Supporting Information for

Exploring the structural details of Cu(I) binding to alpha-synuclein by NMR spectroscopy

Andres Binolfi[†], Ariel A. Valiente Gabioud[†], Rosario Duran[¶,¤], Markus Zweckstetter[‡], Christian Griesinger[‡] and Claudio O. Fernandez[†,‡,]*

[†]Instituto de Biología Molecular y Celular de Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas (IBR-CONICET), Universidad Nacional de Rosario, Suipacha 531, S2002LRK, Rosario, Argentina

[¶]Institut Pasteur de Montevideo (IPM), Mataojo 2020, 11400 Montevideo, Uruguay.

[¤]Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Avenida Italia 3318. C.P. 11.600. Montevideo - Uruguay

[‡]Department of NMR–based Structural Biology, Max Planck Institute for Biophysical Chemistry (MPIbpc), Am Fassberg 11, D-37077 Göttingen, Germany

[§]DFG Research Center for the Molecular Physiology of the Brain (CMPB), Göttingen, Germany

Experimental details

Protein and reagents. The protein AS was prepared as previously described.[1-2] The purified protein was dialyzed against Buffer A (20 mM MES, 100 mM NaCl, pH 6.5) supplemented with Chelex (Sigma). The peptide ^{1}MDVFMK^{6} was synthesized in solid-phase (Rink amide resin) using F-moc chemistry in a peptide synthesizer (CEM Liberty). Peptide was purified by reversed phase HPLC (Waters Delta 600) and characterized by HPLC and electrospray ionization mass spectrometry (Agilent). This peptide was amidated at the C-terminal carboxylate group, while the α-NH_2 terminal was left unmodified. The absorption extinction coefficient of the peptide ^{1}MDVFMK^{6} was determined using a calibration curve prepared in Buffer A (20 mM MES, 100 mM NaCl, pH 6.5), weighting out the purified dry peptide samples. The absorption extinction coefficient for the peptide was 11500 $cm^{-1}M^{-1}$ (214 nm), and it was used to determine the peptide concentration in each sample.

Generation of the Cu(I) complexes. To obtain the Cu(I) complexes with AS and ^{1}MDVFMK^{6}, the Cu(II) complexes were first prepared and then reduced with ascorbate. Reduction of Cu(II) complexes with ascorbate was followed by the decrease of the characteristic d-d transition band in the UV-Vis spectrum[3] (Figure S1). Spectra were recorded at 15^{o}C on a Jasco V-550 spectrophotometer.

MALDI-TOF Mass Spectrometry. Peptide samples were purified using C18 reverse phase micro-columns (OMIX Pipette tips, Varian) and eluted directly into the mass spectrometer sample plate with 2 μL of matrix solution (α-cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile containing 0.2% trifluoroacetic acid). Molecular masses of native and modified peptides were determined in a 4800 MALDI TOF/TOF instrument (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as the matrix. External calibration was performed with a mixture of peptide standards (Applied Biosystems).

NMR spectroscopy. NMR spectra were acquired on a Bruker Avance II 600 MHz spectrometer using a triple-resonance probe equipped with z-axis self-shielded gradient coils. The ^{1}H-^{15}N HSQC, ^{1}H-^{13}C (H)CCH TOCSY and ^{1}H-^{1}H TOCSY spectra were registered at 15^{o}C on 300 μM protein (isotopically enriched in ^{15}N and ^{13}C) and

peptide samples dissolved in Buffer A to minimize solvent exchange effects on amide resonances. The ^{1}H-^{13}C HSQC and HMBC spectra were registered on 2.5 mM peptide samples dissolved in deuterated Buffer A. Proton and carbon resonances of ^{1}MDVFMK^{6} were assigned by ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC experiments (Table S1). Chemical shifts were referenced to DSS as standard.

