Electronic Supplementary Material (ESI) for Metallomics.

Supporting Information

The N-terminal 14-mer model peptide of human Ctr1 can collect Cu(II) from albumin. Implications for copper uptake by Ctr1

Ewelina Stefaniak, Dawid Płonka, Simon C. Drew, Karolina Bossak-Ahmad, Kathryn L. Haas, M. Jake Pushie, Peter Faller, Nina E. Wezynfeld, and Wojciech Bal

Experimental section

1. Peptide synthesis, purification and preparation of reagent stock solutions

The MDHSHHMGMSYMDS-am ($hCtr1_{1-14}$) peptide was synthesized in our laboratory on a Prelude™ peptide synthesizer (Protein Technologies, Inc.), according to the solid phase Fmoc strategy.[1] Crude peptides were analyzed and purified by HPLC on an Empower system (Waters) equipped with ACE 5 C18-300 analytical and semi-preparative columns (5 mm particle size, 4.6 × 250 mm and 8 × 250 mm, respectively). The mobile phase consisted of (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in 90% acetonitrile in water. The pure lyophilized peptides were characterized on a ESI-Q-ToF Premier mass spectrometer (Waters), exhibiting correct molecular masses. Stock solution of peptide was made by dissolving lyophilized peptides in 1 mL of Nanopure water. Concentration of peptide was determined using the Edelhoch method.[2] In short, 5−10 μL of peptide stock was diluted in 1 mL of Nanopure water to obtain a UV absorbance between 0.1 and 1 absorbance units at 280 nm. The absorbance of amino acid side chains, only tyrosine in this case, at 276, 278, 280, and 282 nm was measured, and the peptide concentration was determined using known extinction coefficients of tyrosine at these wavelengths.[2] The Cu^{II} standardized solution ($CuSO_4$, 0.1 M) and lyophilized, fatty acid free, globulin free human serum albumin (HSA) were purchased from Sigma-Aldrich (Sigma, A3782). The HSA concentrations were determined by using an extinction coefficient at 280 nm of 33000 $cm^{-1}M^{-1}$.[3] About 0–30% of the N terminus of HSA is normally truncated, abolishing the high affinity ATCUN site.[4] Based on the knowledge that the intact N terminus of HSA can bind 1 mol equiv Cu^{II} with the typical absorption maximum at 520 nm, the concentration of N-terminal Cu-binding site was measured by titrating Cu^{II} to HSA.

2. Potentiometry

Potentiometric titrations were performed on a 907 Titrando Automatic Titrator (Metrohm), using a Biotrode combined glass electrode (Metrohm), calibrated daily by nitric acid titrations. A 100 mM NaOH solution (carbon dioxide free) was used as a titrant. Samples (1.5 mL) were prepared by dissolving peptides in 4 mM HNO_3/96 mM KNO_3 to obtain 1 mM peptide concentrations. The Cu^{II} complex formation was studied using samples in which the molar ratios of peptide to Cu^{II} were 1:0.9, 1:0.8, and 1:0.4. The pH range for all potentiometric titrations was 2.5−11.5. All experiments were performed under argon at 25 °C. The data were analyzed using the SUPERQUAD and HYPERQUAD programs.[5,6] Standard deviations provided by these software and reported here have statistical nature and do not include potential systematic errors.

3. UV-vis and CD spectroscopy

The pH-metric titrations were performed on a Lambda 950 UV/vis/NIR (Perkin Elmer) spectrophotometer in the spectral range of 220 nm - 850 nm, with 1 nm steps at 0.5 s/nm scanning rate, and on a J-815 CD spectrometer (JASCO) covering the spectral range of 220 nm - 850 nm, with 1 nm intervals and the scanning rate of 0.5 s/nm. All spectra were recorded in quartz cuvettes with 1 cm path length (Hellma). Samples of 0.6 mM of peptide and 0.5 mM of Cu^{II} were titrated with small amounts of concentrated NaOH solutions up to pH=11.51.

