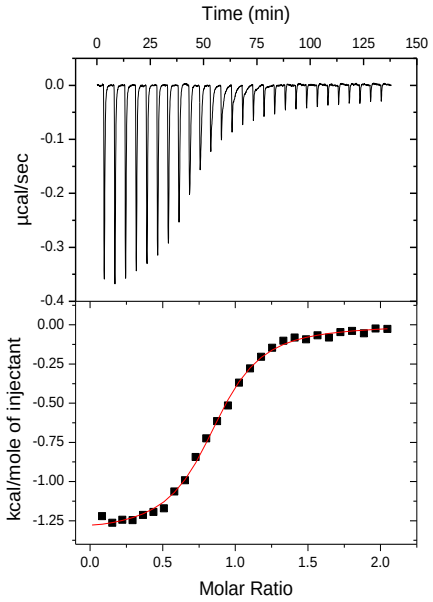

Figure S7. Representative thermogram of 1.0 mM Cu(II) titrated into 100 µM C-peptide in 15 mM MOPSO, pH 7.4 with red fit line: n = 0.852 ± 0.006; K_{ITC} = 3.0 (± 0.2) x 10^5; ΔH_{ITC} = −1.33 ± 0.01 kcal mol^{-1}. Buffer-independent thermodynamics are summarized in Table 2 and indicate a predominantly entropic driving force.

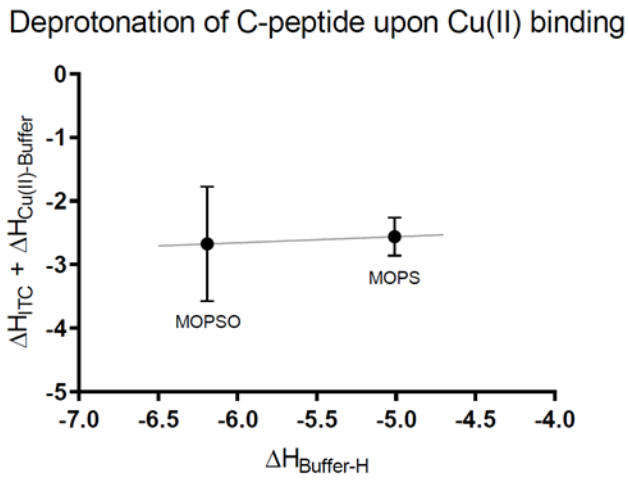

Figure S8. Analysis to determine the number of protons that are displaced from C-peptide upon Cu(II) binding. Protonation plot of $\Delta H_{ITC} + \Delta H_{Cu(II)-Buffer}$ *vs.* $\Delta H_{Buffer-H}$ has a slope equal to the number of protons as detailed by Grossoehme *et al.*[1] that are binding to the buffer and is equal to 0.1 ± 0.2. The error of the slope is estimated from subtraction of the minimum slope from the maximum slope between the error bars divided by two.

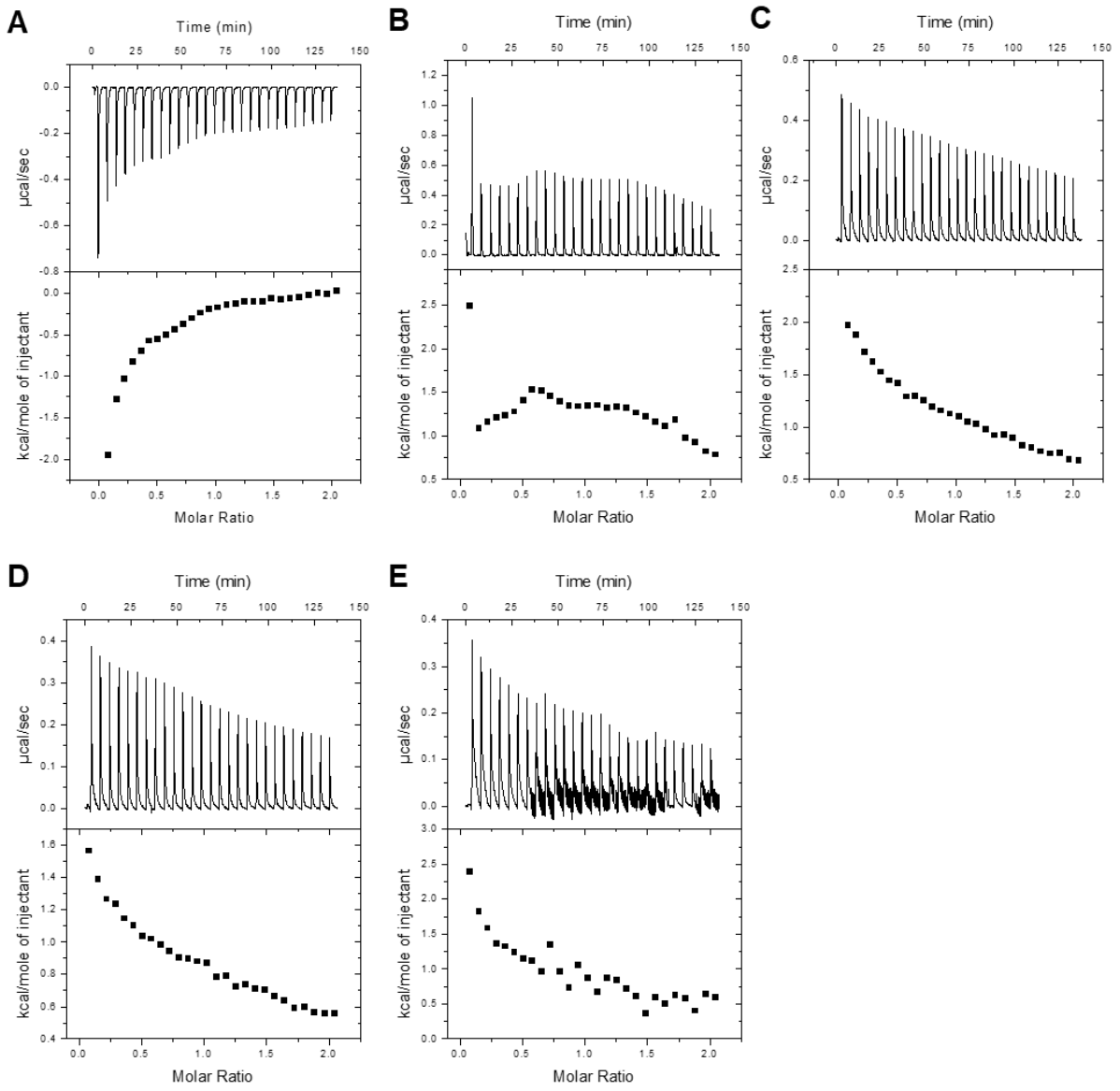

Figure S9. Representative thermograms of 1.0 mM Zn(II) titrated into 100 µM C-peptide in A) 15 mM Tris, pH 7.4, B) 15 mM bisTris, pH 7.4, C) 15 mM MOPS, pH 7.4, D) 15 mM MOPSO, pH 7.4, and E) 15 mM PIPES, pH 7.4. The titrations were inconsistent, do not indicate specific Zn(II)-binding, and are most likely from heat of dilution from the Zn(II) titration into the cell.