^{1}H-^{15}N HSQC, ^{1}H-^{13}C HSQC and ^{1}H-^{1}H TOCSY cross-peaks affected during metal titration experiments were identified by comparing their chemical shift values with those of the same cross-peaks in the data set of samples lacking metal ion. Differences in the mean weighted chemical shift (MWΔCS) displacements for ^{1}H, ^{15}N and ^{13}C were calculated as $[(\Delta\delta^{1}H)^2 + (\Delta\delta N/10)^2]^{1/2}$ and $[(\Delta\delta^{1}H)^2 + (\Delta\delta C/4)^2]^{1/2}$, respectively.[4]

The affinity features of Cu(I) binding to the peptide ^{1}MDVFMK^{6} were determined from ^{1}H-^{1}H TOCSY experiments on 300 μM peptide samples recorded at increasing concentrations of the metal ion. Changes in chemical shifts values of amide resonances of Val3, Phe4 and Met5 were fit to a model incorporating one Cu(I) ion per molecule (with dissociation constant K_d) by using the program DynaFit.[5]

$$\text{P1AS-Cu(I)} \quad \leftrightarrow \quad \text{P1AS + Cu(I)} \qquad\qquad K_d$$

Compared to the mass spectrometry and UV-vis developed assays,[6-7] which also rely on ascorbic acid as a biologically relevant reductant to reduce Cu(II) to Cu(I), the use of this NMR based approach allows to obtain residue-specific structural information related to the binding motifs and affinity features of Cu(I) in proteins and peptides.

Acquisition, processing and visualization of the NMR spectra were performed using TOPSPIN 2.0 (Bruker) and Sparky.

Table S1. ^{1}H and ^{13}C NMR chemical shifts of assigned resonances in the peptide $^{1}MDVFMK^{6}$.[a]

Aminoacid Residue	Nuclei	Chemical Shift (ppm)
Met 1	HN	n.d.
	Hα	4.20
	Hβ	2.22
	Hγ	2.69
	Hε	2.19
	CO	172.29
	Cα	55.22
	Cβ	33.01
	Cγ	30.88
	Cε	16.91
Asp 2	HN	n.d.
	Hα	4.81
	Hβ1	2.74
	Hβ2	2.68
	CO	175.48
	Cα	54.29
	Cβ	41.64
	COO^{-}	179.75
Val 3	HN	8.31
	Hα	4.15
	Hβ	2.12
	Hγ	0.94
	CO	175.80
	Cα	62.43
	Cβ	32.69
	Cγ	20.66
Phe 4	HN	8.42
	Hα	4.73
	Hβ1	3.25
	Hβ2	3.17
	Hδ	7.39
	Hε	7.46
	Hz	7.43
	CO	175.42
	Cα	57.75
	Cβ	39.40
	Cγ	138.67
	Cδ	132.03
	Cε	131.7
	Cz	130.11
Met 5	HN	8.29
	Hα	4.52
	Hβ	2.12
	Hγ	2.61
	Hε	2.21
	CO	175.41
	Cα	55.3
	Cβ	32.74
	Cγ	31.96
	Cε	16.87
Lys 6	HN	8.31
	Hα	4.33
	Hβ	1.83
	Hγ	1.55
	Hδ	1.83
	Hε	3.12
	Hz	n.d.
	CO	178.81
	Cα	56.29
	Cβ	33.02
	Cγ	24.89
	Cδ	29.12
	Cε	42.15

n.d.: not detected.

[a]Reported at 25°C on 2.5 mM peptide samples dissolved in deuterated buffer A, pH 6.5.

