Supplementary Information for:

How trimerization of CTR1 N-terminal model peptides tune Cu-binding and redox-chemistry

Thibaut Galler,[a] Vincent Lebrun,[a] Laurent Raibaut,[a] Peter Faller,*[a] Nina E. Wezynfeld *[a,b]

[a] Institut de Chimie, UMR 7177, CNRS-Universitéde Strasbourg, 4 rue Blaise Pascal, 67000 Strasbourg, France. E-mail: pfaller@unistra.fr

[b] Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, 00-664 Warsaw, Poland. E-mail: nwezynfeld@ch.pw.edu.pl

Contents

Experimental section:

1. Abbreviations

Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, High Performance Liquid Chromatography; RP, Reverse Phase; UHPLC, Ultra High Performance Liquid Chromatography; ESI, electrospray ionization; MS, mass spectrometry; LCQ, liquid chromatography quadripole; TBMB, 1,3,5-Tris(bromomethyl)benzene; TIS, triisopropylsilane; ACN, acetonitrile; Asc, ascorbate; DCM, Dichloromethane; Ac_2O, acetic anhydride; DIC, Diisopropylcorbodiimide; TFA, Trifluoroacetic acid; DIEA, Diisopropylethylamine; DMF, N,N-Dimethylformamide; Fmoc, fluorenylmethoxycarbonyl protecting group; Oxyma, Ethyl cyanohydroxyiminoacetate; NH_4HCO_3, Ammonium Hydrogenocarbonate; BCA, Bicinchoninic acid. DTT, 1,4-Dithiothreitol.

2. Reagents and solvents

All reagents and solvents were purchased from commercial suppliers and used without further purification.
Fmoc amino acids were purchased from Novabiochem (Merck). TFA, piperidine, Oxyma, TIS, thianisole, and DIEA were purchased from Merck and DIC were purchased from IRIS Biotech. Ac_2O, DTT, TBMB, BCA, ether and NH_4HCO_3 was purchased from Sigma-Aldrich. Fmoc-Rink Amide AM resin was obtained from Rapp Polymer GmbH. Asc was purchased from Alfa Easar and DCM were obtained from Carlo Erba. DMF and ACN were purchased from Fischer Chemical.

3. Peptide synthesis of MDHS-NH_2, MDHSHH-NH_2, MDHSGGGC-NH_2 and MDHSHHGGGC-NH_2

Peptide synthesis of MDHS-NH_2, MDHSGGGC-NH_2 and MDHSHH-NH_2, MDHSHHGGGC-NH_2 peptides was performed using Fmoc/*t*Bu protocol on Biotage® Initiator+ Alstra™ Automated Microwave Peptide synthesizer on a Fmoc-Rink Amide AM resin (Br-1330 Iris Biotech GMBH, 0.74 mmol/g).

Double couplings (60 min) were performed using a 4-fold molar excess of Fmoc-L-amino acid, a 3.8-fold molar excess of DIC/Oxyma, and a 20-fold molar excess of DIEA at room temperature. A capping step was performed after each coupling with Ac_2O/DIEA in DMF (2 min). Fmoc removal was performed using 20% piperidine in DMF (3 × 5 min). A final cleavage was performed for 2-3 h using a mixture of 93% TFA, 3% TIS, 1% thioanisole, 3% water with addition of 0.1 M DTT to avoid Cys oxidation. Crude peptides were precipitated by the addition of cold diethyl ether.

Then, the crudes were purified by preparative liquid chromatography with a XBridge peptide BEH C18 column (130 Å, 5 µm, 19 mm × 150 mm) and the eluent was a mix of solutions A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN/H_2O 9:1). The purification method consisted in a gradient of eluent from 2% to 30% of B within 15 min, and then from 30% to 100% within the next 15 min (flow: 14 ml/min). The purity of the peptides was checked using an analytic RP-HPLC on a C18 column (XBridge® Peptide BEH C18 OBD™ Prep Column from Waters, 4.6 mm x 150 mm, pore size 300 Å, particle size 3.5 µm) using a Primaide Diode Array Detector instrument with UV-vis detection at 214 nm and 254 nm and LCQ Fleet ESI-MS equipped with a Vanquish UHPLC (Thermo-Scientific).