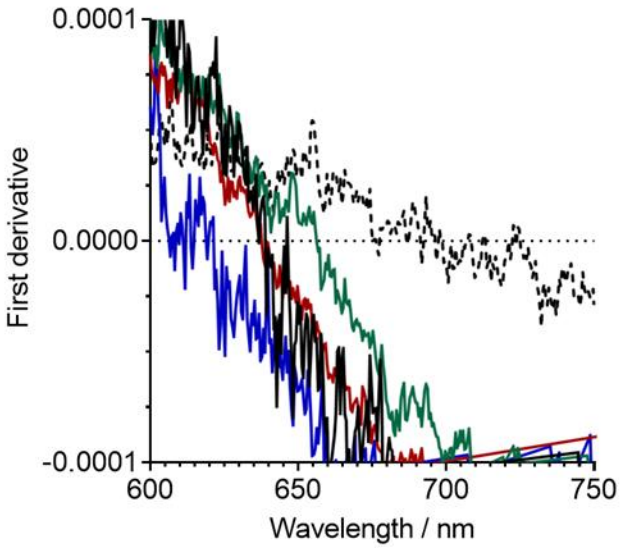

Figure S10. First derivatives of the spectra shown in Figure 7 corresponding to WT C-peptide (black), E3A C-peptide (red), D4A C-peptide (blue), E3A D4A C-peptide (green), and Cu(II) in 15 mM MOPS, pH 7.4 (black, dashed). λ_{max} for the d-d band as determined by the first derivative of WT, E3A, D4A, E3A D4A C-peptide, and Cu(II) in buffer are 638, 637, 620, 658, and 696 nm, respectively. Derivatives of spectra were processed by a 20-point smoothing. The similar energy of the d-d band from Cu(II) suggests that the Cu(II) is bound to similar ligands in all peptides. The λ_{max} of E3A D4A C-peptide is red-shifted, is most similar to buffer alone, and exhibits the largest change in λ_{max} among the mutants relative to WT C-peptide.

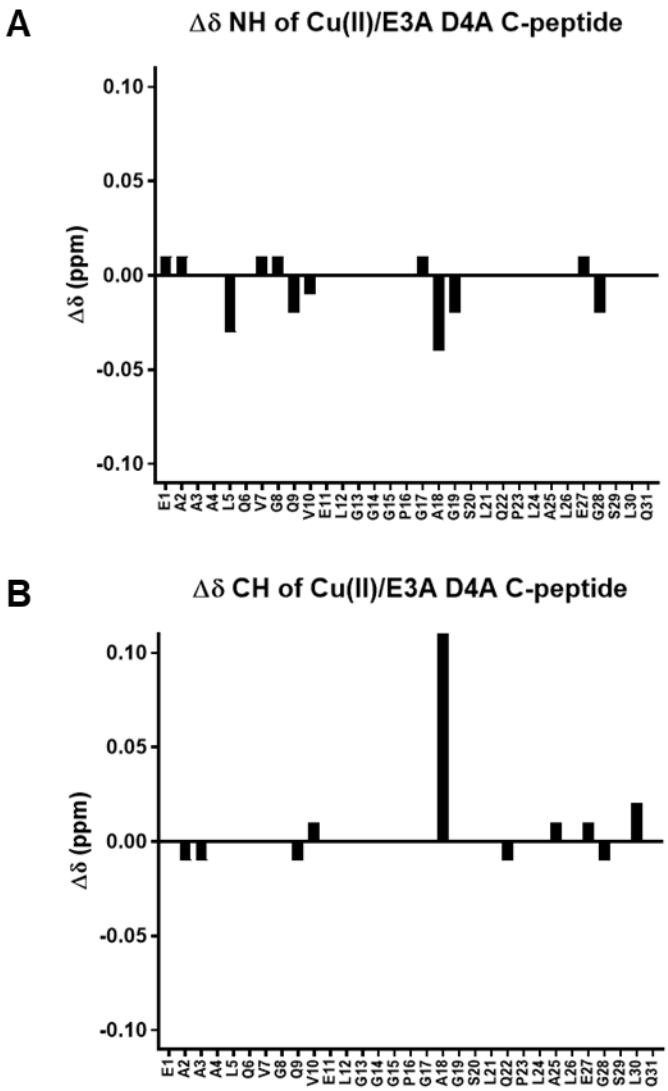

Figure S11. The Δδ (δ(Cu(II)/E3A D4A C-peptide) − δ(apo E3A D4A C-peptide)) of ^{1}H chemical shifts (ppm) of the A) backbone amide (NH) and B) CHα protons plotted against peptide sequence. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Δδ plots for the backbone side chain protons are shown in Figure S12. The small Δδ indicate minimal changes in peptide structure upon Cu(II) binding. ^{1}H chemical shift assignments are tabulated in Tables S7-S9.

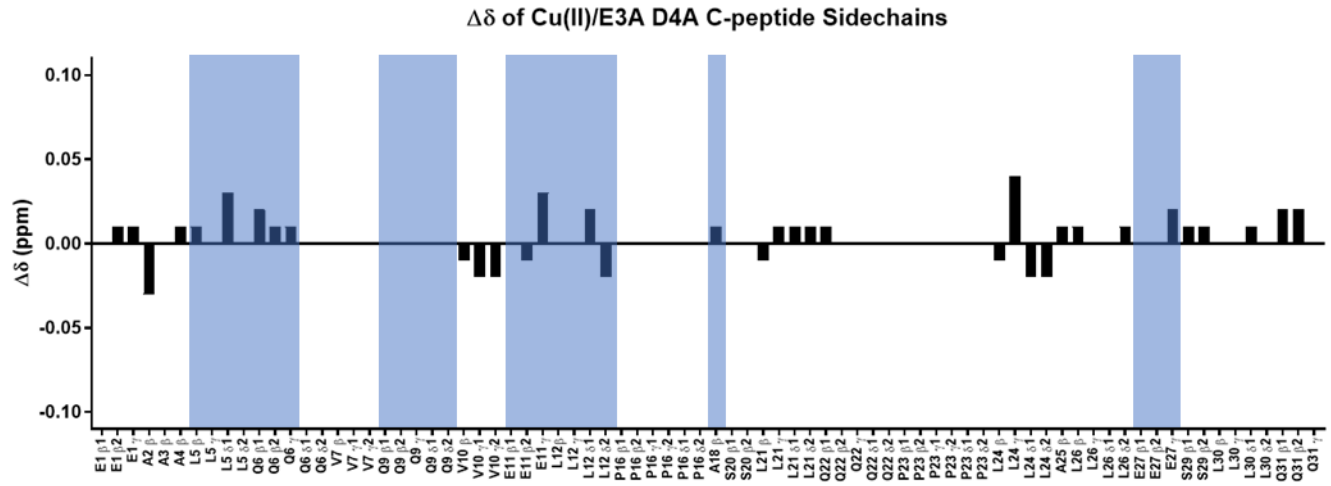

Figure S12. The Δδ (δ(Cu(II)/E3A D4A C-peptide) − δ(apo E3A D4A C-peptide)) of ^{1}H chemical shifts (ppm) of amino acid side chains plotted against peptide sequence. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Δδ plots for the backbone amide and CHα protons are in the Figure S11. The proton resonances with shading indicate reduced (blue) intensities. Mutation of the Cu(II) binding site (E3 and D4) to alanines shifts Cu(II)-binding to other residues (Q6, E11, and E27) that also had reduced intensities in WT and indicate multiple modes of Cu(II) binding where the preferential is E3 and D4. ^{1}H chemical shift assignments are tabulated in Tables S7-S9.