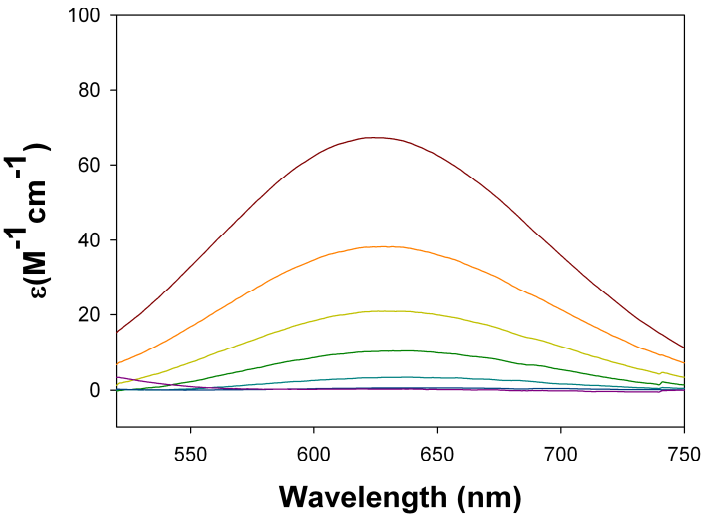

Figure S1. Electronic absorption spectra of the reduction of 300 μM AS-Cu(II) by ascorbate. Colour lines correspond to the following ascorbate concentrations: dark red (0), orange (2.5 mM), yellow (5.0 mM), green, (10.0 mM), cyan, (20.0 mM), blue (30.0 mM) and magenta (40.0 mM).

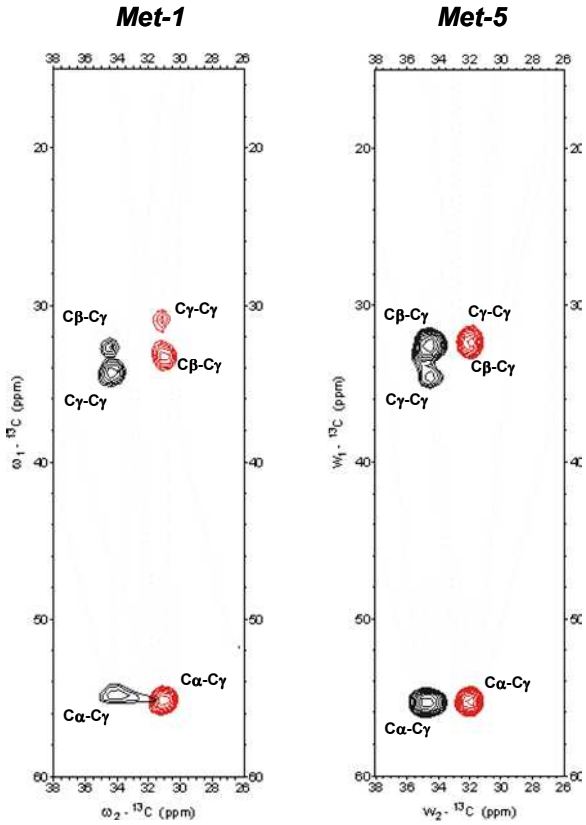

Figure S2. Strips for the Hγ of Met1 and Hγ of Met5, taken from the ^{1}H-^{13}C (H)CCH TOCSY experiment of AS (300 μM) in the absence (red) and presence (black) of 1 equivalent of Cu(II) and 35 mM ascorbate.