4. Trimer synthesis

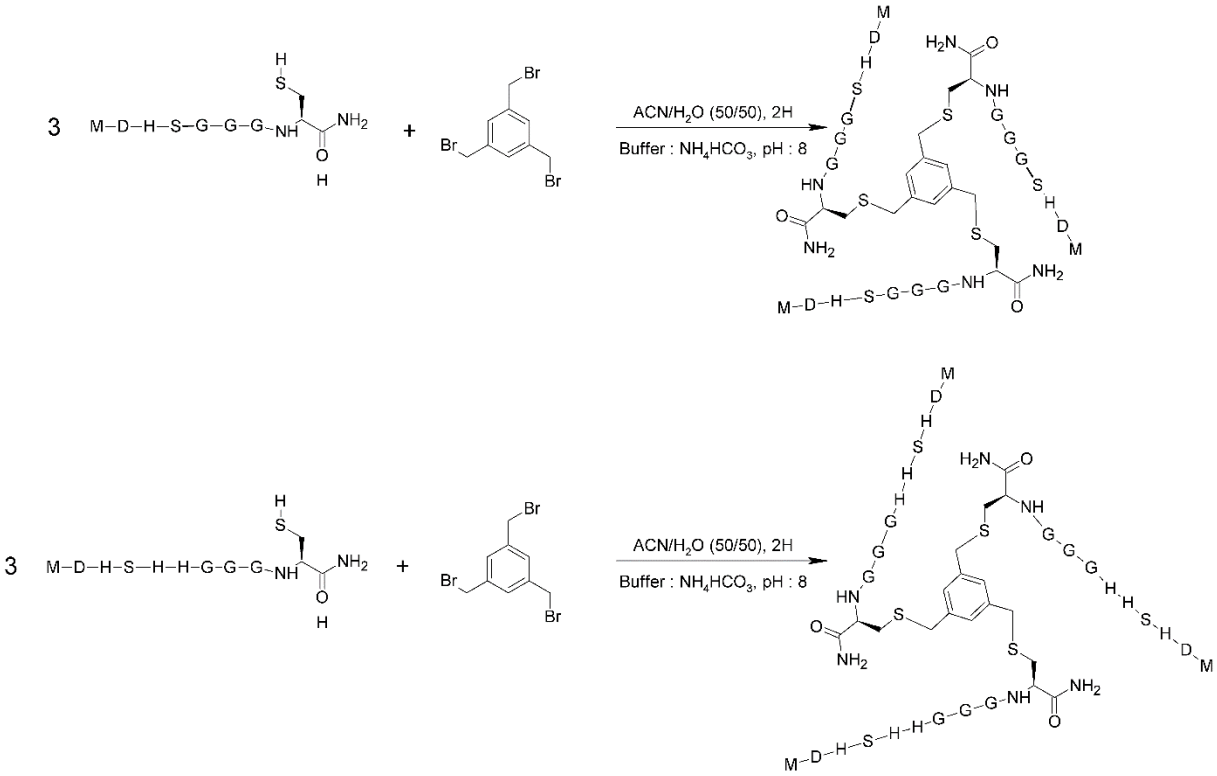

Figure S1. Scheme of synthesis of *tri*-MDHS (top) and *tri*-MDHSHH (bottom).

All solvents were initially bubbled with argon to remove oxygen. A stock solution of 10 mM TBMB was prepared in ACN, as well as a stock solution of 20 mM peptide in 0.2 M NH_4HCO_3 pH 8. Then, 500 µL of the peptide stock, 200 µL of the TBMB stock, and 300 µL of ACN were mixed under argon and stirred at room temperature for 2 h. The reaction was followed by RP-HPLC every 30 min. After complete disappearance of the TBMB peak, the reaction was stopped by freezing with dry ice, and the crude product was lyophilized.

The trimer purification was performed by RP-HPLC with the XBridge peptide BEH C18 column (130 Å, 5 µm, 19 mm × 150 mm) and the eluent was a mix of solutions A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN/H_2O 9:1). The purification method consisted in a gradient of eluent from 2% to 30% of B within 15 min, and then from 30% to 100% within the next 15 min (flow: 14 ml/min). The identity of the obtained fraction was verified by ESI-MS.

5. Determination of Cu(II) and peptide concentration

$CuCl_2$ stock concentration was determined using extinction coefficient ε=12 $M^{-1}cm^{-1}$ at 780 nm. Stock solutions of the peptides were prepared based on weight including the TFA counter ion. Then, the concentrations of these stock peptide were verified by UV-vis titration of about 1 mM peptide solution with Cu(II) in 50 mM HEPES, pH 7.4 monitoring on an Agilent Cary 60 UV-Vis spectrophotometer the changes in the d-d bands at 525 nm and based on the well-established 1:1 stoichiometry for Cu(II)-ATCUN complexes.

6. UV-vis titration of trimers with Cu(II)

Trimer solutions (0.5 mM) in 50 mM HEPES pH 7.4 were titrated by multiple additions of aqueous Cu(II) solution (0.1 M). UV-vis spectra were recorded on the Agilent Cary 60 UV-Vis spectrophotometer at 230-900 nm. The spectra of the copper complexes of trimers were compared with those of monomers for selected Cu (II): monomer/trimer ratios.

7. Copper (II) reduction

All experiments were performed in Milli-Q water and all solutions were bubbled with argon. For each analysis, a 0.1 M ascorbate stock solution was freshly prepared in Milli-Q water under argon. Then, the ascorbate stock solution was added to a sample of 0.3 mM monomer/0.1 mM trimer and 0.09 mM Cu(II) in 50 mM HEPES buffer pH 7.4 under argon to final ascorbate concentrations of 0.2 mM for *mono*-MDHSHH, *tri*-MDHSHH and of 5 mM for *mono*-MDHS, *tri*-MDHS. The changes in UV-Vis spectra were monitored every 2 min. over 1 h. The kinetics were repeated at least 2-3 times. The statistics performed by two-tailed Student's t-test.

8. Bicinchoninic acid (BCA) competition

Model peptides were added to samples of 5 µM of BCA, 1.8 µM Cu(I) and 1.5 mM of dithionite to the final concentration of 25 µM for *mono*-MDHS and *mono*-MDHSHH or to final concentration of 8.3 µM for *tri*-MDHS and *tri*-MDHSHH. All BCA competitions were performed using a 1 mL cuvette (1 cm optical path length) at atmospheric pressure. Sodium dithionite stock solution of 0.1 M was freshly prepared in Milli-Q water under argon.

9. NMR experiments

^{1}H NMR spectra were recorded in D_2O at 25 °C with a Varian Unity Plus 400 spectrometer at 400 MHz using standard pulse programs from the Varian library.