4. Cu^{II} transfer from HSA to $hCtr1_{1-14}$ by CD

The kinetic experiment was performed on a J-815 CD spectrometer (JASCO) covering the spectral range of 220 nm - 850 nm, with 1 nm intervals and the scanning rate of 0.5 s/nm in quartz cuvette with 1 cm path length (Helma). Concentrated solution of $hCtr1_{1-14}$ (final concentration in the incubated sample 0.4 mM) was added to the solution of Cu^{II} complex of HSA ([HSA] = 0.4 mM, [Cu(II) = 0.32 mM]) at the beginning of the kinetic measurement. The sample was incubated at 25 °C 144 cycles, 5 mins each. The sample was prepared in a 50 mM HEPES buffer.

5. Cu^{II} transfer from HSA to $hCtr1_{1-14}$ by EPR

Lyophilized, fatty acid free, globulin free, human serum albumin and lyophilized $hCtr1_{1-14}$ were resuspended in milliQ grade water (Millipore) and their concentrations determined by UV absorbance as above. A concentrated stock of $^{65}CuSO4$ was made by dissolving ^{65}CuO (>99%, Cambridge Isotope Laboratories) in concentrated H_2SO_4, followed by removal of excess acid under heat and addition of milliQ grade water. $Copper^{II}$, protein and concentrated HEPES were combined and diluted in milliQ water to final concentrations of 0.5, 0.625 and 20 mM respectively. The final pH was measured using a microprobe (Metrohm) and adjusted to 7.4 using concentrated NaOH. Continuous-wave, first-harmonic EPR spectra were obtained at 22 °C using a Bruker Elexsys E500 spectrometer fitted with a Bruker super-high-Q probehead (ER 4122SHQE), with samples contained within a quartz flat cell (Wilmad, WG-808-Q). Baseline correction was achieved by weighted subtraction of a spectrum 20 mM HEPES. For the competition experiment between $hCtr1_{1-14}$ and HSA, spectra were recorded every 10 min following the addition of 1 equiv $hCtr1_{1-14}$ to a solution containing Cu^{II}/HSA 0.8:1. Instrumental settings: microwave frequency, 9.86 GHz; microwave power, 20 mW; magnetic field modulation amplitude, 5 G; field modulation frequency, 100 kHz; receiver time constant, 82 ms; receiver gain, 80 dB; sweep rate, 10 gauss s^{-1}; averages, 5. The double integral of each first-harmonic spectrum was used as a normalization factor.

6. Cu K-edge X-ray Absorption Spectroscopy

X-ray absorption spectroscopic data was collected on beamline 7-3 at the Stanford Synchrotron Radiation Lightsource, with the 3 GeV SPEAR3 storage ring operating at 500 mA in top-off mode. A closed cycle helium cryostat (Oxford Instruments, Abingdon, UK) was used to maintain a temperature of 10 K for the duration of data collection in order to minimize photo-damage and minimize thermal disorder. A Si(220) double crystal monochromater in the ϕ = 90° orientation was employed and was detuned to 50% for rejection of higher energy harmonics. X-ray fluorescence was collected using a 30-element solid-state Ge detector (Canberra Ltd., Meriden, CT) with a 6 absorption lengths-thick Ni holder placed between the Soller slits and the sample. The total incoming count rate was maintained under 100 kHz per channel. Monochromator energy was calibrated to the first inflection point in the Cu K-edge spectrum of a Cu foil (9080.3 eV), recorded simultaneously during data acquisition. To minimize photoreduction of Cu(II) to Cu(I) during data acquisition the sample was repositioned after each sweep through the energy so that a region of previously unexposed sample was in the beam path. To further minimize photoreduction we implemented a quick EXAFS scan, which minimizes the duration the sample is exposed to beam at the expense of slightly poorer statistics, however, the signal-to-noise is improved with signal averaging from multiple