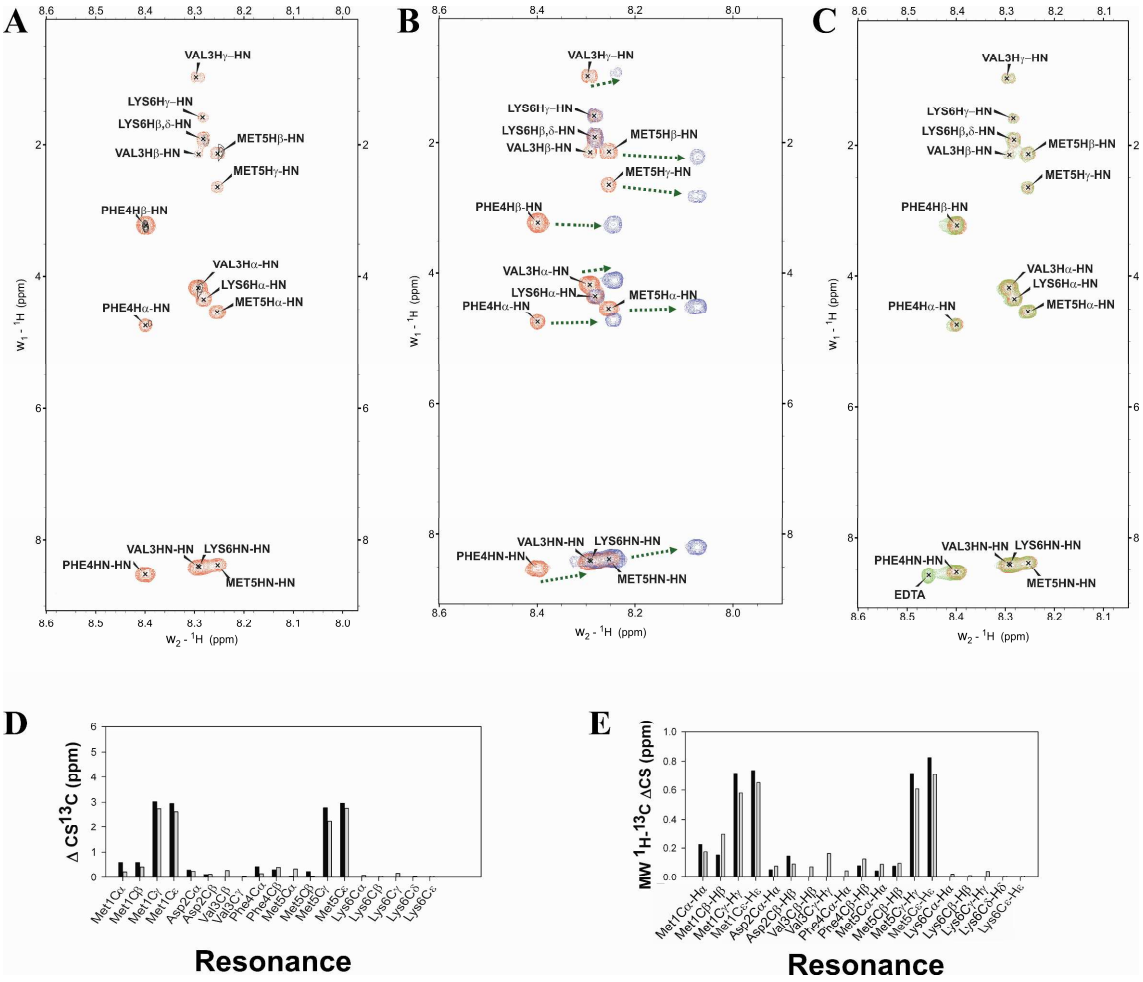

Figure S3. NMR analysis of Cu(I) binding to peptide ^{1}MDVFMK^{6}. (A) Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} (300 μM) in the absence (red) and presence (black) of 1 Cu(II) equivalent. (B) Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} in the absence (red) and presence (blue) of 1 Cu(II) equivalent followed by the addition of 35 mM ascorbate. (C) Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} in the absence (red) and presence (green) of 1 Cu(II) equivalent and 35 mM ascorbate followed by the addition of 5 mM EDTA. (D) ^{13}C and (E) ^{1}H-^{13}C mean weighted chemical shifts displacements (MW^{1}H-^{13}CΔCS) of ^{1}MDVFMK^{6} (gray) and AS (black) resonances in the presence of Cu(I), measured from ^{1}H-^{13}C HSQC and ^{1}H-^{13}C (H)CCH TOCSY experiments. Chemical shifts changes on Val3 and Lys6 resonances could not be measured accurately due to severe signal overlapping in the ^{1}H-^{13}C spectra of full length AS.