A solution of 1 mM MDHS/MDHSHH monomer/trimer was prepared in 0.2 M phosphate buffer pH 7.4 in 500 µL of Milli-Q water. This solution was lyophilized and then dissolved in 500 µL of D_2O and freeze-dried again. This step was repeated two times.

The freeze-dried product was dissolved in D_2O and then bubbled with argon. The stock solution of sodium dithionite was prepared by weighting 17.4 mg of the compound in a vial flushed under argon, dissolved in 500 µL of bubbled Milli-Q water to obtain a final concentration of 0.2 M, and then again bubbled under argon.

The NMR tube was flushed with argon, filled with the MDHS/MDHSHH monomer/trimer and Cu(II) solutions, and closed using a septum. 1.5 mM dithionite was added to the tube using a Hamilton syringe to reduce Cu(II) to Cu(I). NMR measurements were performed for the monomer/trimer without Cu(I) and after addition of $CuCl_2$ (from 1 eq. to 3 eq.).

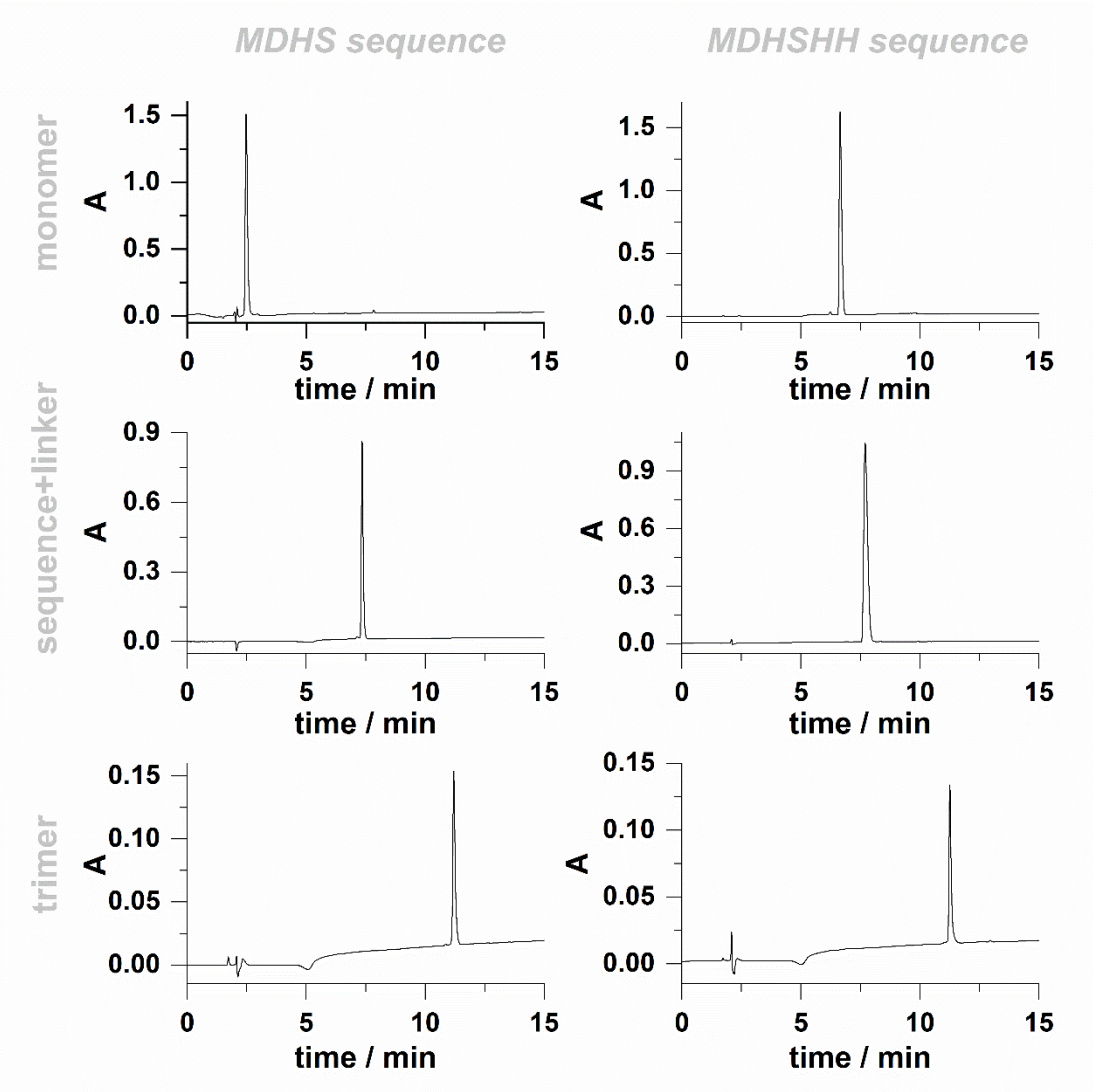

Figure S2. HPLC chromatograms of pure monomers, *mono*-MDHS and *mono*-MDHSHH (top); pure peptides for trimer synthesis – sequence + linker, MHDSGGGC-NH_2 and MHDSHHGGGC-NH_2 (middle); pure trimers, *tri*-MDHS and *tri*-MDHSHH (bottom). The separation was performed using a linear gradient of 0.1% TFA in H_2O (solution A) and 0.1% TFA/90% ACN (solution B) from 2% to 30% solution B within 15 min, flow 1ml/min, and UV detection at 214 nm.