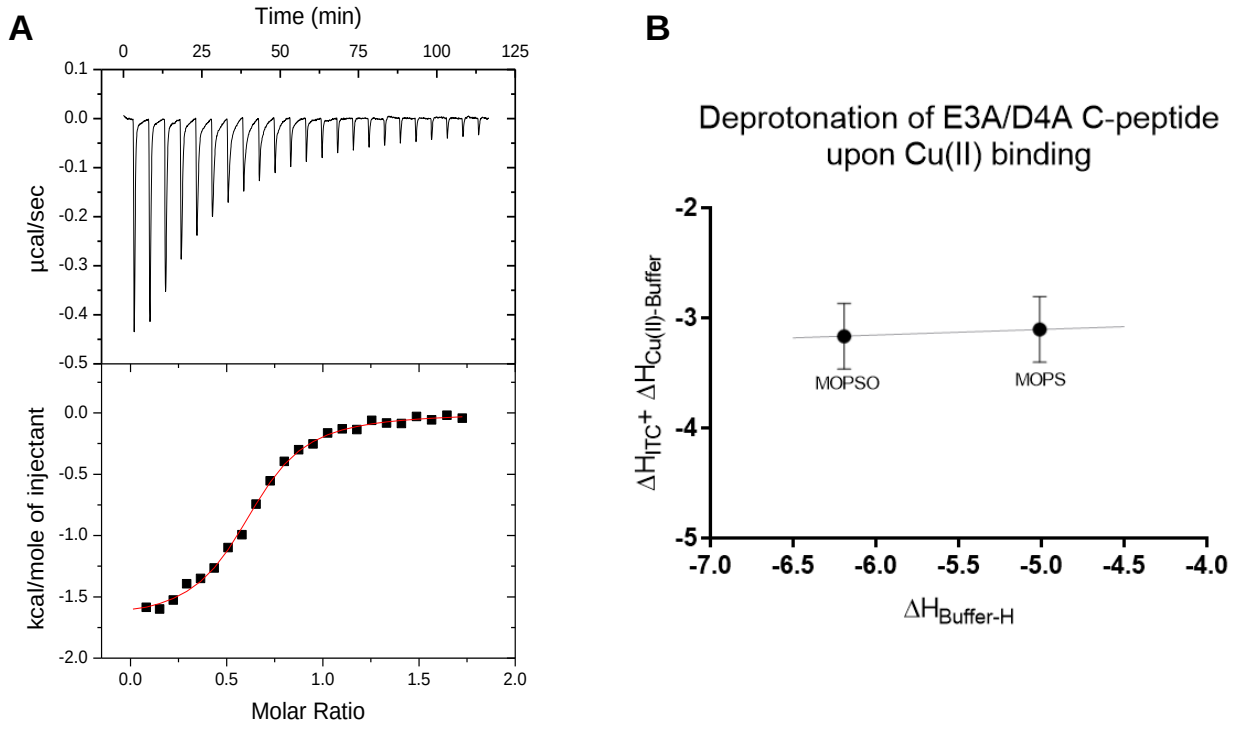

Figure S13. A) Representative thermogram of 1.0 mM Cu(II) titrated into 100 µM E3A/D4A C-peptide in 15 mM MOPSO, pH 7.4 with red fit line: n = 0.621 ± 0.006; K_{ITC} = 2.6 (± 0.2) x 10^5; $\Delta H_{ITC} = -1.70 \pm 0.02$ kcal mol^{-1}. B) Protonation plot of $\Delta H_{ITC} + \Delta H_{Cu(II)\text{-}Buffer}$ *vs.* $\Delta H_{Buffer\text{-}H}$ has a slope equal to the number of protons as detailed by Grossoehme *et al.*[1] that are binding to the buffer and is equal to 0.1 ± 0.6. The large error of the slope is estimated from subtraction of the minimum slope from the maximum slope between the error bars divided by two. Buffer-independent thermodynamics are summarized in Table S10 and indicate a predominantly entropic driving force.

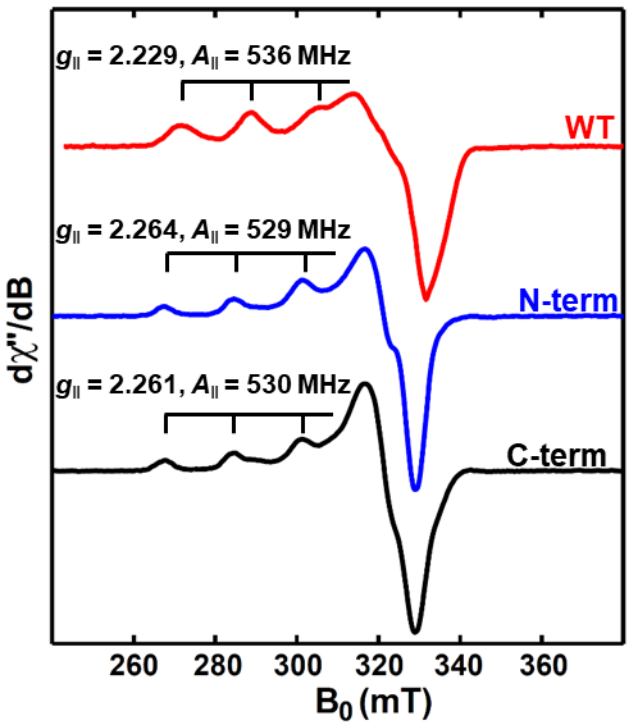

Figure S14. X-band EPR of Cu(II) bound to WT C-peptide (red), N-term (blue), and C-term (black) and collected at 20 K. WT C-peptide from Figure 5 is shown here for clarity of comparisons to truncations. Samples were prepared with 0.5 mM Cu(II) and 1.0 mM C-peptide in 15 mM MOPS, pH 7.4 with 20% ethylene glycol as a glassing agent. These spectra indicate that Cu(II) is bound in a square planar geometry to either 4O or 3O1N coordination.[2]

