Triggering Cu-coordination change in Cu(II)-Ala-His-His by external ligands

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SUPPLEMENTARY INFORMATION

Experimental Section

Materials and Methods:

Synthesis of peptides.

The AHH-OH, AAH-OH and AH-OH peptides (in short, AHH, AAH and AH, respectively) were synthesized manually with standard 9-fluorenylmethoxycarbonyl (Fmoc) Chemistry on a Fmoc-L-His(Trt)-Wang resin (0.63 mmol/g from Iris Biotech GMBH), through solid phase peptide synthesis protocols. HBTU (Novabiochem, Merck Millipore) ((1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) was used as the coupling reagent, DIPEA (Merck) (N,N-diisopropylethylamine) as the base, and DMF (Merck) (dimethylformamide) as the solvent. The coupling reactions were performed by using a fourfold excess of amino acid, 3.8 fold excess of HBTU, 6 fold excess of DIPEA in DMF under mixing for 30 minutes; Fmoc deprotection was carried out using 20 % of 4 methylpiperidine in DMF for 20 minutes. Coupling reactions were controlled by the Kaiser test and repeated if the test was positive. Crude peptides were cleaved and the side chain deprotected at the same time by treatment with 95% TFA (Sigma Aldrich), 2.5% TIPS (Sigma Aldrich, 2.5% water for 2 hours. From the cleaving solution, the peptides were precipitated with cold ether, centrifuged and dissolved in a CH_3CN/H_2O (1:1), 0.1% TFA solution, filtered from the resin and lyophilized. Purity of the peptides was assessed by NMR.

Potentiometry

Potentiometric titrations were carried out on a 907 Titrando Automatic Titrator (Metrohm), using a Biotrode combined glass electrode (Metrohm), calibrated daily by nitric acid titrations prior to each series of experiments.[1] All samples were titrated with 0.1 M standardized NaOH, carbon dioxide free (POCH S.A.). All experiments were performed under argon at 25 °C. The data were analyzed using the SUPERQUAD and HYPERQUAD programs.[2,3]

Concentrations of stock solutions.

Stock solutions of AHH and imidazole were prepared by diluting respective ligands in a 4 mM HNO_3/96 mM KNO_3 (Sigma-Aldrich) to reach roughly 5 mM and 50 mM concentrations, respectively. Precise concentrations of stock solutions were calculated using HYPERQUAD on the basis of four consecutive titrations of each ligand (1.5 mL samples containing ~0.5-2 mM of imidazole or AHH). Protonation constants of AHH were used as obtained in our previous paper[14] whereas imidazole protonation constants where chosen from the literature.[4,5] However, due to different measurements conditions, protonation constants of imidazole were re-calculated from these titrations along with the determination of its concentration.

Binary complexes.

A total of six 1.5 mL samples of imidazole with $CuCl_2$ (Sigma-Aldrich) were prepared in 1:4, 1:3, 1:2 and 1:1 molar ratios. All the samples but one contained 1 mM imidazole and a respective amount of Cu(II).

Binary AHH/Cu(II) complexes where described previously,[6] but for the purpose of experimental consistence we titrated one control sample with a 1:1 molar ratio containing 1 mM AHH. Current results were in a good agreement with the previously observed stability constants, so that there was no need to correct them.

Ternary complexes.

To calculate stability constants for the ternary system of AHH/Cu(II)/imidazole titrations were performed of the following three samples (i) 2mM Im/0.65 mM AHH/ 0.58 mM Cu(II), (ii) 2mM Im/ 0.5 mM AHH/ 0.45 mM Cu(II) and (iii) 2mM Im/0.4 mM AHH/0.36 mM Cu(II).

UV-vis and CD spectroscopies

All solutions were prepared with Milli-Q (18 MΩ) water. Stock solutions of peptides were prepared by dissolving the peptides in Mili-Q, and concentrations were determined by titration followed by UV-vis absorption spectroscopy as indicated below. This experimental determination led to concentration values that are 10-20% below the expected value compared to those obtained using the molecular mass of the peptide and its counterions, thereby suggesting that counterion salts co-precipitate during peptide synthesis. Stock solutions were stored at −20 °C. The Cu(II) ion source was $CuCl_2$ $2H_2O$. The Cu(II) concentration was determined by dissolving the Cu salt in acidified Milli-Q water to prepare a 100 mM solution considering an extinction coefficient of ϵ_{816} = 12.6 $mol^{-1}cm^{-1}$. For CD measurements, the peptide stock solution concentration was measured by potentiometry.