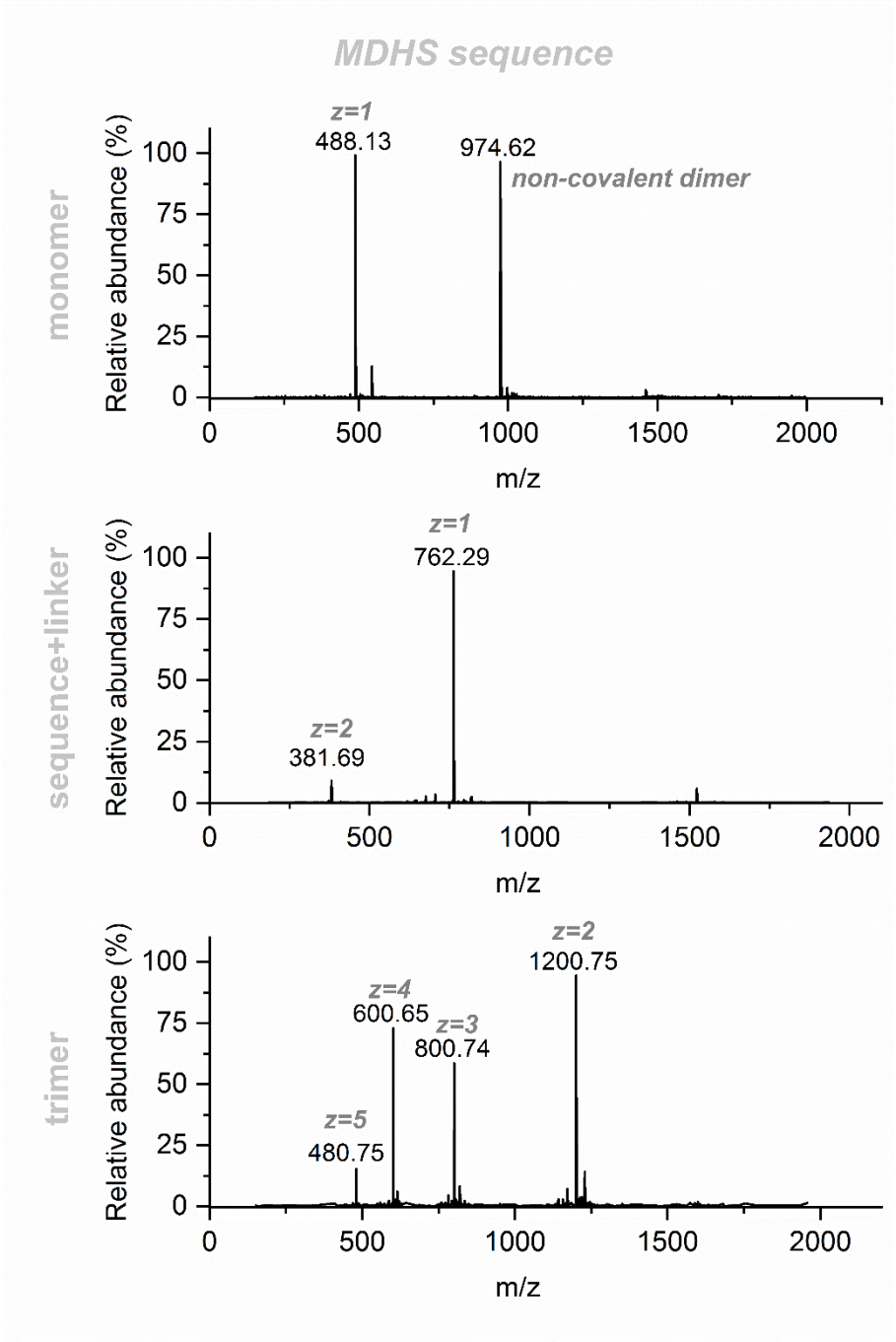

Figure S3. ESI-MS spectra of peptides containing a MDHS sequence: *mono*-MDHS (top), a peptide used for the trimer synthesis (sequence + linker) MDHSGGGC-NH_2 (middle), and *tri*-MDHS (bottom). A dimer detected for *mono*-MDHS is non-covalent and the similar dimers are often observed in ESI-MS spectra for low-molecular peptides. The comparison of theoretical and observed m/z values for the presented models are provided in Table S1.