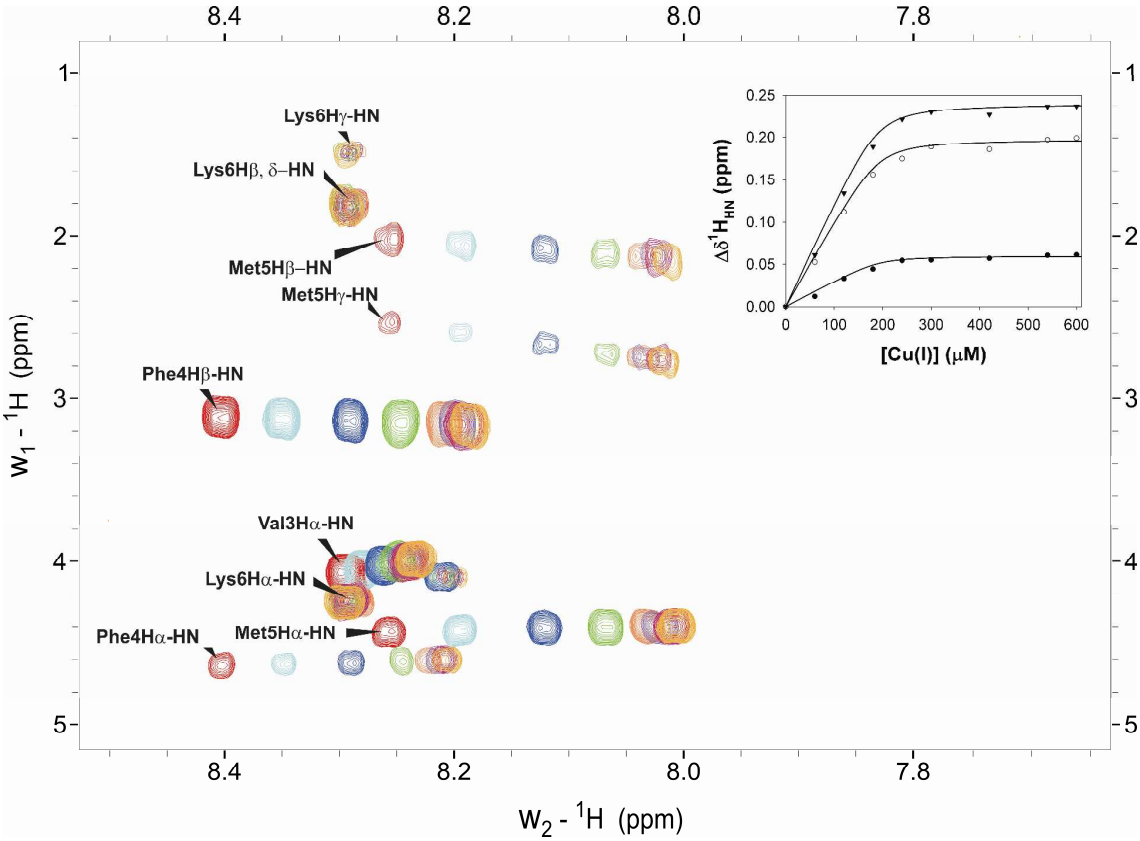

Figure S4. Affinity features of Cu(I) binding to ^{1}MDVFMK^{6}. Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} (300 μM) in the presence of increasing Cu(II) concentrations followed by the addition of an excess of ascorbate: red (0), cyan (60 μM), blue (120 μM), green (180 μM), light brown (240 μM), gray (300 μM), purple (420 μM), dark red (540 μM) and orange (600 μM). Inset shows the binding curves of Cu(I) to AS as monitored by changes in the amide protons chemical shifts ($\Delta\delta^{1}H_{HN}$) of Val3 (●), Phe4 (○) and Met5 (▼) residues.

