Supplementary Information for:

Interaction of Cu(I) with the Met-X_3-Met motif of alpha-synuclein: binding ligands, affinity and structural features

Iñaki Gentile,[1#] Hugo A. Garro,[1#] Susana Delgado Ocaña,[1] Nazareno Gonzalez,[1] Timo Strohäker,[2] Daniela Schibich,[1] Liliana Quintanar,[3] Luis Sambrotta,[1] Markus Zweckstetter,[2,4] Christian Griesinger,[2] Mauricio Menacho Márquez [1] and Claudio O. Fernández[*,1,2]*

[1]Max Planck Laboratory for Structural Biology, Chemistry and Molecular Biophysics of Rosario (MPLbioR, UNR-MPIbpC) and Instituto de Investigaciones para el Descubrimiento de Fármacos de Rosario (IIDEFAR, UNR-CONICET), Universidad Nacional de Rosario, Ocampo y Esmeralda, S2002LRK Rosario, Argentina.

[2]Department of NMR-based Structural Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany.

[3]Department of Chemistry, Centro de Investigación y de Estudios Avanzados (Cinvestav), Av. Instituto Politécnico Nacional 2508, 07360, D.F., México.

[4]Deutches Zentrum für Neurodegenerative Erkrankungen (DZNE), von-Siebold-Str. 3a, 37075, Göttingen, Germany.

# These authors contributed equally to the manuscript.

** To whom correspondence should be addressed at: Max Planck Laboratory for Structural Biology, Chemistry and Molecular Biophysics of Rosario (MPLbioR, UNR-MPIbpC) and Instituto de Investigaciones para el Descubrimiento de Fármacos de Rosario (IIDEFAR, UNR-CONICET), Universidad Nacional de Rosario, Ocampo y Esmeralda, S2002LRK Rosario, Argentina. Phone: ++54 341 4237868 (ext. 752). E-mail: fernandez@iidefar-conicet.gob.ar / cfernan@gwdg.de*

Protein and reagents. ^{15}N isotopically enriched N-terminally acetylated αS (AcαS) and its M5I/H50A variant were obtained by co-transfecting *E. coli* BL21 cells with the plasmid harboring the protein gene and a second one that encodes for the components of yeast NatB acetylase complex.[1] Both plasmids carried different antibiotic resistance, namely Ampicillin and Chloramphenicol to select the doubly transformed *E coli* colonies. Since NatB acetylase targets Met-Glu or Met-Asp sequences,[1] the D2A variant of αS studied in this work lacks such a post-translational modification. Purification was carried out as previously reported,[2] with the exception that when required both antibiotics were included in the growth flasks to avoid plasmid purge during growth and expression. The final purity of the protein samples was determined by SDS-PAGE. Copper sulfate, L-ascorbic acid, MES buffer and D_2O were purchased from Merck or Sigma; ^{15}N NH_4Cl was purchased from Cambridge Isotope Laboratories. Purified protein samples were dissolved in 20 mM MES buffer supplemented with 100 mM NaCl at pH 6.5 (Buffer A). Protein concentrations were determined spectrophotometrically by measuring absorption at 274 nm and using an epsilon value of 5600 $M^{-1}cm^{-1}$. Peptide ^{1}MDVFMK^{6} (P1AS) and its variants were synthesized in the solid phase (Rink amide resin) using F-moc chemistry. The absorption extinction coefficient for all of the peptides used was (11500) cm^{-1} M^{-1} at 214 nm and was used to determine the peptide concentration in each sample. The absorption extinction coefficient of the peptides was determined using a calibration curve prepared in Buffer A, weighting out the purified dry peptide samples.

Generation of peptide and protein Cu(I) complexes. To generate the Cu(I) complexes of the model peptides and the protein variants, the respective Cu(II) complexes were prepared first and then reduced with ascorbate under anaerobic conditions. In all cases,

the final concentration of ascorbate used to generate each Cu(I) complex was 50:1 relative to the amount of added Cu(II). The reduction of Cu(II) protein/peptide complexes with ascorbic acid was followed by a decrease of the characteristic d−d transition band in the UV−vis spectrum, as previously described.[3] Spectra were recorded at 15 °C on a Jasco V-550 spectrophotometer. Before and after ascorbate addition, samples were treated with a flow of N_2 during 5 minutes to generate an N_2 atmosphere. In all cases, NMR tubes sealed under N_2 atmosphere were used, as previously described.[3,4]