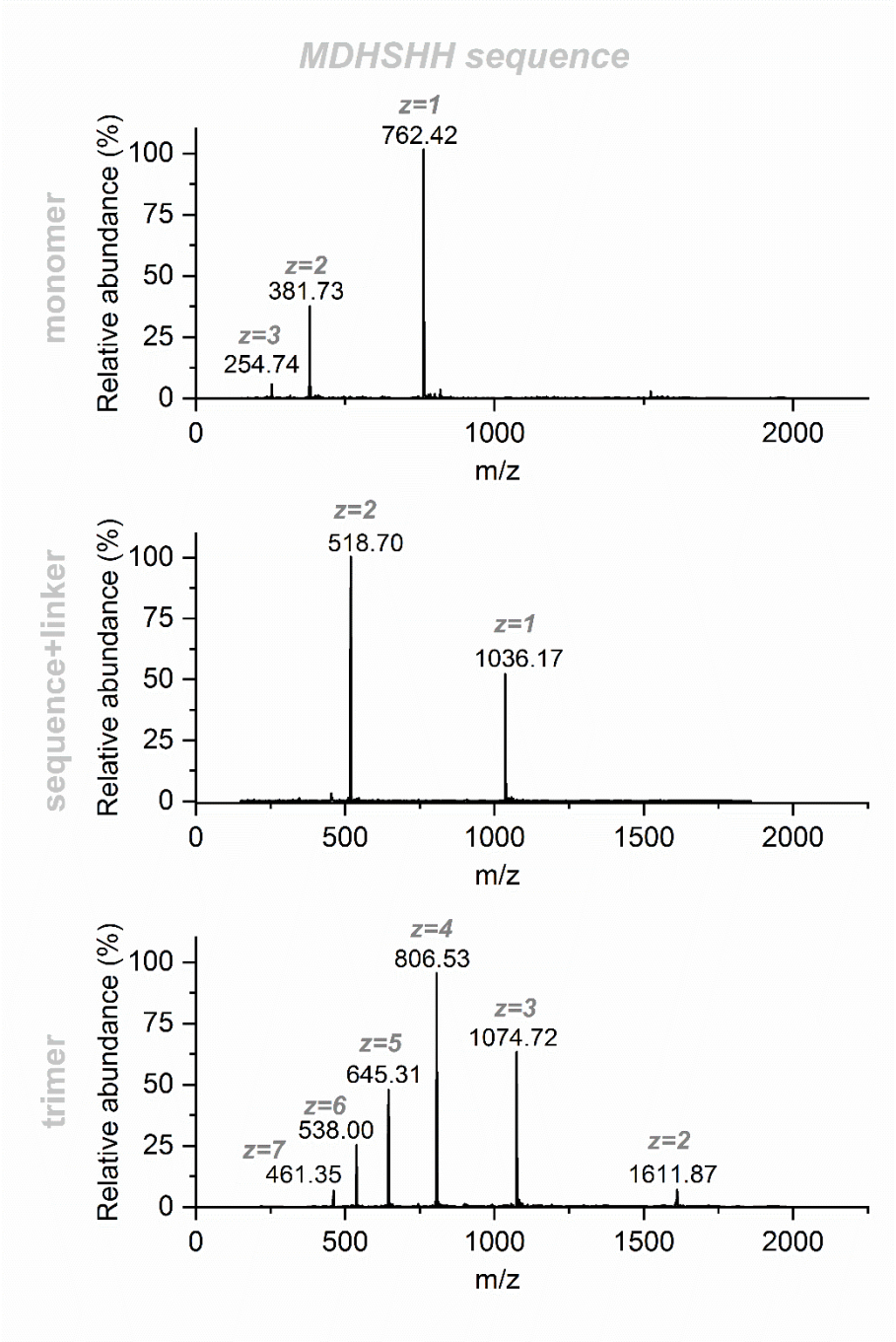

Figure S4. ESI-MS spectra of peptides containing a MDHSHH sequence: *mono*-MDHSHH (top), a peptide used for the trimer synthesis (sequence + linker) MDHSHHGGGC-NH_2 (middle), and *tri*-MDHSHH (bottom). The comparison of theoretical and observed m/z values for the presented models are provided in Table S2.

Table S1. Comparison of theoretical and observed m/z values for peptides containing a MDHS sequence

z	*mono*-MDHS		sequence + linker (MDHSGGGC-NH_2)		*tri*-MDHS	
	theoretical m/z	observed m/z	theoretical m/z	observed m/z	theoretical m/z	observed m/z
1	488.54	488.13	762.27	762.29	2398.82	
2			381.64	381.69	1200.83	1200.75
3					800.89	800.74
4					600.92	600.65
5					480.93	480.75

Table S2. Comparison of theoretical and observed m/z values for peptides containing a MDHSHH sequence

z	*mono*-MDHSHH		sequence + linker (MDHSHHGGGC-NH_2)		*tri*-MDHSHH	
	theoretical m/z	observed m/z	theoretical m/z	observed m/z	theoretical m/z	observed m/z
1	762.81	762.42	1036.38	1036.17	3221.18	
2	381.91	381.73	518.69	518.70	1611.87	1611.87
3	254.95	254.74			1075.16	1075.72
4					806.62	806.53
5					645.50	645.31
6					538.09	538.00
7					461.36	461.35