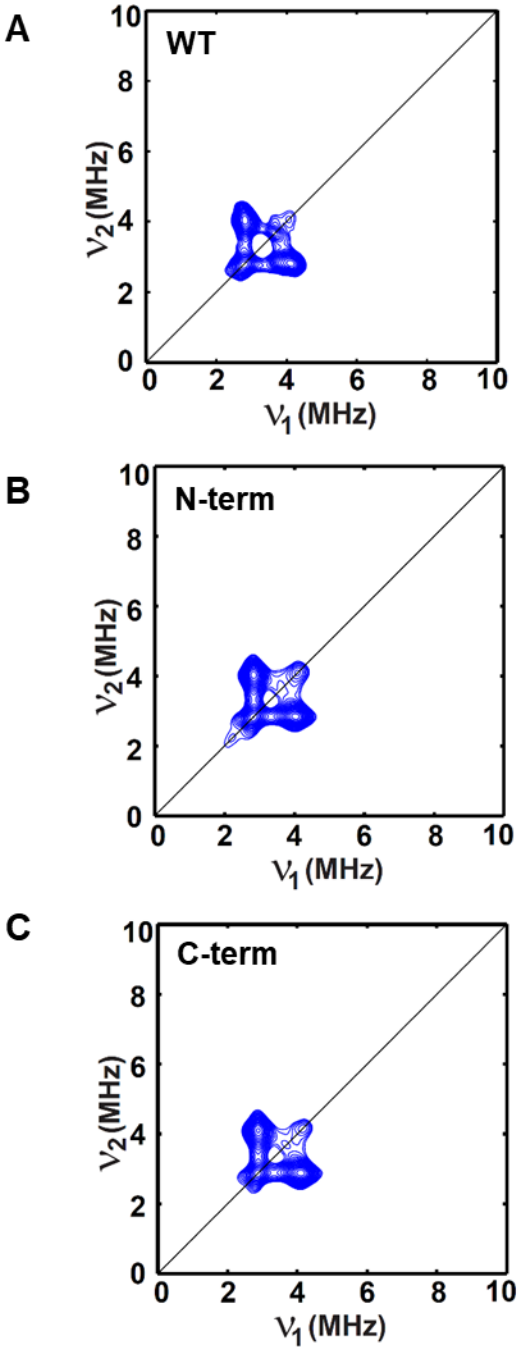

Figure S15. HYSCORE spectra of Cu(II) bound to A) WT C-peptide, B) N-term, and C) C-term and collected at 20 K. WT C-peptide from Figure 5 is shown here for clarity of comparisons to truncations.

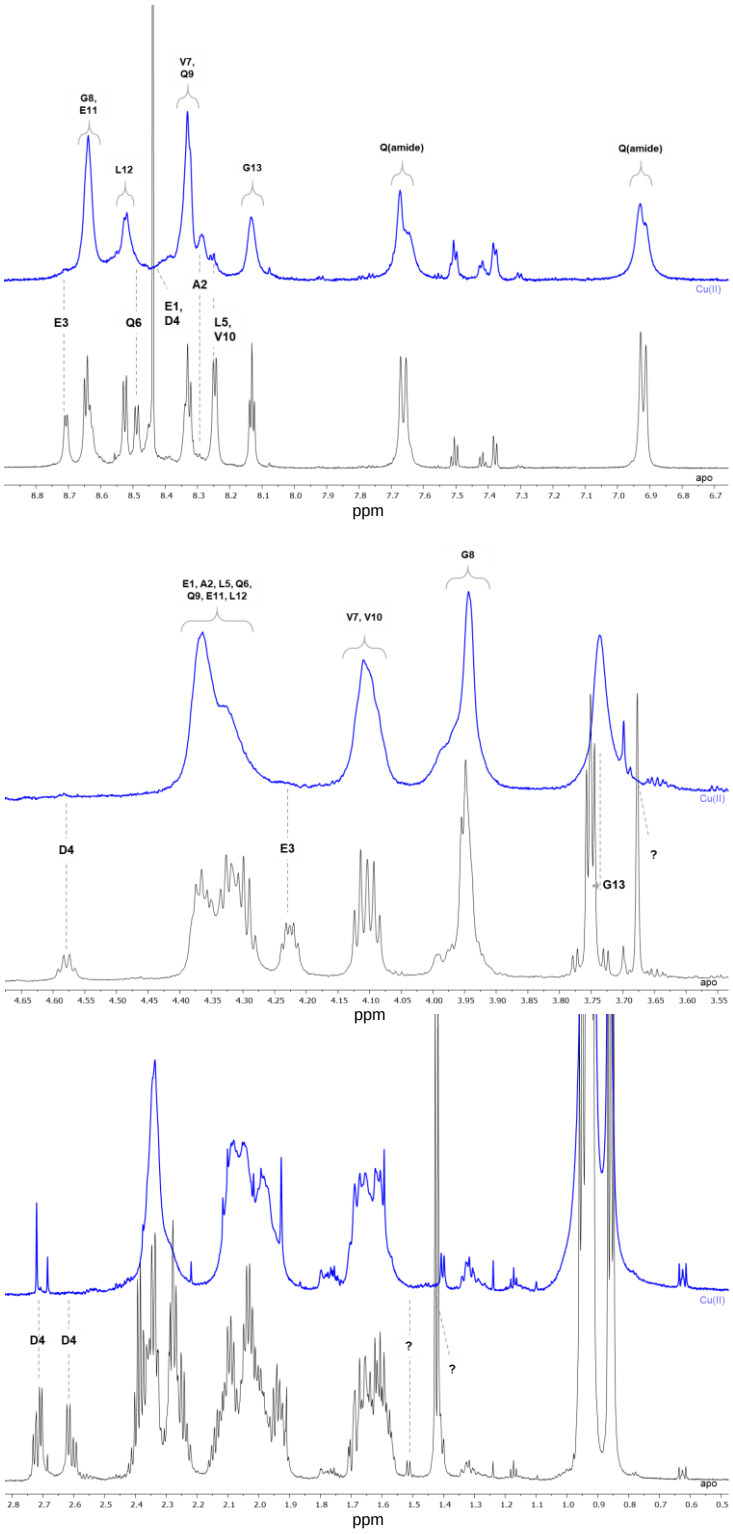

Figure S16. NMR spectra of apo and Cu(II) bound N-term C-peptide with assigned peaks and noted differences when Cu(II) is bound. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C.