NMR experiments. NMR spectra were recorded on a Bruker 600 MHz HD Avance III spectrometer, equipped with a cryogenically cooled triple resonance $^{1}H(^{13}C/^{15}N)$ TCI probe. 1D ^{1}H experiments and 2D $^{1}H-^{1}H$ TOCSY were all recorded on 50−200 μM peptide samples dissolved in Buffer A at 15ºC. 2D ^{1}H-^{15}N HSQC and ^{15}N R_1/R_2 relaxation rates were all recorded on 25−200 μM protein (isotopically enriched in ^{15}N) samples dissolved in Buffer A at 15ºC. Sequence-specific assignments for the backbone of unbound and Cu(I)-bound AcαS were obtained from our previous works. In the case of the M5I/H50A and the D2A mutants assignment of backbone resonances in the vicinity of the mutation was confirmed by 3D TOCSY-HSQC and NOESY-HSQC experiments. In all cases, standard pulse sequences from the Topspin suite (Bruker) library were used. Three-bond HN-Hα coupling constants ($^{3}J_{HN-H\alpha}$) were obtained from the ratio between the intensities of the diagonal peaks and cross-peaks in the HNHA experiment.[5] Three-bond HN-Hα coupling constants ($^{3}J_{HN-H\alpha}$) are sensitive to the torsion angle ϕ populated by each residue in the protein sequence and thus report on secondary structure content. This coupling falls in the range 3.0–6.0 Hz for an α-helix and 8.0–11.0

Hz for a β-sheet structure. For a random-coil, a weighted average of these values is observed, that typically ranges between 6.0 and 8.0 Hz for most residues.[6,7] Mean weighted chemical shifts differences (mwΔδ) were calculated as $[(\Delta\delta^{1}H)^2 + (\Delta\delta^{15}N/10)^2]^{1/2}$.[8] Acquisition and processing of NMR spectra were performed using TOPSPIN 3.1 (Bruker Biospin). 2D spectra analysis and visualization were performed with CCPN.

Protein-Cu(I) complex affinities. The affinity features of Cu(I) binding to the protein and peptide variants were determined from 1D ^{1}H NMR and 2D ^{1}H-^{15}N HSQC experiments using protein/peptide samples recorded at increasing concentrations of the metal ion. In the case of the peptides, the Hε protons of Met-1 and Met-5, in close proximity to the sulfur atoms coordinating Cu(I) were used to estimate the affinity features of the αS-Cu(I) complex according to a single site model (with apparent dissociation constant K_{d1}) :

$$P1AS\text{-}Cu(I) \leftrightarrow P1AS + Cu(I) \qquad K_{d1}$$

For the protein variants, changes in ^{1}H-^{15}N mwΔδ values of amide resonances in the sequence comprising residues 1 to 10 were fit to ta model incorporating one Cu(I) ion per site, as previously described.[9,10] This model assumes that the different sites located at the N- and C-terminus of the protein are independent and that the availability of free Cu(I) for one site at each titration point depends on the amount of metal bound to the others.

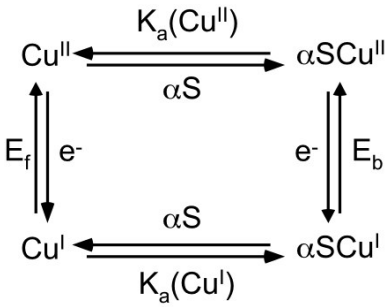
The apparent K_d values estimated by using the program DynaFit.[9,10] These values were then corrected to calculate the conditional dissociation constants ($^{c}K_d$). The correction of the apparent ($^{a}K_d$) values calculated from the NMR experiments to obtain the conditional ($^{c}K_d$) values was performed as reported previously.[10–12] The main

components of the buffer that could act as Cu(I) binding competitors are MES buffer and Cl^- ions. According to previously reported studies, MES competition for Cu(I) may be considered negligible in the presence of 100 mM Cl^-.[13–15] Thus, the equation used to calculate the ${}^{c}K_d$ was:

$$ {}^{c}_{\beta}K_d = \frac{{}^{a}_{\beta}K_d}{\left(1 + \beta_2 \cdot [Cl^-]^2_\beta + \beta_3 \cdot [Cl^-]^3_\beta\right)} $$

Since Cl^- ions are in large excess (100 mM) as compared to Cu(I), the $[Cl^-]$ in the equation was considered to be the total $[Cl^-]_o$. The major Cu(I)-Cl^- complexes involved are $CuCl_2^-$ and $CuCl_3^{2-}$ ($\beta_2 = 3x10^5\ M^{-2}$ and $\beta_3 = 6.4x10^4\ M^{-2}$, respectively).[16]