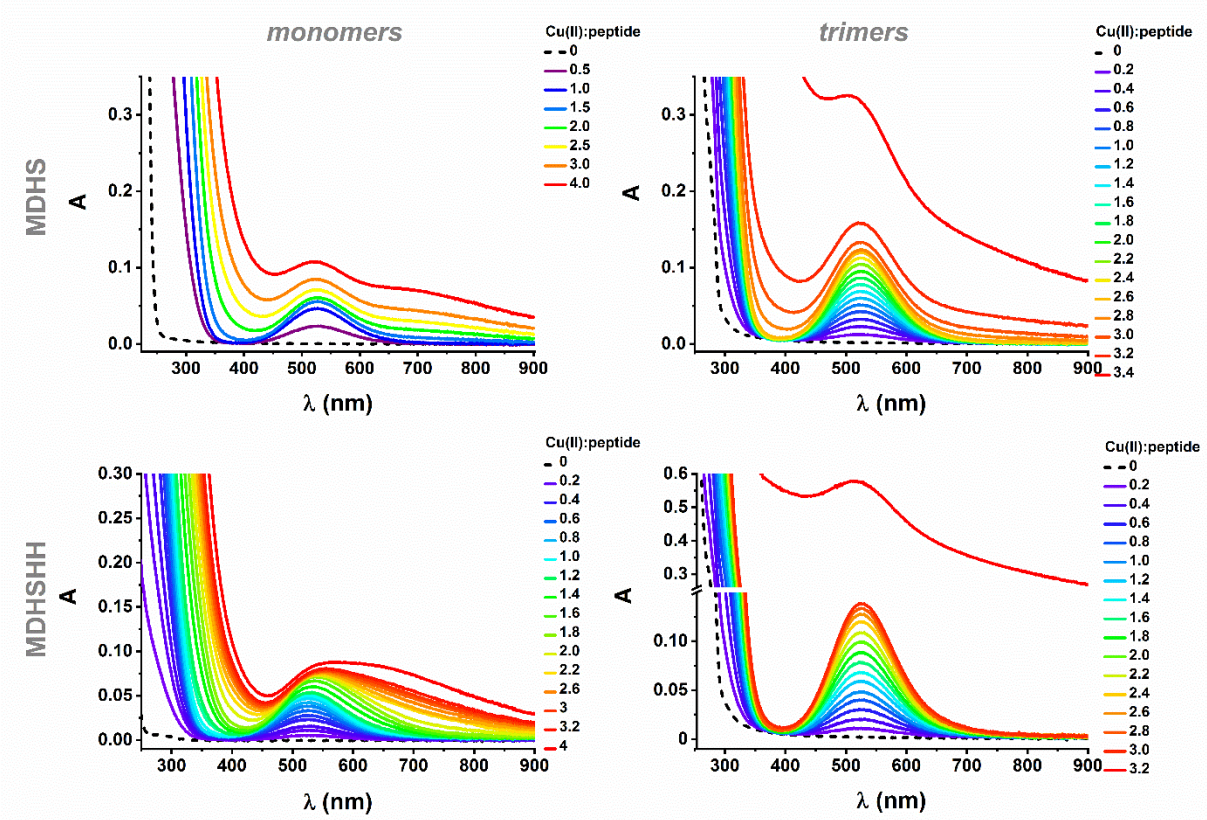

Figure S5. UV-vis spectra of titrations of monomeric (left) and trimeric (right) models of the CTR1 N-terminus, containing a MDHS sequence (top) or a MDHSHH sequence (bottom) with Cu(II) codded in rainbow colours from the lowest (blue) to the highest (red) Cu(II) to peptide ratio. The spectrum of the peptide alone is present as a dashed black line. Experiments performed for 0.5 mM monomer/trimer in 50 mM HEPES pH 7.4.

