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# Copper(I)- $\alpha$ -Synuclein Interaction: Structural Description of Two Independent and Competing Metal Binding Sites

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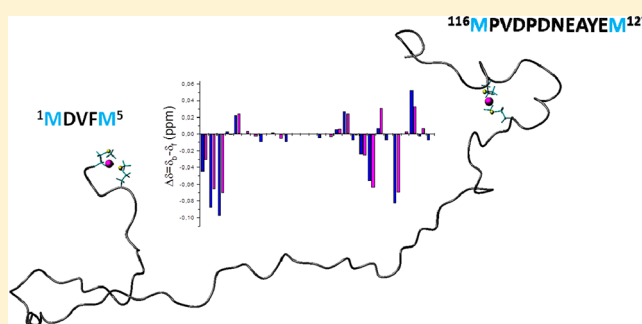
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## S Supporting Information

**ABSTRACT:** The aggregation of  $\alpha$ -synuclein ( $\alpha$ S) is a critical step in the etiology of Parkinson's disease. Metal ions such as copper and iron have been shown to bind  $\alpha$ S, enhancing its fibrillation rate in vitro.  $\alpha$ S is also susceptible to copper-catalyzed oxidation that involves the reduction of  $\text{Cu}^{\text{II}}$  to  $\text{Cu}^{\text{I}}$  and the conversion of  $\text{O}_2$  into reactive oxygen species. The mechanism of the reaction is highly selective and site-specific and involves interactions of the protein with both oxidation states of the copper ion. The reaction can induce oxidative modification of the protein, which generally leads to extensive protein oligomerization and precipitation.  $\text{Cu}^{\text{II}}$  binding to  $\alpha$ S has been extensively characterized, indicating the N terminus and His-50 as binding donor residues. In this study, we have investigated  $\alpha$ S– $\text{Cu}^{\text{I}}$  interaction by means of NMR and circular dichroism analysis on the full-length protein ( $\alpha\text{S}_{1-140}$ ) and on two, designed *ad hoc*, model peptides:  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$ . In order to identify and characterize the metal binding environment in full-length  $\alpha$ S, in addition to  $\text{Cu}^{\text{I}}$ , we have also used  $\text{Ag}^{\text{I}}$  as a probe for  $\text{Cu}^{\text{I}}$  binding. Two distinct  $\text{Cu}^{\text{I}}/\text{Ag}^{\text{I}}$  binding domains with comparable affinities have been identified. The structural rearrangements induced by the metal ions and the metal coordination spheres of both sites have been extensively characterized.



## INTRODUCTION

Parkinson's disease (PD) is a devastating progressive neurodegenerative disease whose major clinical symptoms are tremors, movement impairments, postural instability, gait difficulty, and rigidity. PD is characterized by the loss of dopaminergic neurons<sup>1,2</sup> and by the presence of intracellular proteinaceous inclusions (Lewy bodies) mainly consisting of aggregated forms of  $\alpha$ -synuclein ( $\alpha$ S).<sup>3–5</sup>

$\alpha$ S is a 140-amino acid presynaptic protein of still unknown function. It is constituted by three different domains: (i) the amphipathic N terminus comprising the first 60 residues, (ii) the nonamyloidogenic component (NAC), highly hydrophobic and encompassing residues between 61 and 95, and (iii) the acidic C-terminal region (96–140) rich in Glu and Asp residues.  $\alpha$ S is classified as an intrinsically disordered protein,<sup>6,7</sup> but it undergoes dramatic conformational transitions from its natively unstructured state to  $\alpha$ -helix-rich conformations upon interaction with lipid membranes.<sup>8,9</sup>

In addition to the formation of  $\alpha$ S aggregates, metal-induced oxidative stress and the directly or indirectly related formation of reactive oxygen species (ROS) have also been implicated in the pathogenesis of PD and other neurodegenerative diseases.<sup>1,10</sup> ROS can damage cells at different levels by causing (i) lipid membrane peroxidation,<sup>11</sup> (ii) oligomerization and/or

inactivation of oxidized proteins, and (iii) cleavage of DNA and RNA molecules. The formation of ROS is accelerated in the presence of redox-active metals such as copper and iron,<sup>12</sup> and there is much evidence that abnormal metal homeostasis has a significant impact on the development of age-related neurodegenerative diseases.<sup>13–15</sup>