sweeps. Data for the quick EXAFS acquisition used 10 eV steps from 200 to 10 eV below the K edge (averaged over 1 s per data point), 0.35 eV steps through the edge region (10 eV below to 30 eV above the K edge, averaged over 1 s per data point), and an additional 283 data points over the *k*-range 1.62 – 15.75 $Å^{-1}$, in 0.05 $Å^{-1}$ steps (averaged from 1-6 s per data point from the beginning to the end of the *k*-range). The total number of data points over the entire scan range is 436. Due to a large monochromator crystal glitch just beyond 15.75 $Å^{-1}$ the energy range was not extended further. A fast automated pneumatic shutter was also employed during data acquisition to further minimize exposure to unwanted radiation during monochromator move and settle times. Data was averaged and processed using the EXAFSPAK suite of XAS data analysis programs developed by GN George.[7]

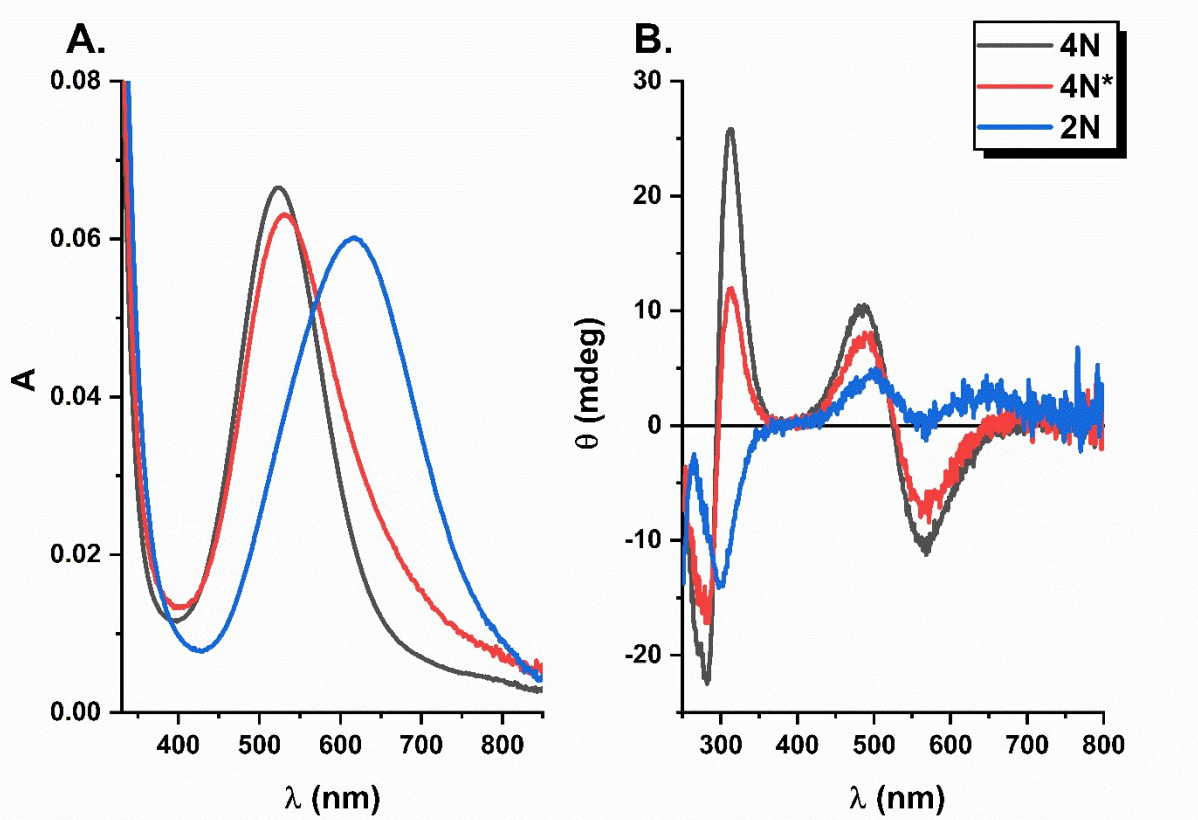

Fig. S1. UV-vis (A) and CD (B) spectra of different coordination modes of Cu^{II} complexes of $hCtr1_{1-14}$ (MDHSHHMGMSYMDS-am), calculated from the experimental spectra by linear combination using concentrations of species calculated on the basis of potentiometric stability constants (as plotted in Fig.2). The 4N spectrum includes the CuL, $CuH_{-1}L$ and $CuH_{-2}L$ species, 4N* includes CuHL and CuH_2L, whereas 2N includes CuH_3L and CuH_4L. The spectra were calculated for 0.5 mM concentration of the complexes.

Table S1. Spectral parameters of different coordination modes of Cu^{II} complexes of $hCtr1_{1-14}$ (MDHSHHMGMSYMDS-am), calculated based on potentiometric and spectroscopic results presented in Fig.2.

Coordination mode	Species involved	UV-vis		CD	
		λ_{max} (nm)	ε (M^{-1} cm^{-1})	λ_{max} (nm)	Δε (M^{-1} cm^{-1})
2N	CuH_4L CuH_3L	616[a]	120	640[a] 499[a] 299[b]	0.16 0.25 -0.83
4N*	CuH_2L CuHL	533[a]	126	570[a] 490[a] 312[c]	-0.42 0.46 0.72
4N	CuL $CuH_{-1}L$ $CuH_{-2}L$	524[a]	133	567[a] 485[a] 312[c]	-0.61 0.58 1.56

[a] *d-d* band, [b] $NH_2/N^{im} \rightarrow Cu^{2+}$ CT band, [c] $N^- \rightarrow Cu^{2+}$ CT band