Ligand titrations

Titrations of ligands were performed on two types of UV-vis spectrophotometers: Cary 60 (Agilent Technologies) and Lambda 950 UV/vis/NIR spectrophotometers (PerkinElmer), and on J-815 Circular Dichroism spectrometer (JASCO). Samples containing 1 mM peptide (AHH, AH, or AAH) and 0.8 mM $CuCl_2$ at pH 7.4 were titrated with concentrated stocks of imidazole or azide ensuring that the final dilution of the sample did not exceed 10% .

To eliminate any external factors influencing ternary complex formation the buffer was omitted in all experiments, therefore each titration point was re-checked for pH stability and adjusted with

minute amounts of concentrated NaOH/HCl as needed. All the dilutions were taken into account during calculations.

pH-metric titrations

The UV-vis and CD measurements were performed in the pH range 2 to 11, for samples containing 1:0.9 peptide to metal ratios (2 mM peptide and 1.8 mM $CuCl_2$ for UV-vis, and 1 mM peptide and 0.9 mM $CuCl_2$ for CD). The absorption spectra were recorded in the 200-800 spectral range, while CD was done in a range of 250-800 nm. Quartz cuvettes with a path length of 10 mm were used. Titrations were made by addition of small amounts of concentrated NaOH.

Calculations of spectroscopic and potentiometric data

Titration curves for spectroscopic titrations of peptides with imidazole at the constant pH 7.4 were generated by recording the UV-vis and CD spectral intensities at the maxima of respective *d-d* bands (CD: 565 nm, 461 nm and 313 nm; UV-vis: 620 nm, 570 nm, 520 nm, 480nm). The *d-d* and CT bands were used for azide titrations. The titration curves were fitted to the apparent binding constant equation 1, where L is a given peptide and A denotes imidazole or azide), using the least squares algorithm implemented in Origin 2016 software.

$$CuL + A = CuLA \qquad (1)$$

The imidazole titration of Cu(AHH) was additionally analyzed for its conformity with the potentiometric results. For each point of this titration a full species distribution was calculated with the use of a Newton−Raphson algorithm implemented in Microsoft Excel. The output of these calculations is equivalent to that of the Species module of the Soleq software suite[7]. In these caclulations the log β values derived from potentiometry were used, except for the log β for CuLA, which was varied freely to reproduce the relative concentration of this complex yielded by the titration.

EPR spectroscopy.

X band EPR spectra (9.4 GHz) were obtained on a continuous-wave EMX-plus spectrometer (Bruker Biospin GmbH, Germany) equipped with a high sensitivity resonator (4119HS-W1, Bruker). The g factor was calibrated in the experimental conditions using the Bruker strong pitch (g = 2.0028). All samples were supplemented by 30% v/v glycerol to ensure homogenous protein distributions, and avoid water crystallization-induced phase separation. Then, samples were introduced to 4 mm outer diameter quartz tubes (Wilmad-Labglass) and freeze-quenched into liquid nitrogen prior to their introduction into the pre-cooled cavity (100 K, achieved by continuous flow liquid nitrogen cryostat). The principal experimental parameters values were: modulation amplitude 5 G; microwave power *ca.* 0.1 mW; conversion time and time constant were setup at *ca*. 310 and 80 ms respectively; 1500 G were swept in 5 min and 1-4 scans were accumulated to achieve reasonable signal to noise (S/N) ratio. Simulations based on experimental data were performed under Matlab environment using Easyspin Toolbox.[8] For the sample preparation, 2 mM peptide concentration was used, 0.8 equivalents of ^{63}Cu/ in 100 mM HEPES buffer at pH 7.4 with a final volume of the sample of 50 μL (For the EPR with N-methyl-imidazole, natural abundance $CuCl_2$ was used. The solutions of the external ligand were used the same as described in the UV experiments.