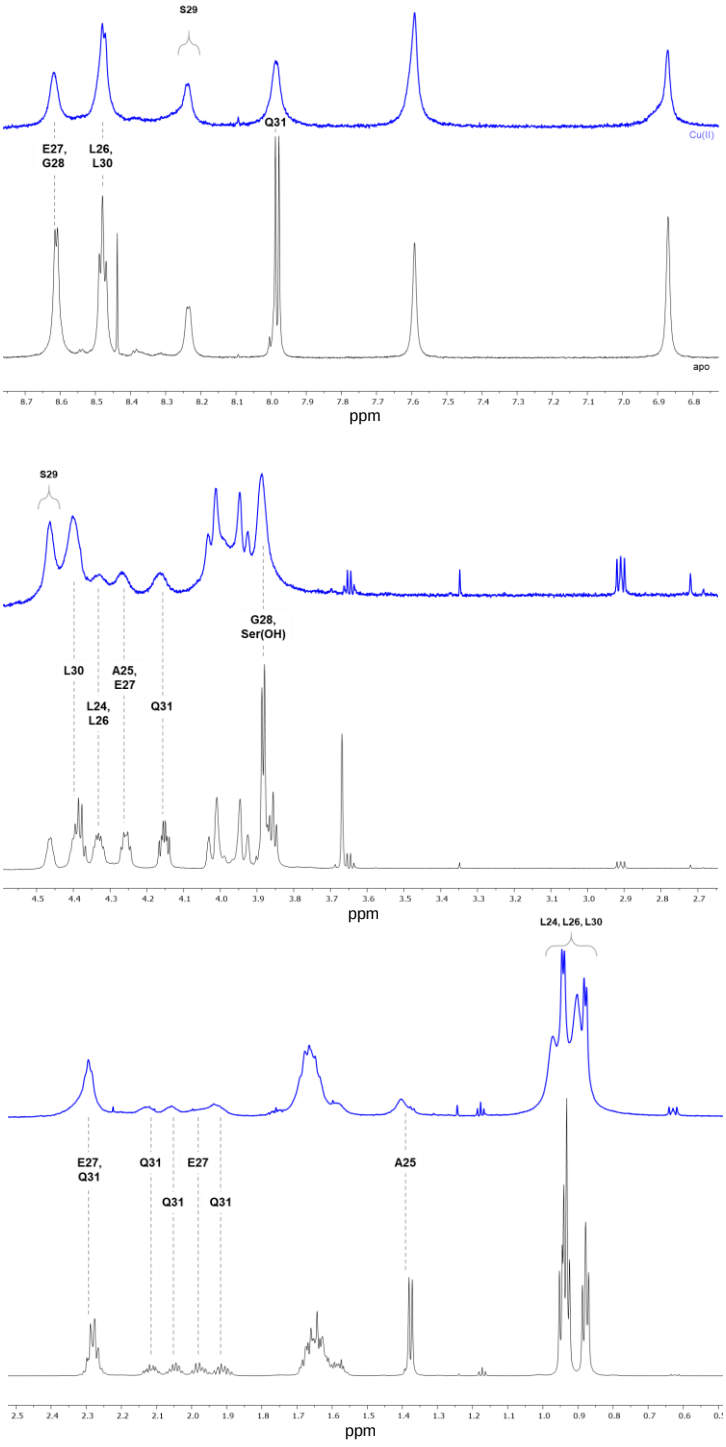

Figure S17. NMR spectra of apo and Cu(II) bound C-term C-peptide with assigned peaks and noted differences when Cu(II) is bound. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 10 °C.

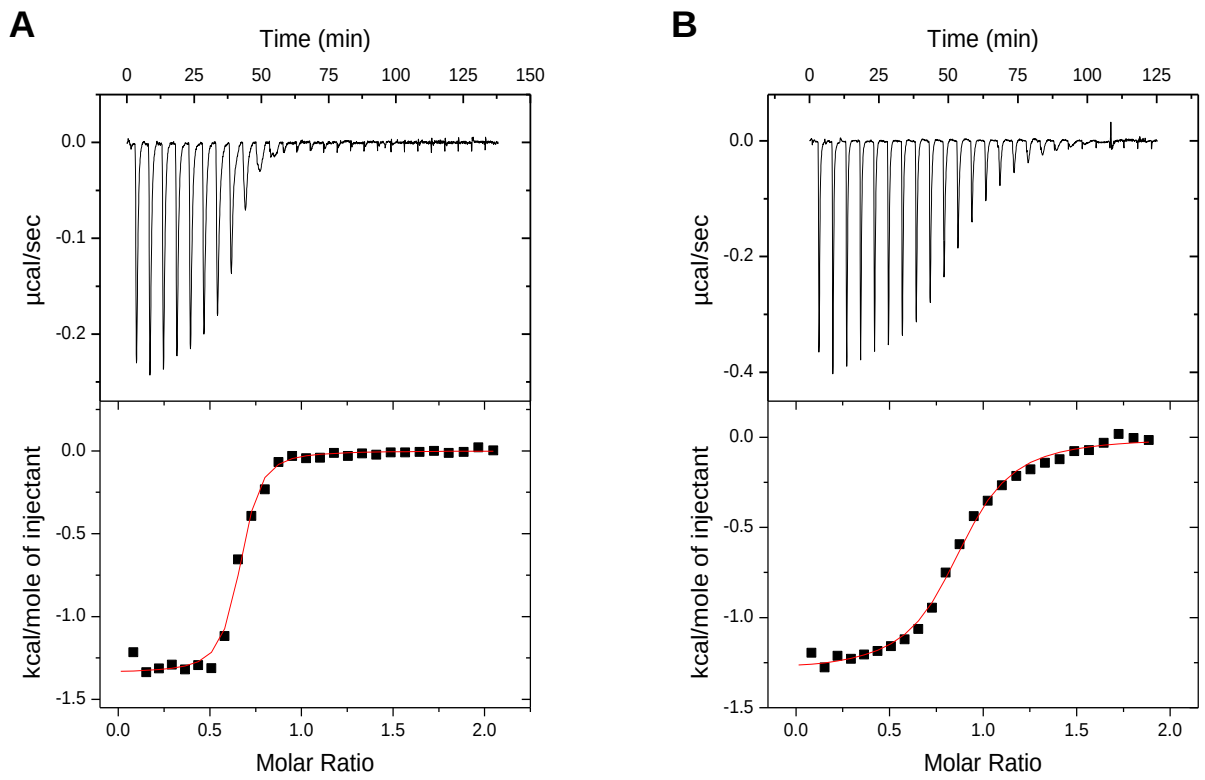

Figure S18. Representative thermogram of 1.0 mM Cu(II) titrated into A) 100 µM N-term and B) 100 µM C-term in 15 mM MOPSO, pH 7.4. Red fit line for A) n = 0.64 ± 0.01; K_{ITC} = 2.2 (± 0.5) x 10^6; ΔHITC = −1.34 ± 0.02 kcal mol-1, and B) n = 0.860 ± 0.009; K_{ITC} = 4.0 (± 0.4) x 10^5; ΔH_{ITC} = −1.30 ± 0.02 kcal mol-1. The average best fit data are summarized in Table S10.

Table S1. Chemical shifts of assigned proton NMR resonances of apo WT C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C.

Chemical Shifts of Assigned ^{1}H NMR Resonances of Apo C-peptide at pH 7.4						
	Chemical Shifts at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	8.04	4.34	1.84, 2.04	2.33	-	-
A2	8.38	4.32	1.37	-	-	-
E3	8.71	4.22	1.94, 2.03	2.28	-	-
D4	8.46	4.57	2.60, 2.72	-	-	-
L5	8.25	4.31	1.64	1.62	0.86, 0.93	-
Q6	8.56	4.25	1.98, 2.04	2.28	-	6.86, 7.59
V7	8.32	4.11	2.03	0.93	-	-
G8	8.62	3.97	-	-	-	-
Q9	8.33	4.36	2.00, 2.09	2.34	-	6.91, 7.66
V10	8.23	4.09	2.09	0.94, 0.96	-	-
E11	8.60	4.30	1.93, 2.02	2.25	-	-
L12	8.54	4.34	1.68	1.63	0.88, 0.91	-
G13	8.40	3.99	-	-	-	-
G14	8.39	3.83	-	-	-	-
G15	8.58	3.97	-	-	-	-
P16	-	4.40	1.90, 2.29	2.02	3.77	-
G17	8.32	3.81	-	-	-	-
A18	8.45	3.98	1.35	-	-	-
G19	8.48	3.98	-	-	-	-
S20	8.24	4.46	3.87			
L21	8.40	4.37	1.63	1.63	0.90, 0.90	-
Q22	8.41	4.60	1.93, 2.09	2.39	-	6.95, 7.61
P23	-	4.41	1.90, 2.28	2.03	3.66	-
L24	8.35	4.33	1.64	1.59	0.89, 0.93	-
A25	8.28	4.31	1.40	-	-	-
L26	8.43	4.28	1.64	1.58	0.90, 0.93	-
E27	8.48	4.33	2.00, 2.12	2.35	-	-
G28	8.67	3.95	-	-	-	-
S29	8.20	4.44	3.86	-	-	-
L30	8.45	4.40	1.66	1.65	0.90, 0.90	-
Q31	7.99	4.16	1.92, 2.12	2.28	-	6.92, 7.66

Table S2. Chemical shifts of assigned proton NMR resonances of Zn(II)-bound WT C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Values in parentheses are a minor feature.