Estimation of $E°_{(\alpha S\text{-}Cu(II/I))}$ by a thermodynamic cycle scheme, using binding affinities for Cu(II) and Cu(I): A thermodynamic cycle scheme can be constructed, relating the reduction potential of each αS-Cu complex to that of free Cu and the relative affinities of the αS species for Cu(II) and Cu(I) ions:



$$ E_b - E_f = 0.059x \log\left(\frac{K_a\left(Cu^I\right)}{K_a\left(Cu^{II}\right)}\right) $$

where E_f is the midpoint reduction potential for free Cu ions, K_a (Cu^I) corresponds to the association constant of the αS-Cu(I) complex, K_a (Cu^{II}) corresponds to the association constant of the αS-Cu(II) complex, and E_b is the reduction potential for the αS-bound species. Using E_f =0.158 V/NHE and the dissociation constants for each αS species

towards Cu(I) and Cu(II), a reduction potential $E_{b(\alpha S\text{-}Cu)}$ for the corresponding αS-Cu complex can be calculated. All calculated reduction potentials are listed in Table S1.

Supplementary Figure Legends:

Figure S1. Overlaid 2D $^{1}H-^{1}H$ TOCSY NMR spectra of the Ac-P1AS peptide in the absence (black) and presence (green) of 1 equiv of Cu(I). The experiments were recorded on 50 μM peptide samples dissolved in Buffer A at 15 °C.

Figure S2. Affinity features of Cu(I) binding to αS synthetic model peptides. Panel (A) shows the binding curves of Cu(I) to Ac-P1AS (50 μM), as monitored by changes in the chemical shifts ($\Delta\delta^{1}H$) of the Hε protons of Met-1 (●) and Met-5 (▲) residues upon increased concentrations of Cu(I) . The binding curves of P1AS and D2A P1AS peptide variants are shown in panels B and C, respectively. Curves represent the fit to the model described in the text, using the program DynaFit. All experiments were recorded on peptide samples dissolved in Buffer A at 15 °C.

Figure S3. Impact of Cu(I) binding on the structural properties of αS variants. $^{3}J_{HN-H\alpha}$ couplings measured for free (black) and Cu(I) complexed (green). Panels show the $^{3}J_{HN-H\alpha}$ profiles measured for the proteins AcαS, M5I/H50A AcαS and D2A αS and its Cu(I) forms. Orange box contains the first 10 residues of protein sequence. Experiments were recorded at 15 °C using ^{15}N isotopically enriched protein samples (200 μM) dissolved in Buffer A, in the absence and presence of 2 equivalents of Cu(I).

Table S1. Calculated reduction potentials of αS-Cu complexes using binding affinities for Cu(II) and Cu(I) [a]

αS species	K_d for Cu(I) [a]	K_d for Cu(II) [a]	Calculated $E_{b(\alpha S\text{-}Cu)}$ vs. NHE (mV)
Ac-P1AS	4.8 nM[b]	50 μM[c]	395 mV
P1AS	8.5 nM[b,f]	4.1 nM[d]	140 mV
D2A P1AS	13.4 nM[b]	488 nM[d]	250 mV[e]
M5I P1AS	65 nM[b,f]	4.1 nM[g]	87 mV
M1I P1AS	163 nM[b,f]	4.1 nM[g]	64 mV
AcαS	3.9 nM[b,h,i]	50 μM[c]	400 mV
αS	7.8 nM[b,j]	4.1 nM[d]	142 mV
AcαS M5I	63 nM[b]	50 μM[c]	329 mV
αS D2A	14 nM[b]	488 nM[k]	249 mV

[a] Conditional K_d values are listed for both redox forms. Conditional K_d values for Cu(II) have been calculated considering competition for the metal ion from the buffer and chloride ions, as previously reported.[17]

[b] This study.

[c] Estimated lower limit from relative affinities to the different Cu(II) binding sites in acetylated protein.[18]

[d] Published work.[19]

[e] The calculated $E_{b(\alpha S\text{-}Cu)}$ considers also the decreased affinity for Cu(II), since Asp-2 also serves as a ligand for Cu(II). The estimated $E_{b(\alpha S\text{-}Cu)}$ considering only the decreased affinity for Cu(I) (assuming no change in affinity for the oxidized form) would be 128 mV, i.e. the drop in binding affinity for Cu(I) upon D2A substitution (as compared to P1AS) would cause a drop in $E_{b(\alpha S\text{-}Cu)}$ of 12 mV.

[f] Published work.[1]

[g] Assumed to be the same as P1AS, since Met to Ile substitutions do not have an impact in Cu(II) binding features.[20]

[h] Published work.[10]

[i] Published work.[21]

[j] Published work.[4]

[k] Assumed to be the same as the D2A P1AS peptide.[19]

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