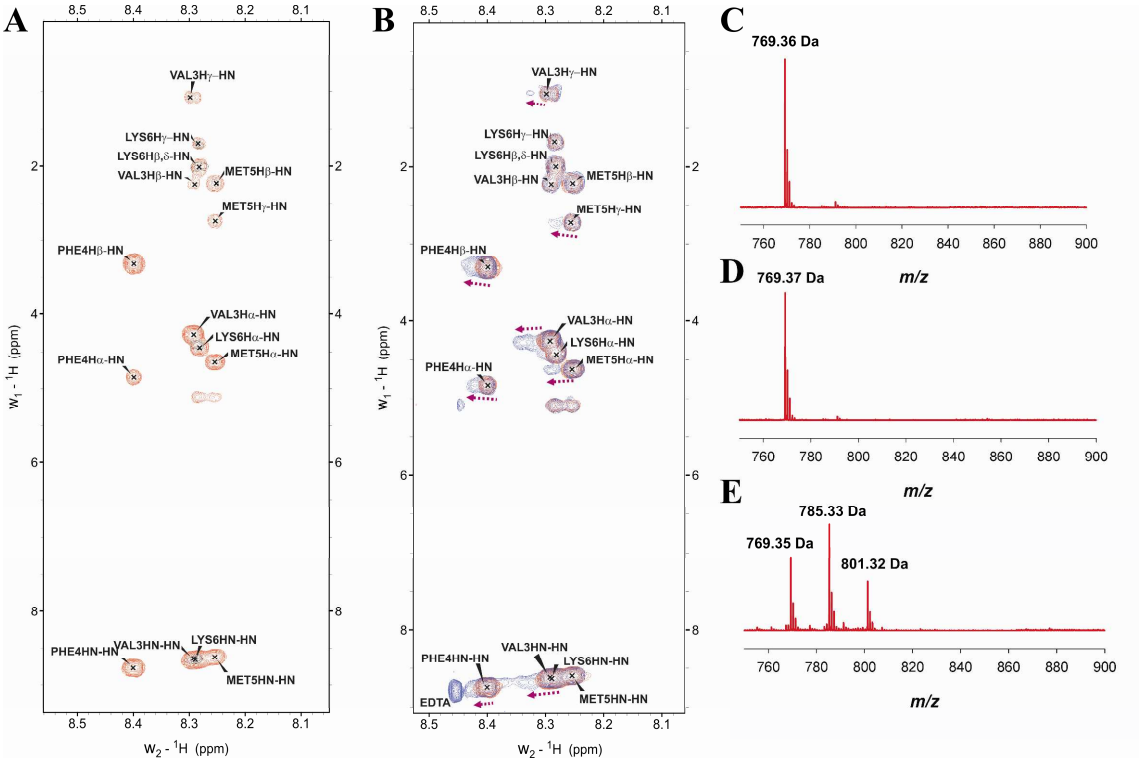

Figure S5. Metal catalyzed oxidation of ^{1}MDVFMK^{6} as monitored by NMR and MALDI-TOF MS. (A) Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} (300 μM) in the absence (red) and presence (black) of 1 Cu(II) equivalent. (B) Overlaid ^{1}H-^{1}H TOCSY spectra of ^{1}MDVFMK^{6} in the absence (red) and presence (blue) of 1 Cu(II) equivalent and 35 mM ascorbate followed by 5 hs of air exposure and addition of 5 mM EDTA. Two species corresponding to unmodified and modified ^{1}MDVFMK^{6} are clearly detected under these conditions, indicating that peptide modification constitute a non-reversible change (resonances corresponding to the modified species are identified by purple arrows). (C-E) MALDI-MS spectra of ^{1}MDVFMK^{6} (300 μM) in the absence (C) and presence of 35 mM ascorbate (D) or 35 mM ascorbate with 1 equivalent of Cu(II) added (E). In all cases, samples were exposed to air (5 hs) before recording of MS spectra. The peak at *m/z* 769.35 was assigned to the unmodified peptide ^{1}MDVFMK^{6}. On the basis of collision induced dissociation MS/MS experiments, peaks at *m/z* 785.33 (mass shift of ~16 Da) and 801.32 (mass shift of ~32 Da) were assigned to the peptide containing one or two methionine sulfoxides in the first or/and fifth (Met1, Met5) positions of the ^{1}MDVFMK^{6} sequence, demonstrating that the modified species detected by NMR corresponds to the oxidized peptide. Oxidation of methionine residues to sulfone was not observed under the experimental conditions of our studies.

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Complete reference 4 in the manuscript text:

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