Chemical Shifts of Assigned ^{1}H NMR Resonances of Zn(II)/C-peptide at pH 7.4						
	Chemical Shifts at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	8.04	4.34	1.84, 2.04	2.32	-	-
A2	8.38	4.31	1.36	-	-	-
E3	8.62	4.25	1.95, 2.03	2.27	-	-
D4	8.36 (8.32)	4.58 (4.62)	2.63, 2.70 (2.68)	-	-	-
L5	8.18	4.31	1.64	1.60	0.85, 0.91	
Q6	8.56	4.25	1.98, 2.04	2.28	-	6.87, 7.59
V7	8.32	4.11	2.03	0.92	-	-
G8	8.61	3.97	-	-	-	-
Q9	8.31	4.36	2.00, 2.09	2.34	-	6.92, 7.66
V10	8.27	4.08	2.08	0.95	-	-
E11	8.66	4.30	1.92, 2.01	2.26	-	-
L12	8.55	4.34	1.67	1.63	0.86, 0.91	-
G13	8.40	3.98	-	-	-	-
G14	8.36	3.89	-	-	-	-
G15	8.58	3.97	-	-	-	-
P16	-	4.40	1.89, 2.29	2.01	3.78	-
G17	8.32	3.81	-	-	-	-
A18	8.44	3.98	1.36	-	-	-
G19	8.47	3.98	-	-	-	-
S20	8.24	4.45	3.88	-	-	-
L21	8.40	4.36	1.63	1.60	0.87, 0.91	-
Q22	8.41	4.61	1.92, 2.09	2.39	-	6.94, 7.61
P23	-	4.41	1.90, 2.28	2.02	3.66	-
L24	8.35	4.33	1.64	1.60	0.90, 0.91	-
A25	8.28	4.30	1.40	-	-	-
L26	8.42	4.28	1.64	1.60	0.89, 0.93	-
E27	8.49	4.35	1.98, 2.11	2.36	-	-
G28	8.65	3.95	-	-	-	-
S29	8.20	4.44	3.86	-	-	-
L30	8.45	4.39	1.67	1.64	0.89, 0.92	-
Q31	7.99	4.15	1.92, 2.11	2.28	-	6.92, 7.64

Table S3. Chemical shifts of assigned proton NMR resonances of Cu(II)-bound WT C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. obl = obliterated signal; sm = reduced signal.

Chemical Shifts of Assigned ^{1}H NMR Resonances of Cu(II)/C-peptide at pH 7.4						
	Chemical Shifts at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	8.04	4.35	1.84, 2.04	2.32	-	-
A2	8.38	4.32	1.37 (1.25, 1.47 sm)	-	-	-
E3	8.71	4.23	obl	obl	-	-
D4	8.46	4.59	obl	-	-	-
L5	8.25	4.32	1.64 sm	1.60 sm	obl	-
Q6	8.56	4.25	2.00, 2.06	2.28	-	6.88, 7.59
V7	8.32	4.11	2.03	0.92	-	-
G8	8.62	3.96	-	-	-	-
Q9	8.33	4.35	2.03, 2.03	2.34	-	6.92, 7.66
V10	8.23	4.10	2.09	0.95	-	-
E11	8.66	4.30	1.94, 2.03 sm	2.26 sm	-	-
L12	8.54	4.35	1.68	1.62	0.87, 0.92	-
G13	8.40	3.98	-	-	-	-
G14	8.39	3.88	-	-	-	-
G15	8.58	3.98	-	-	-	-
P16	-	4.40	1.89, 2.29	2.02	3.78	-
G17	8.32	3.82	-	-	-	-
A18	8.45	3.98	1.35	-	-	-
G19	8.48	3.98	-	-	-	-
S20	8.24	4.45	3.88	-	-	-
L21	8.40	4.37	1.65	1.60	0.88, 0.92	-
Q22	8.41	4.60	1.93, 2.09	2.39	-	6.94, 7.61
P23	-	4.41	1.90, 2.28	2.02	3.66	-
L24	8.35	4.32	1.64	1.60	0.89, 0.92	-
A25	8.28	4.31	1.40	-	-	-
L26	8.42	4.28	1.65	1.61	0.91, 0.93	-
E27	8.49	4.34	2.00 sm, 2.00 sm	2.37	-	-
G28	8.65	3.95	-	-	-	-
S29	8.21	4.44	3.87	-	-	-
L30	8.45	4.39	1.65	1.65	0.88, 0.93	-
Q31	7.99	4.16	1.92, 2.12	2.29	-	6.88, 7.59

Table S4. Δδ of assigned proton NMR resonances of Zn(II)-bound WT C-peptide and apo WT C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Visual representations shown in Figures S4 and S5. Values in parentheses are a minor feature.

Δδ of the Assigned ^{1}H NMR Resonances of Zn(II)/C-peptide at pH 7.4						
	Δδ = δ(Zn(II)/C-peptide) - δ(apo C-peptide) at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	0.00	0.00	0.00, 0.00	-0.01		
A2	0.00	-0.01	-0.01			
E3	-0.09	0.03	0.01, 0.00	-0.01		
D4	-0.10 (-0.14)	0.01 (0.05)	0.03, -0.02 (0.08, -0.02)			
L5	-0.07	0.00	0.00	-0.02	-0.01, -0.02	
Q6	0.00	0.00	0.00, 0.00	0.00		0.01, 0.00
V7	0.00	0.00	0.00	-0.01, -0.01		
G8	-0.01	0.00				
Q9	-0.02	0.00	0.00, 0.00	0.00		0.01, 0.00
V10	0.04	-0.01	-0.01	0.01, -0.01		
E11	0.06	0.00	-0.01, -0.01	0.01		
L12	0.01	0.00	-0.01	0.00	-0.02, 0.00	
G13	0.00	-0.01				
G14*	-0.03	0.06				
G15*	0.00	0.00				
P16		0.00	-0.01, 0.00	-0.01	0.01	
G17	0.00	0.00				
A18	-0.01	0.00	0.01			
G19	-0.01	0.00				
S20	0.00	-0.01	0.01			
L21	0.00	-0.01	0.00	-0.03	-0.03, 0.01	
Q22	0.00	0.01	-0.01, 0.00	0.00		-0.01, 0.00
P23		0.00	0.00, 0.00	-0.01	0.00	
L24	0.00	0.00	0.00	0.01	0.01, -0.02	
A25	0.00	-0.01	0.00			
L26	-0.01	0.00	0.00	0.02	-0.01, 0.00	
E27	0.01	0.02	-0.02, -0.01	0.01		
G28	-0.02	0.00				
S29	0.00	0.00	0.00			
L30	0.00	-0.01	0.01	-0.01	-0.01, 0.02	
Q31	0.00	-0.01	0.00, 0.01	0.00		0.00, -0.02

Table S5. Δδ of assigned proton NMR resonances of Cu(II)-bound WT C-peptide and apo WT C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Visual representations shown in Figures S4 and S5. obl = obliterated signal; sm = reduced signal.