Absorption: Titrations of Im to Cu(AHH), Cu(AAH) and Cu(AH)

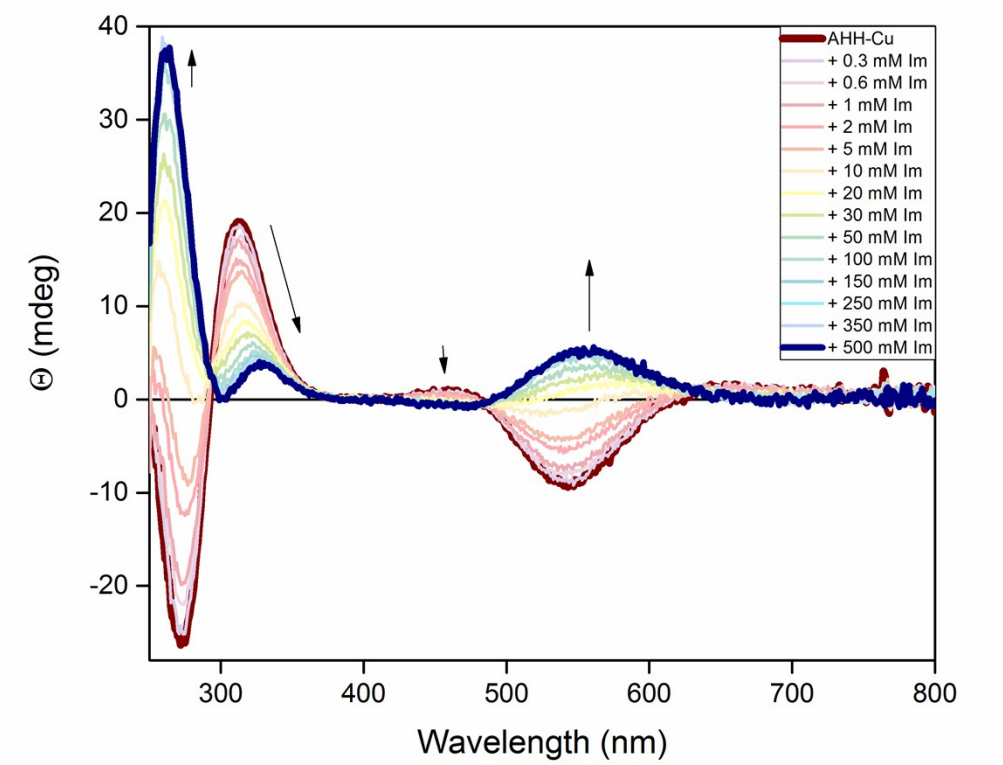

Figure S1: CD titration of 1 mM AHH, 0.8 mM CuCl2 with imidazole at pH 7.4. Arrows shows the direction of pattern changes in the characteristic regions.