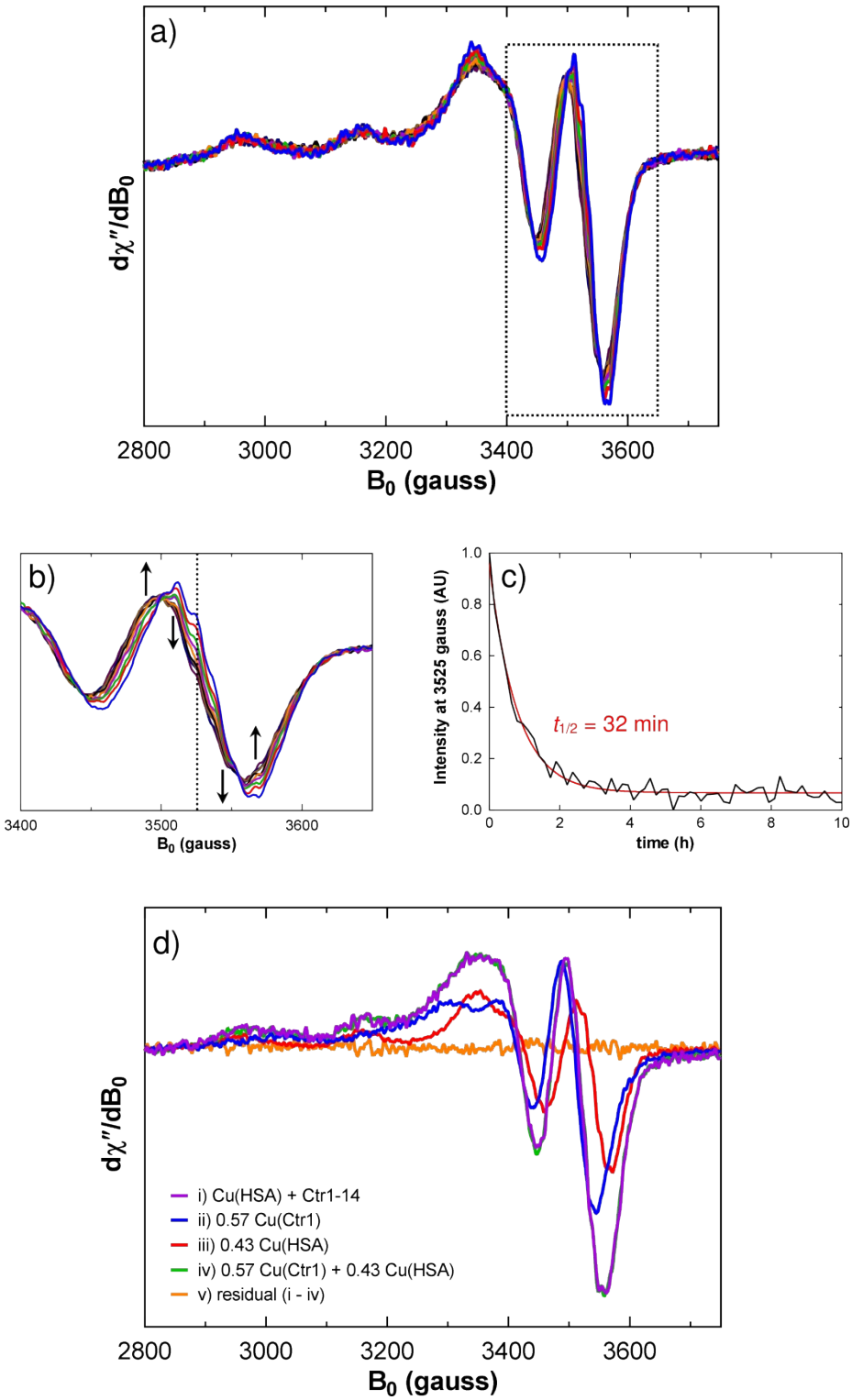

Fig. S2. Exchange of Cu^{II} from Cu(HSA) to $hCtr1_{1\text{-}14}$ measured by EPR spectroscopy at 22 °C. **(a)** Spectral changes observed between 0–75 min after the addition of 1 equiv $hCtr1_{1\text{-}14}$ to $^{65}Cu^{II}$/HSA 0.8:1, in HEPES pH 7.4. **(b)** Expansion of the region enclosed within the dashed box in *a*, showing the isosbestic points near 3500 and 3600 gauss. **(c)** Time-dependent variation in the EPR signal intensity at 3525 gauss (field location indicated by the dashed line in *b*), with a mono-exponential rate constant of $k = 0.0216 \pm 0.0009$ s^{-1} (red). **(d)** Decomposition of the equilibrium spectrum obtained 8 hours after addition of 1 equiv $hCtr1_{1\text{-}14}$ to Cu^{II}/HSA 0.8:1, indicating 57% copper transfer from HSA to $hCtr1_{1\text{-}14}$. The basis spectra *ii* and *iii* were