Δδ of the Assigned ^{1}H NMR Resonances of Cu(II)/C-peptide at pH 7.4						
	Δδ = δ(Cu(II)/C-peptide) - δ(apo C-peptide) at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	0.00	0.01	0.00, 0.00	-0.01		
A2	0.00	0.00	0.00 (-0.12 sm, 0.10 sm)			
E3	0.00	0.01	obl	obl		
D4	0.00	0.02	obl			
L5	0.00	0.01	0.00 sm	-0.04 sm	obl	
Q6	0.00	0.00	0.02, 0.02	0.00		0.02, 0.00
V7	0.00	0.00	0.00	-0.01, -0.01		
G8	0.00	-0.01				
Q9	0.00	-0.01	0.03, -0.06	0.00		0.01, 0.00
V10	0.00	0.01	0.00	0.01, -0.01		
E11	0.06	0.00	0.01, 0.01 sm	0.01 sm		
L12	0.00	0.01	0.00	-0.01	-0.01, -0.01	
G13	0.00	-0.01				
G14*	0.00	0.05				
G15*	0.00	0.01				
P16		0.00	-0.01, 0.00	0.00	0.01	
G17	0.00	0.01				
A18	0.00	0.00	0.00			
G19	0.00	0.00				
S20	0.00	-0.01	0.01			
L21	0.00	0.00	0.02	-0.03	-0.02, 0.02	
Q22	0.00	0.00	0.00, 0.00	0.00		-0.01, 0.00
P23		0.00	0.00, 0.00	-0.01	0.00	
L24	0.00	-0.01	0.00	0.01	0.00, -0.01	
A25	0.00	0.00	0.00			
L26	-0.01	0.00	0.01	0.03	0.01, 0.00	
E27	0.01	0.01	0.00 sm, 0.00 sm	0.02		
G28	-0.02	0.00				
S29	0.01	0.00	0.01			
L30	0.00	-0.01	-0.01	0.00	-0.02, 0.03	
Q31	0.00	0.00	0.00, 0.00	0.01		-0.04, -0.07

Table S6. Experimental fit parameters from at least three titrations in each buffer.

Buffer	n_{ITC}	K_{ITC}	ΔH_{ITC} (kcal mol^{-1})
MOPS	0.8 ± 0.2	2 (± 1) × 10^6	-1.3 ± 0.2
MOPSO	1.0 ± 0.1	5 (± 2) × 10^5	-1.31 ± 0.03

Table S7. Chemical shifts of assigned proton NMR resonances of apo E3A D4A C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C.

Chemical Shifts of Assigned ^{1}H NMR Resonances of Apo E3A D4A C-peptide at pH 7.4						
	Chemical Shifts at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	8.04	4.34	1.84, 2.03	2.31	-	-
A2	8.44	4.31	1.38	-	-	-
A3	8.40	4.28	1.37	-	-	-
A4	8.37	4.31	1.36	-	-	-
L5	8.30	4.31	1.62	1.56	0.89, 0.92	-
Q6	8.56	4.25	1.98, 2.04	2.28	-	6.87, 7.59
V7	8.31	4.12	2.05	0.92	-	-
G8	8.66	3.96	-	-	-	-
Q9	8.32	4.36	2.00, 2.09	2.34	-	6.92, 7.67
V10	8.35	4.09	2.06	0.94, 0.94	-	-
E11	8.66	4.30	1.94, 2.04	2.25	-	-
L12	8.54	4.34	1.67	1.61	0.88, 0.92	-
G13	n.d.	n.d.	-	-	-	-
G14	n.d.	n.d.	-	-	-	-
G15	n.d.	n.d.	-	-	-	-
P16	-	n.d.	n.d.	n.d.	n.d.	-
G17	8.39	3.98	-	-	-	-
A18	8.54	4.25	1.37	-	-	-
G19	8.58	3.97	-	-	-	-
S20	8.24	4.45	3.87	-	-	-
L21	8.40	4.37	1.63	1.61	0.88, 0.93	-
Q22	8.41	4.61	1.93, 2.09	2.39	-	6.94, 7.61
P23	-	4.61	1.92, 2.39	2.09	3.65, 3.77	-
L24	8.35	4.32	1.63	1.58	0.95, 0.95	-
A25	8.27	4.30	1.40	-	-	-
L26	8.42	4.28	1.63	1.58	0.91, 0.93	-
E27	8.47	4.37	1.97, 2.09	2.35	-	-
G28	8.61	3.98	-	-	-	-
S29	8.20	4.44	3.86	-	-	-
L30	8.45	4.38	1.66	1.66	0.88, 0.93	-
Q31	7.99	4.15	1.9, 2.11	2.28	-	6.87, 7.59

Table S8. Chemical shifts of assigned proton NMR resonances of Cu(II)-bound E3A D4A C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C.

Chemical Shifts of Assigned ^{1}H NMR Resonances of Cu(II)/E3A D4A C-peptide at pH 7.4						
	Chemical Shifts at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	8.05	4.34	1.84, 2.04	2.32	-	-
A2	8.45	4.30	1.35	-	-	-
A3	8.40	4.27	1.37	-	-	-
A4	8.37	4.31	1.37	-	-	-
L5	8.27	4.31	1.63	1.56	0.92, 0.92	-
Q6	8.56	4.25	2.00, 2.05	2.29	-	n.d.
V7	8.32	4.12	2.05	0.92	-	-
G8	8.67	3.96	-	-	-	-
Q9	8.30 and 8.32	4.35	2.00, 2.09	2.34	-	n.d.
V10	8.34	4.10	2.05	0.92	-	-
E11	8.66	4.30	1.94, 2.03	2.28	-	-
L12	8.54	4.34	1.67	1.61	0.90, 0.90	-
G13	n.d.	n.d.	-	-	-	-
G14	n.d.	n.d.	-	-	-	-
G15	n.d.	n.d.	-	-	-	-
P16	-	n.d.	n.d.	n.d.	n.d.	-
G17	8.40	3.98	-	-	-	-
A18	8.50	4.37	1.38	-	-	-
G19	8.56	3.97	-	-	-	-
S20	8.24	4.45	3.87	-	-	-
L21	8.40	4.37	1.62	1.62	0.89, 0.94	-
Q22	8.41	4.60	1.94, 2.09	2.39	-	6.94, 7.61
P23	-	n.d.	n.d.	n.d.	n.d.	-
L24	8.35	4.32	1.62	1.62	0.93, 0.93	-
A25	8.27	4.31	1.41	-	-	-
L26	8.42	4.28	1.64	1.58	0.91. 0.94	-
E27	8.48	4.38	1.97, 2.09	2.37	-	-
G28	8.59	3.97	-	-	-	-
S29	8.20	4.44	3.87	-	-	-
L30	8.45	4.40	1.66	1.66	0.89, 0.93	-
Q31	7.99	4.15	1.92, 2.13	2.28	-	n.d.