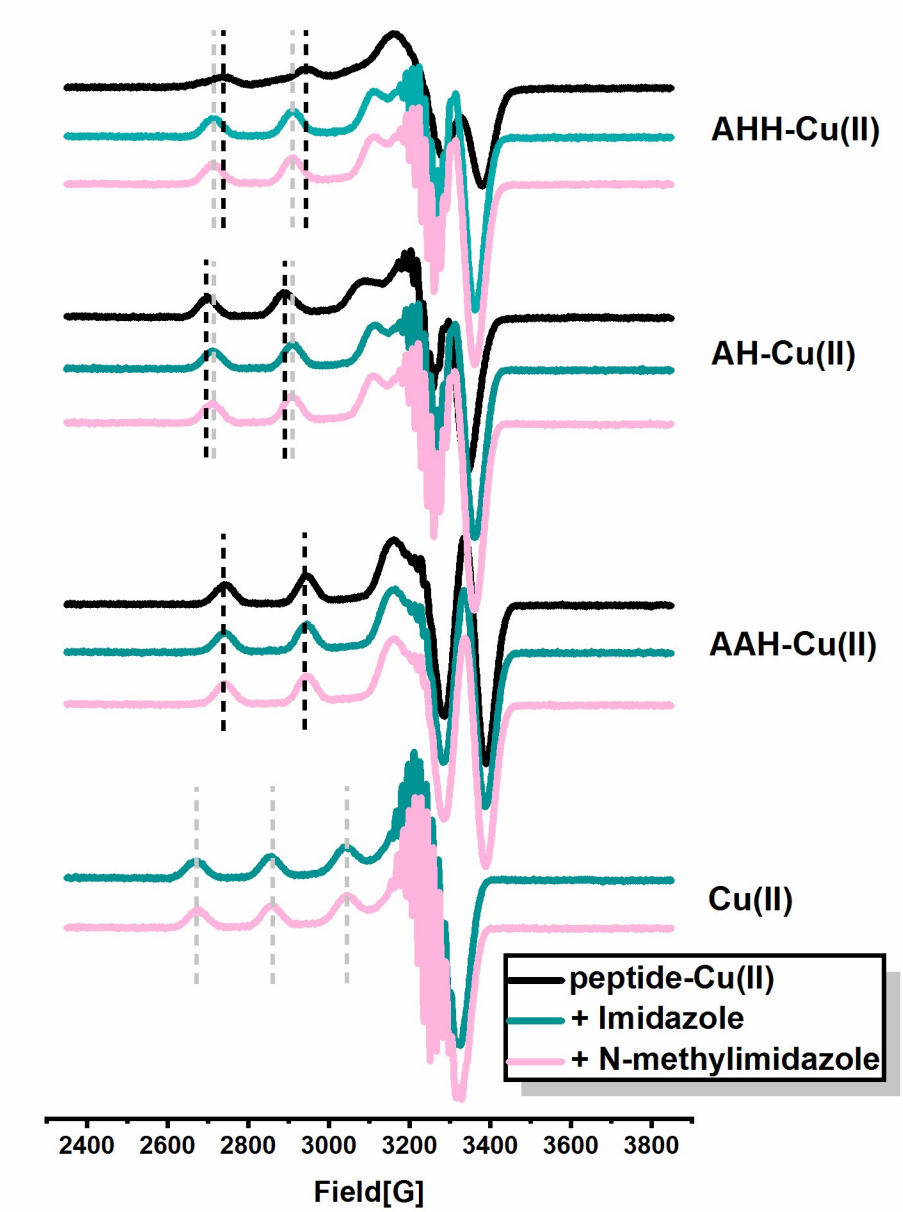

Figure S2a: EPR spectra of AHH-Cu(II), AH-Cu(II), and AAH-Cu(II) before (black lines) and after addition of 50 mM Imidazole (green lines) or 50 mM N-methylimidazole (pink lines). Experimental conditions: 1.6 mM $CuCl_2$, 2 mM peptide, 100 mM HEPES pH 7.4, 30% glycerol at 100 K. Dotted black lines indicate the lowest field peaks of the Cu(II) parallel hyperfine splitting constant of Cu(II)-peptide binary complex, whereas gray dotted lines refer to the shift after addition of Imidazole/N-methylimidazole. For comparison control spectra of 1.6 mM $CuCl_2$ in 50 mM Imidazole/N-methylimidazole were added.