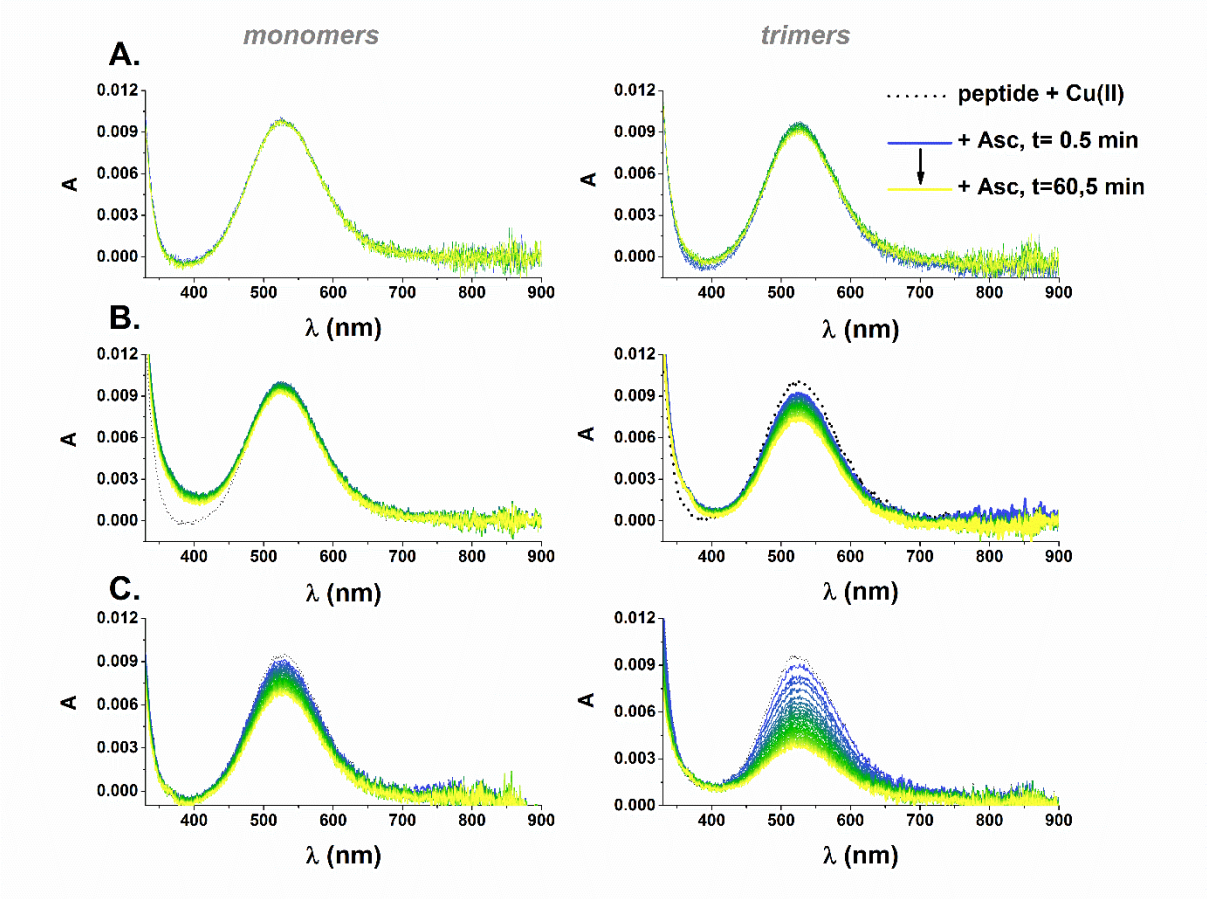

Figure S6. UV-vis spectra following the reduction of Cu(II) complexes of monomeric (left) and trimeric models of CTR1 N-terminus (right) by ascorbate (Asc). The kinetic was followed for 1 h (from blue, green to yellow lines) and the initial spectrum of Cu(II) complexes of peptides is given as a dotted line. Conditions: (A.) 0.3 mM *mono*-MDHS or 0.1 mM *tri*-MDHS, 0.09 mM Cu, 0.2 mM Asc; (B.) 0.3 mM *mono*-MDHS or 0.1 mM *tri*-MDHS, 0.09 mM Cu, 5 mM Asc; (C.) 0.3 mM *mono*-MDHSHH or 0.1 mM *tri*-MDHSHH, 0.09 mM Cu, 0.2 mM Asc. All experiments were performed in 50 mM HEPES buffer pH 7.4 under argon.

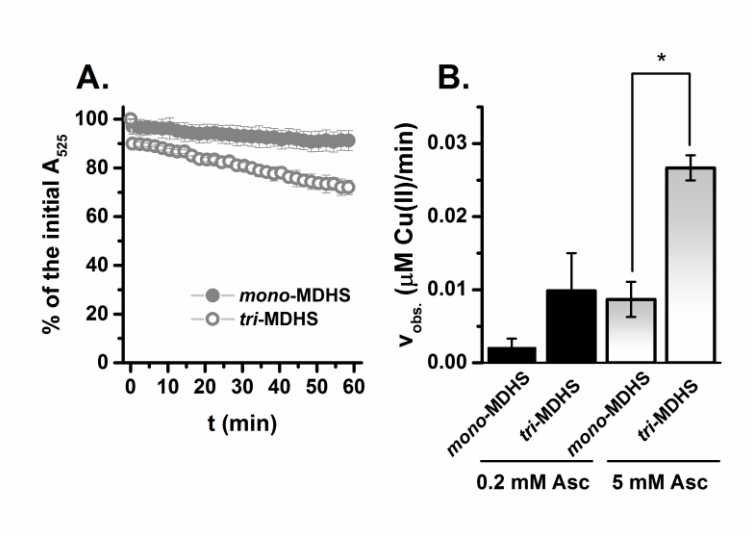

Fig. S7. (A.) Kinetics of reduction of Cu(II) bound to monomeric (full symbols) and trimeric (open symbols) models of the CTR1 N-terminus containing the shorter MDHS sequence in the presence of 5 mM Asc based of the decrease in A_{525}. (B.) Comparison of the kinetics for Cu(II) complexes of MDHS models in the presence of 0.2 mM Asc or 5 mM Asc calculated over the first 1 h. Experiments performed at least three times for 0.09 mM Cu, 0.1 mM trimer or 0.3 mM monomer in 50 mM HEPES pH 7.4 under argon. *indicates $p<0.001$.