$\alpha$ S, like other proteins associated with neurodegeneration ( $A\beta$ , prion protein, etc.), interacts with copper, which can modulate  $\alpha$ S aggregation and oligomerization.<sup>16,17</sup> Copper is an essential trace element and is a key component of many important enzymes involved in vital biochemical processes, such as iron acquisition, respiration, and free-radical detoxification.<sup>18</sup> On the other hand, high cellular levels of copper may result in toxicity for the cell. The toxicity has been attributed to  $\text{Cu}^{\text{I}}/\text{Cu}^{\text{II}}$  redox cycling, which leads to the production of ROS through Haber–Weiss or Fenton reactions.<sup>19</sup> To prevent metal-catalyzed oxidative stress, cells have developed sophisticated systems for copper storage and transport. An imbalance in copper homeostasis has been associated with genetic disorders such as Menkes disease and

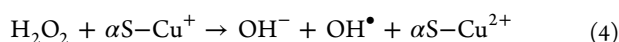
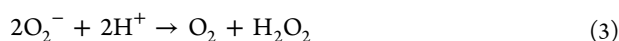
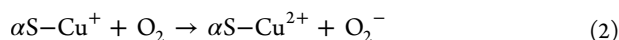
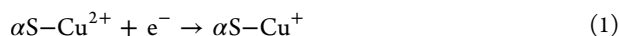
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Wilson syndrome but also with neurodegenerative and prion diseases.<sup>20</sup>

It has been shown that  $\alpha$ S, as well as the A $\beta$  peptide, is highly susceptible to copper-catalyzed oxidation by ROS.<sup>21–23</sup> The reaction requires the ability of the protein to bind both Cu<sup>II</sup> and Cu<sup>I</sup> ions and leads to oligomerization and precipitation of the protein itself.<sup>24</sup> Cu<sup>II</sup> complexed by both full-length  $\alpha$ S and its N-terminal fragment  $\alpha$ S<sub>1–19</sub> can be reduced to Cu<sup>I</sup> by cellular reductants [such as NADH, ascorbic acid (AA), GSH, or the protein itself] to form stable species with features similar to those of copper complexes with A $\beta$  peptides.<sup>25</sup> The  $\alpha$ S–Cu<sup>I</sup> complex can then catalyze the reduction of molecular oxygen O<sub>2</sub> into ROS, such as H<sub>2</sub>O<sub>2</sub> and OH• (eqs 1–4), which results in protein damage through oxidation of specific amino acid residues:



Cu<sup>II</sup>– $\alpha$ S binding has been extensively elucidated by using both full-length protein, point mutants,<sup>22,27–36</sup> and ad hoc designed model peptides.<sup>37–41</sup> All of the obtained findings suggest the N-terminus and His-50 as Cu<sup>II</sup> donor residues. On the contrary, very few reports describing the nature of Cu<sup>I</sup>– $\alpha$ S binding sites are available.<sup>27,42</sup>  $\alpha$ S contains two regions,  $\alpha$ S<sub>1–5</sub> and  $\alpha$ S<sub>116–127</sub>, located at the N and C termini, respectively, containing two Met residues and arranged as [M(X)<sub>n</sub>M], motifs which are commonly found in other proteins involved in copper homeostasis.<sup>43–48</sup> NMR investigations of full-length  $\alpha$ S and its derived fragment,  $\alpha$ S<sub>1–6</sub>, have revealed that the main Cu<sup>I</sup> anchoring site involves Met-1 and Met-5 sulfur-donor atoms,<sup>42</sup> whereas the C-terminal site was proposed to be less effective in metal binding.<sup>27</sup>

In this study, we have investigated  $\alpha$ S–Cu<sup>I</sup> interaction by means of NMR and circular dichroism (CD) analysis on the full-length  $\alpha$ S ( $\alpha$ S<sub>1–140</sub>) and on the fragments  $\alpha$ S<sub>1–15</sub> (<sup>1</sup>MDVFMKGLSKAKEGV<sup>15</sup>–NH<sub>2</sub>) and  $\alpha$ S<sub>113–130</sub> (Ac-<sup>113</sup>LEDMPVDPDNEAYEMPSE<sup>130</sup>–NH<sub>2</sub>), both containing the [M(X)<sub>n</sub>M] motif found at the N and C termini, respectively. In order to further identify and characterize the metal binding environment in full-length  $\alpha$ S, besides Cu<sup>I</sup>, we have also used Ag<sup>I</sup> as a probe for Cu<sup>I</sup> binding. In fact, Ag<sup>I</sup>, being isoelectronic with Cu<sup>I</sup>, is amenable to NMR studies and redox-inactive and may be commonly used to investigate Cu<sup>I</sup> sites, as demonstrated by the structural similarity of complexes formed by Ag<sup>I</sup> and Cu<sup>I</sup>, including ligands with methionine-rich sites.<sup>49–51</sup> A comparison between the copper binding abilities of all of the investigated systems, together with an estimation of apparent K<sub>d</sub> values at physiological pH, is discussed with the aim to getting helpful information on the bioinorganic chemistry of  $\alpha$ S.