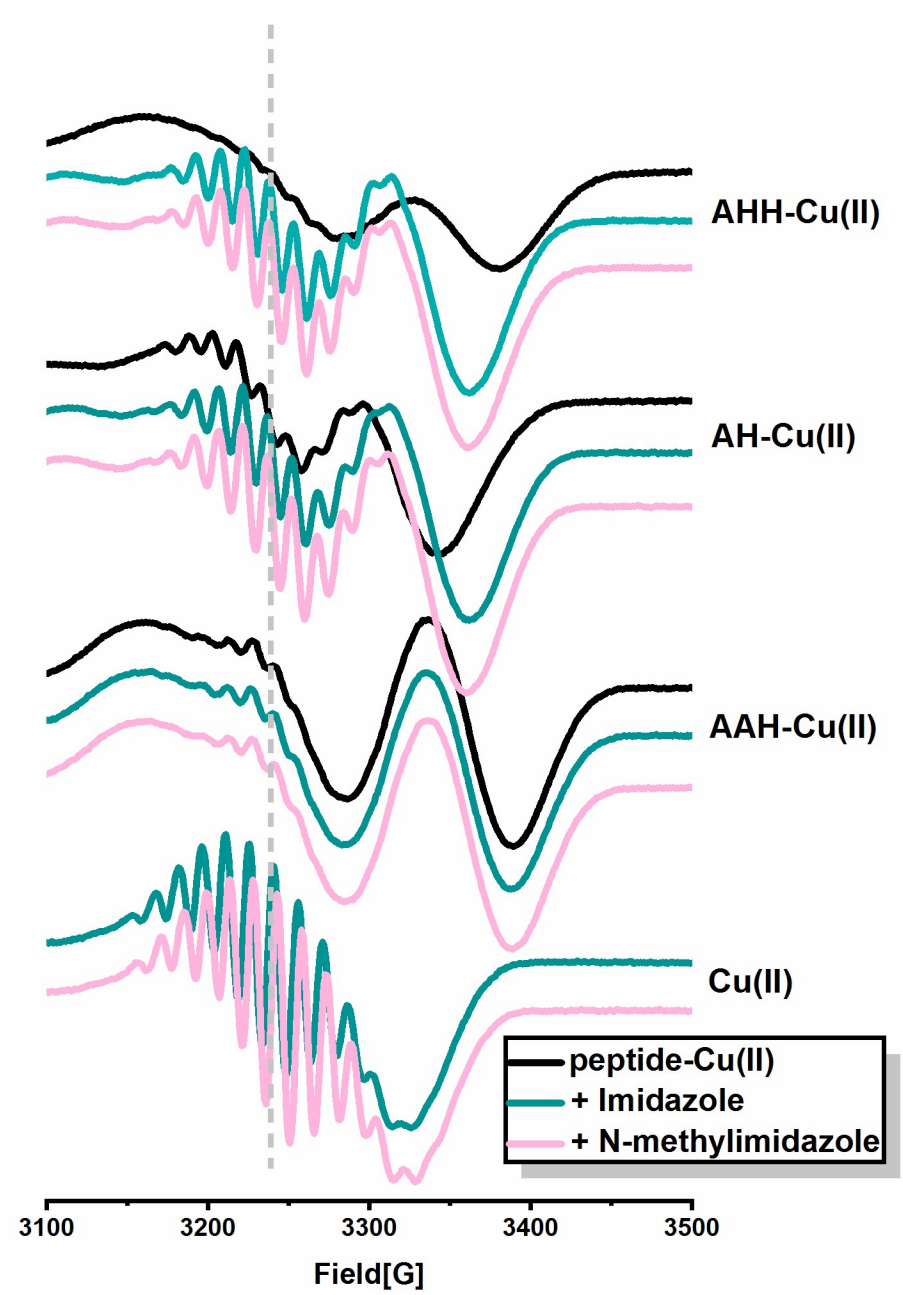

Figure S2b: Zoom of the superhyperfine splitting area of the EPR spectra from Figure S2a.

EPR spectra of AHH-Cu(II), AH-Cu(II), and AAH-Cu(II) before (black lines) and after addition of 50 mM Imidazole (green lines) or 50 mM N-methylimidazole (pink lines). Experimental conditions: 1.6 mM $CuCl_2$, 2 mM peptide, 100 mM HEPES pH 7.4, 30% glycerol at 100 K. For comparison control spectra of 1.6 mM $CuCl_2$ in 50 mM Imidazole/N-methylimidazole were added.