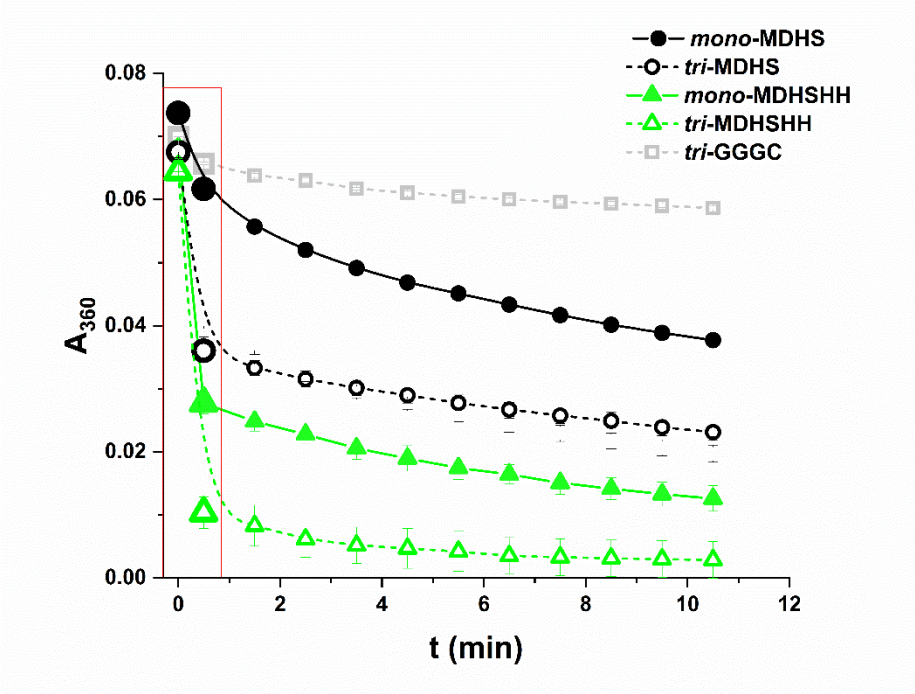

Figure S8. The kinetic of changes of absorbance at 360 nm assigned to Cu(I)-$(BCA)_2$ after additions of model peptides *mono*-MDHS, *tri*-MDHS, *mono*-MDHSHH and *tri*-MDHSHH as well as the platform of trimers alone (*tri*-GGGC). In the red box: the signals of Cu(I)-$(BCA)_2$ alone and the first point after the additions of the peptides taken for the calculations and further analysis. Conditions: 5 µM BCA, 1.8 µM Cu(I), 1.5mM dithionite, 25 µM monomer or 8.3 µM trimer CTR1 models in 50 mM HEPES pH 7.4. The experiments were repeated at least three times.

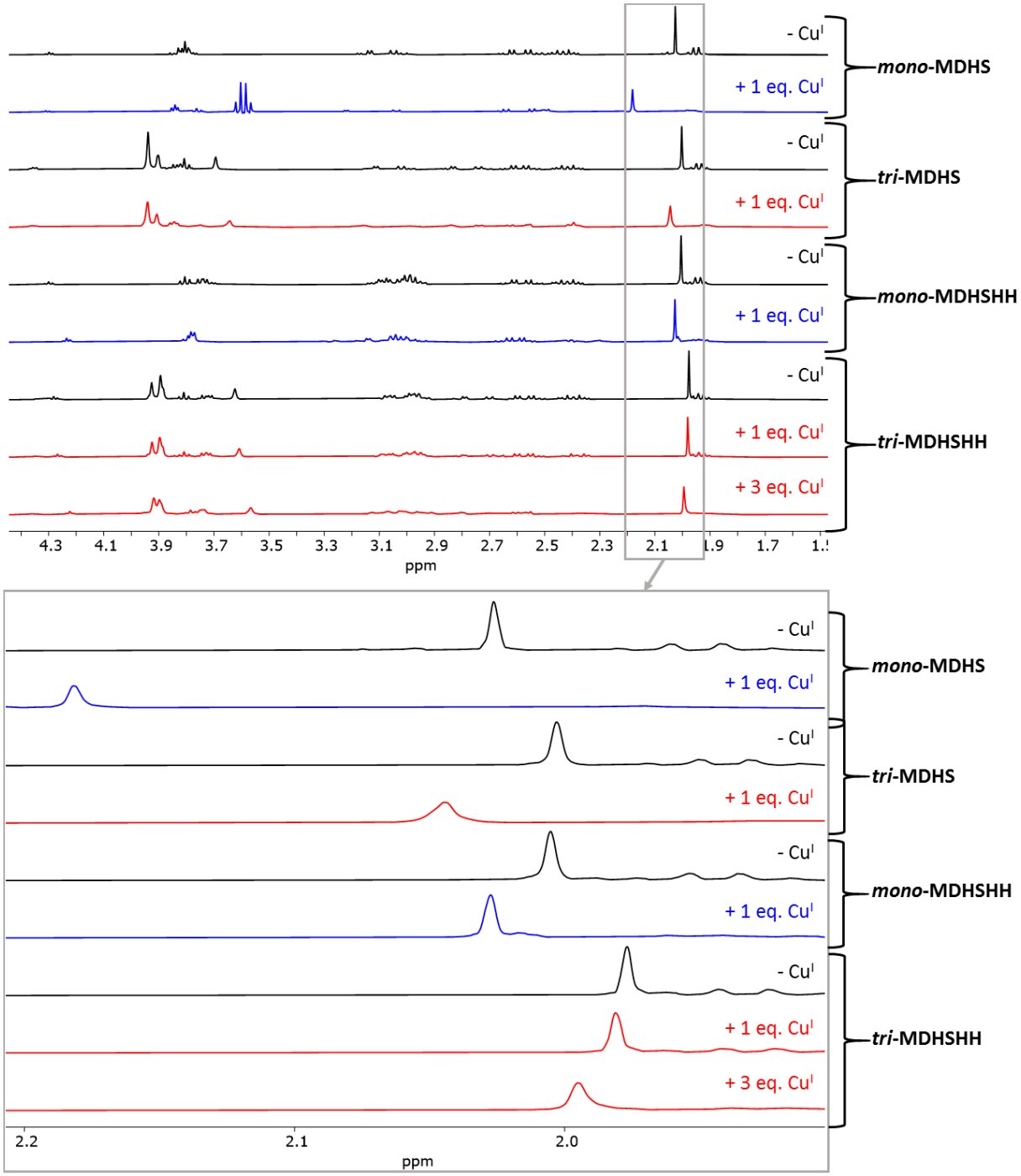

Figure S9: The 1H-NMR spectra in the aliphatic region (top) with the magnified fragment of CH_3 of Met (bottom) for *mono*-MDHS, *tri*-MDHS, *mono*-MDHSHH, and *tri*-MDHSHH with and without Cu(I). Conditions: 1 mM peptide (monomer or trimer), 1 mM or 3 mM Cu(I), 1.5 mM dithionite in 0.2M phosphate buffer pH 7.4 in D_2O saturated by argon.