obtained from (normalized) spectra of $^{65}Cu^{II}$/ $hCtr1_{1\text{-}14}$ 0.8:1 and $^{65}Cu^{II}$/HSA 0.8:1, respectively.

References:

1 W. C. Chan and P. D. White, *Fmoc Solid Phase Peptide Synthesis*, 2000.

2 C. N. Pace, F. Vajdos, L. Fee, G. Grimsley and T. Gray, *Protein Sci.*, 1995, **4**, 2411–2423.

3 F. Bruce Edwards, R. B. Rombauer and B. J. Campbell, *BBA - Protein Struct.*, 1969, **194**, 234–245.

4 D. Bar-Or, R. Bar-Or, L. T. Rael, D. K. Gardner, D. S. Slone and M. L. Craun, *Crit. Care Med.*, 2005, **33**, 1638–41.

5 P. Gans, A. Sabatini and A. Vacca, *J. Chem. Soc. Dalt. Trans.*, 1985, 1195–1200.

6 P. Gans, A. Sabatini and A. Vacca, *Talanta*, 1996, **43**, 1739–53.

7. G. George, I. Pickering, *EXAFSPAK: a suite of computer programs for analysis of X-ray absorption spectra*, 1995 http://www-ssrl.slac.stanford.edu/exafspak.html.