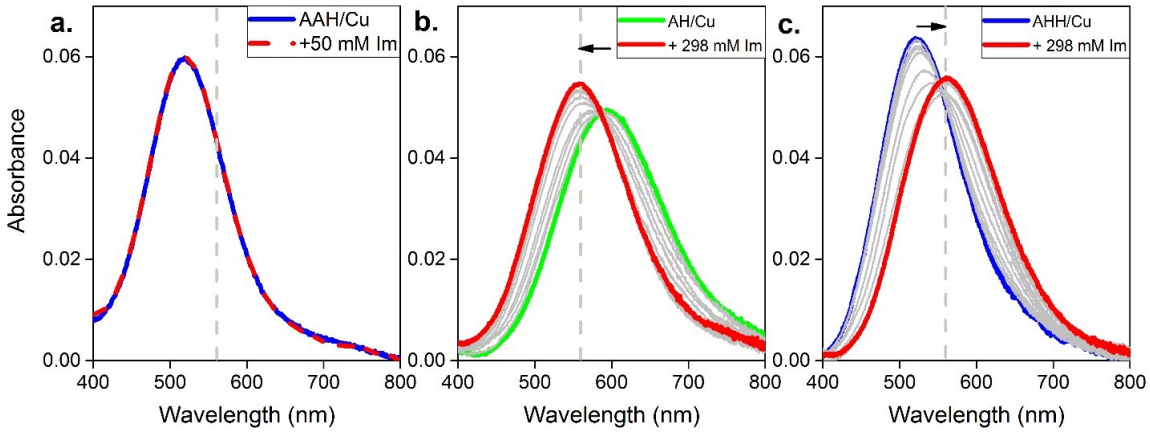

Figure S3: UV-Vis spectra of imidazole titrations of a) Cu-AAH; b) Cu-AH and c) Cu-AHH. Experimental conditions: 1 mM peptide, 0.8 mM $CuCl_2$, titrated with 0 – 298 mM imidazole at pH 7.4.