Table S9. Δδ of assigned proton NMR resonances of Cu(II)-bound E3A D4A C-peptide and apo E3A D4A C-peptide. Solutions were prepared at 1.5 mM peptide in 95:5 (v/v) H_2O:D_2O with 10 mM Tris-d_{11} at pH 7.4 and spectra were collected at 800 MHz and 10 °C. Visual representations shown in Figures S11 and S12.

Δδ of the Assigned ^{1}H NMR Resonances of Cu(II)/E3A D4A C-peptide at pH 7.4						
	Δδ = δ(Cu(II)/C-peptide) - δ(apo C-peptide) at 10 °C (ppm)					
Residue	NH	CHα	CHβ	CHγ	CHδ	NHγ
E1	0.01	0.00	0.00, 0.01	0.01	-	-
A2	0.01	-0.01	-0.03	-	-	-
A3	0.00	-0.01	0.00	-	-	-
A4	0.00	0.00	0.01	-	-	-
L5	-0.03	0.00	0.01	0.00	0.03, 0.00	-
Q6	0.00	0.00	0.02, 0.01	0.01	-	n.d.
V7	0.01	0.00	0.00	0.00	-	-
G8	0.01	0.00	-	-	-	-
Q9	-0.02 and 0.00	-0.01	0.00, 0.00	0.00	-	n.d.
V10	-0.01	0.01	-0.01	-0.02, -0.02	-	-
E11	0.00	0.00	0.00, -0.01	0.03	-	-
L12	0.00	0.00	0.00	0.00	0.02, -0.02	-
G13	n.d.	n.d.	-	-	-	-
G14	n.d.	n.d.	-	-	-	-
G15	n.d.	n.d.	-	-	-	-
P16	-	n.d.	n.d.	n.d.	n.d.	-
G17	0.01	0.00	-	-	-	-
A18	-0.04	0.12	0.01	-	-	-
G19	-0.02	0.00	-	-	-	-
S20	0.00	0.00	0.00	-	-	-
L21	0.00	0.00	-0.01	0.01	0.01, 0.01	-
Q22	0.00	-0.01	0.01, 0.00	0.00	-	0.00, 0.00
P23	-	n.d.	n.d.	n.d.	n.d.	-
L24	0.00	0.00	-0.01	0.04	-0.02, -0.02	-
A25	0.00	0.01	0.01	-	-	-
L26	0.00	0.00	0.01	0.00	0.00, 0.01	-
E27	0.01	0.01	0.00, 0.00	0.02	-	-
G28	-0.02	-0.01	-	-	-	-
S29	0.00	0.00	0.01	-	-	-
L30	0.00	0.02	0.00	0.00	0.01, 0.00	-
Q31	0.00	0.00	0.02, 0.02	0.00	-	n.d.

Table S10. ITC experimental fit parameters from 1.0 mM Cu(II) titrated into 100 µM peptide in 15 mM buffer, pH 7.4 at 25 °C. Titrations were in triplicate for all WT C-peptide, N-term, and C-term, and in duplicate for E3A/D4A C-peptide. The data show that E3A/D4A C-peptide has a smaller n_{ITC} indicating 1:2 Cu(II):peptide stoichiometry and that C-term has a smaller K_{ITC} than the other peptides.

Variant	Buffer	n_{ITC}	K_{ITC}	ΔH_{ITC} (kcal mol^{-1})	$K_{Cu(II)/C\text{-}peptide}$	$\Delta H_{Cu(II)/C\text{-}peptide}$ (kcal mol^{-1})
WT	MOPS	0.8 ± 0.2	2 (± 1) × 10^6	-1.3 ± 0.2	2 (± 1) × 10^8	-2 ± 1
	MOPSO	1.0 ± 0.1	5 (± 2) × 10^5	-1.31 ± 0.03	6 (± 3) × 10^7	-2 ± 1
E3A/D4A	MOPS	0.5 ± 0.2	1 (± 0.6) × 10^6	-1.8 ± 0.2	9 (± 6) × 10^7	-3 ± 1
	MOPSO	0.55 ± 0.07	4 (± 1) × 10^5	-1.80 ± 0.09	4 (± 1) × 10^7	-2.8 ± 0.8
N-term	MOPS	0.6 ± 0.2	2.0 (± 0.7) × 10^6	-1.1 ± 0.4	n.d.	n.d.
C-term	MOPS	0.7 ± 0.1	3 (± 1) × 10^5	-1.28 ± 0.06	n.d.	n.d.

Scheme S1. Determination of $\Delta H_{Cu(II)\text{-}MOPSO}$. Titrations of 1.0 mM Cu(II) into 100 µM EDTA in 15 mM MOPSO, pH 7.4 with fit parameters from three experiments: $n_{ITC} = 0.91 \pm 0.09$; $K_{ITC} = 4 (\pm 1) \times 10^7$; $\Delta H_{ITC} = -7.5 \pm 0.9$ kcal mol^{-1}. $\Delta H_{Cu(II)\text{-}MOPSO}$ is found to be −1.4 kcal mol^{-1}. All ΔH values are found in NIST.[3] All competing equilibria are accounted for as described by Grossoehme *et al.*[1]

Equlibria	**n**	**ΔH (kcal mol^{-1})**	**n×ΔH (kcal mol^{-1})**
Cu(II)-MOPSO → Cu(II) + MOPSO	1	−X	−X
EDTA-2H$^+$ → EDTA-H$^+$ + H$^+$	0.05	4.2	0.2142
EDTA-H$^+$ → EDTA + H$^+$	0.99	5.6	5.544
MOPSO + H$^+$ → MOPSO-H$^+$	1.04	−6.19	−6.4376
Cu(II) + EDTA → Cu(II)-EDTA	1	−8.2	−8.2

$\Delta H_{ITC} = -X - 8.8794$

$X = -\Delta H_{ITC} - 8.8794$

$X = \Delta H_{Cu(II)\text{-}MOPSO} = -1.364$

References

(1) Grossoehme, N. E.; Spuches, A. M.; Wilcox, D. E. Application of Isothermal Titration Calorimetry in Bioinorganic Chemistry. *J. Biol. Inorg. Chem.* **2010**, *15* (8), 1183–1191. https://doi.org/10.1007/s00775-010-0693-3.

(2) Peisach, J.; Blumberg, W. E. Structural Implications Derived from the Analysis of Electron Paramagnetic Resonance Spectra of Natural and Artificial Copper Proteins. *Arch. Biochem. Biophys.* **1974**, *165* (2), 691–708. https://doi.org/Doi: 10.1016/0003-9861(74)90298-7.

(3) NIST Critically Selected Stability Constants of Metal Complexes, Version 8.0.