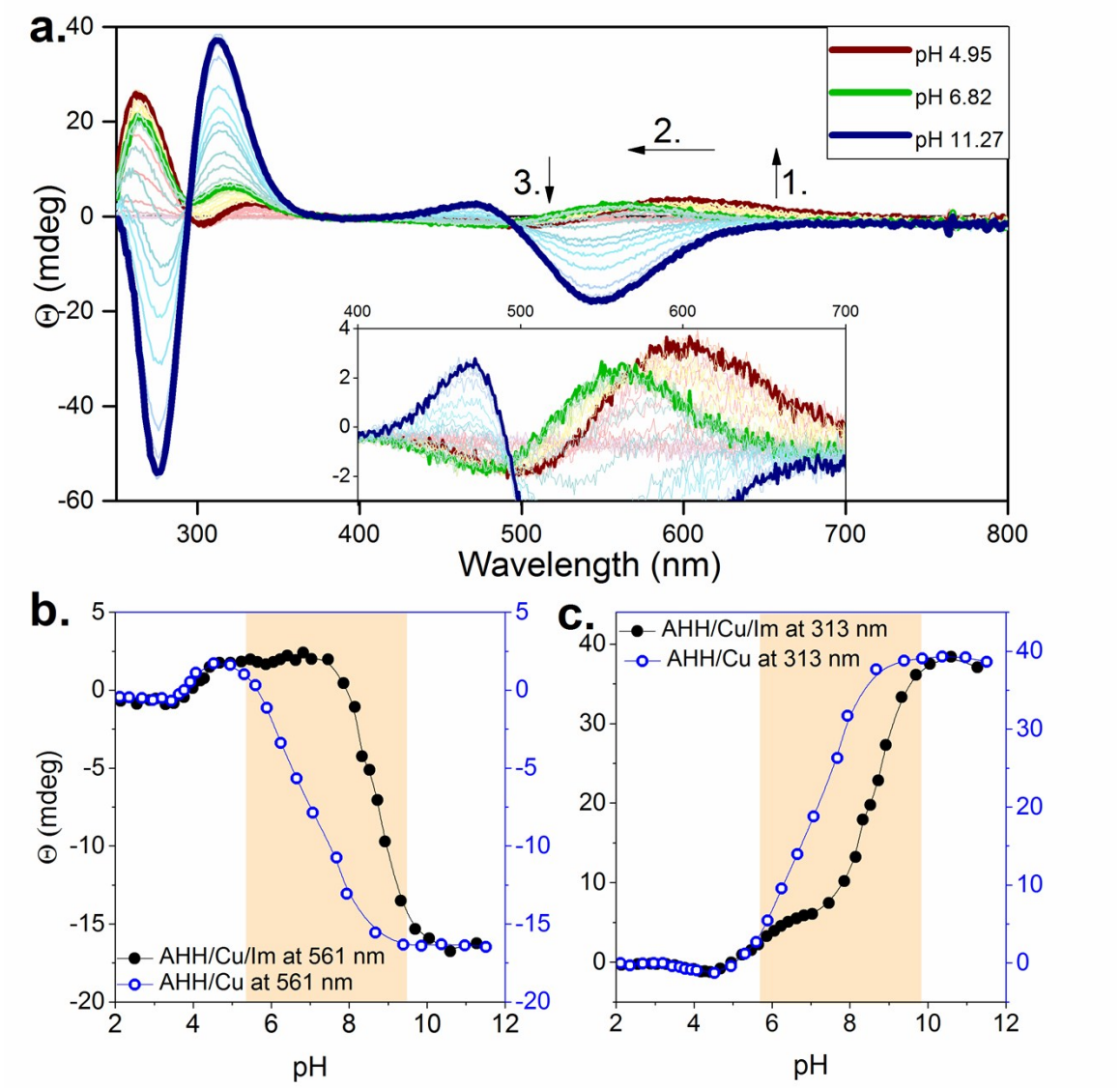

Figure S4: a) CD spectra of pH-metric titration for 1 mM AHH, 0.9 mM $CuCl_2$ and 25 mM Imidazole; inset shows the close-up of the *d-d* band region. Wine color represent the pH that consist mostly of 3N species, green – $3N+N^{Im}$, and blue a 4N species. b) and c) pH-dependence comparison of AHH/Cu/Im and AHH/Cu elipticities at 561 and 313 nm. The highlighted pH range shows the range in which the ternary complex is present in the titration.

Titration of azide to Cu(AHH) Cu(AAH) and Cu(AH)

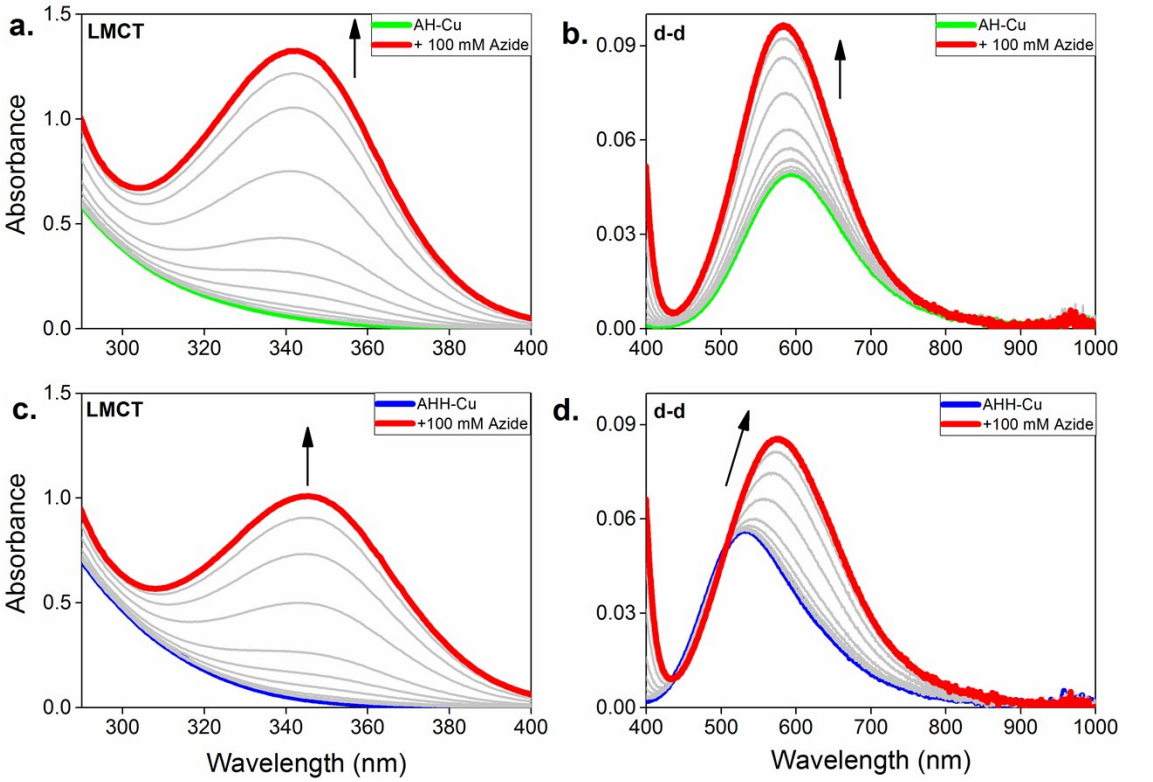

Figure S5: UV-vis spectra of a) LMCT region and b) d-d region of AH/Cu, and c) LMCT region and d) d-d region for AHH/Cu titration with azides in a range of 0-100 mM, at pH 7.4 and RT

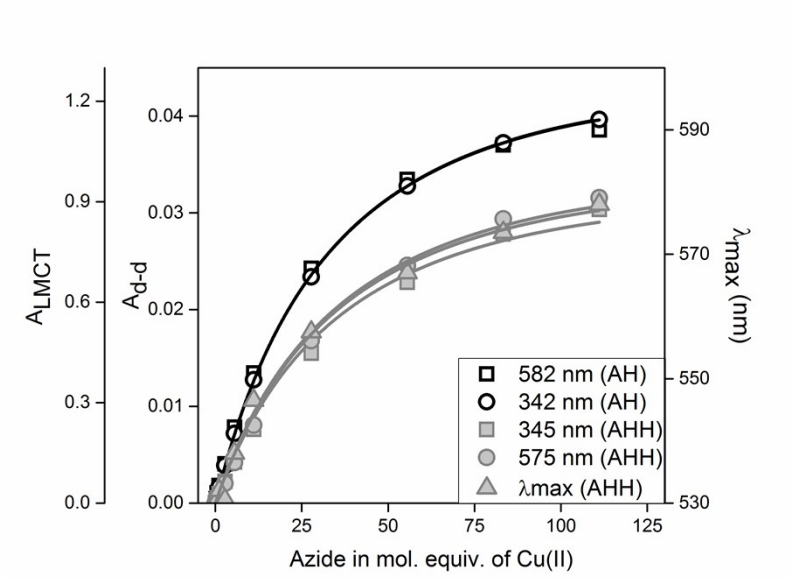

Figure S6: Dependence of absorption changes from amount of added azide represented as a molar equivalents of Cu(II). Experimental conditions: 1 mM peptide, 0.8 mM $CuCl_2$ titrated with 100 mM azide at pH 7.4. Solid lines represent global fitting of each data set.

Table S1: Spectroscopic parameters of AHH/Cu/Im complexes.

Cu(II) Species	Abs (nm)	Ɛ ($L\ mol^{-1}\ cm^{-1}$)	EPR			
			$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ *(MHz)*	$g_{\parallel}/A_{\parallel}$ **(cm)**
3N	600	59	2.230	2.052	580	115
3N + N^{Im}	560	68	2.210	2.047	600	110
4N	513	74	2.183	2.042	611	107

Table S2. Principal EPR simulations parameters [a].

Species	Im (mM)	$g_{//}$	$g_{\perp}$	$A_{//}$ (MHz)	$g_{//}/A_{//}$ (cm)
Cu(II)-AAH	**0**	2.18(2)	2.04(2)	610	107
	50	2.18(3)	2.04(3)	610	107
Cu(II)-AHH	**0**	2.18(0)	2.04(9)	614	106
		2.23(0)	2.048(1)	560	119
	50	2.21(1)	2.04(6)	596	111
Cu(II)-AH	**0**	2.23(1)	2.05(1)	576	116
	50	2.21(2)	2.04(7)	600	110
Cu(II)	**200**	2.25(9)	2.05(4)	565	120

[a] field strain and linewidth parameters were used to account for the experimental line-broadening.

Note that the $g_{\parallel}/A_{\parallel}$ (cm) ratio gives a rough estimate of the coordination geometry of the Cu(II) complex [9, 10]. Planar complexes have a ratio of about 110-120, strongly distorted of about 180-250 and slight to moderate distortion of about 130-150.

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