## MATERIALS AND METHODS

**Protein and Reagents.** Human wild-type  $\alpha$ S cDNA, amplified by polymerase chain reaction with synthetic oligonucleotides (Sigma-Aldrich) containing NcoI and XhoI restriction sites, was cloned into a pET-28a plasmid (Novagen, Merck KGaA, Darmstadt, Germany). Then the protein was expressed in *Escherichia coli* BL21 (DE3) cells. Overexpression of the proteins was achieved by growing cells in a LB medium or, for the <sup>15</sup>N-labeled protein, in a M9 minimal medium

prepared with <sup>15</sup>NH<sub>4</sub>Cl (Cambridge Isotope Laboratories, Inc., Andover, MA) at 37 °C until an appropriate optical density at 600 nm (OD<sub>600</sub> nm) of 0.3–0.4, followed by induction with 0.1 mM isopropyl  $\beta$ -thiogalactopyranoside for 4–5 h. The cells were collected by centrifugation, and recombinant protein was recovered from the periplasm by osmotic shock, as previously described.<sup>26</sup> The periplasmic homogenate was then boiled for 15 min, and the soluble fraction, containing  $\alpha$ S, was subjected to a two-step (35% and 55%) ammonium sulfate precipitation. The pellet was then resuspended, extensively dialyzed against 20 mM Tris-HCl (pH 8.0), loaded into a 6 mL Resource Q column (GE Healthcare, Fairfield, CT), and eluted with a 0–500 mM gradient of NaCl. Finally, the protein was dialyzed against water, lyophilized, and stored at –20 °C.

The N-terminal  $\alpha$ S<sub>1–15</sub> peptide was synthesized in a solid phase using Fmoc chemistry. A rink-amide resin was used as the solid support, so that the resulting peptides would be amidated at the C terminus. After removal of the peptide from the resin and deprotection, the crude product was purified by RP HPLC on a Phenomenex Jupiter Proteo C12 column, using a Jasco PU-1580 instrument with diode-array detection (Jasco MD-1510), with a semilinear gradient of 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in CH<sub>3</sub>CN over 40 min. The identity of the peptide was confirmed by electrospray ionization mass spectrometry (Thermo-Finnigan). The purified peptide was lyophilized and stored at –20 °C until use.

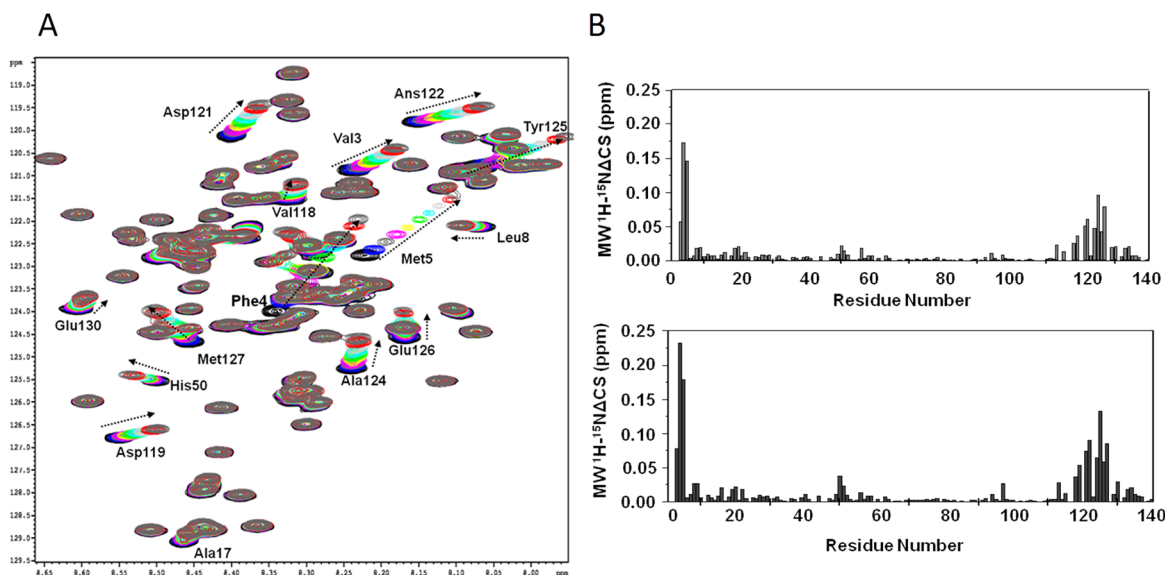
The C-terminal  $\alpha$ S<sub>113–130</sub> peptide was purchased from Biomatik.

**NMR and CD Spectroscopic Measurements.** Full-length  $\alpha$ S protein was dissolved in a 20 mM phosphate buffer in water at pH 7.4, obtaining a final concentration of 240  $\mu$ M for NMR experiments and 10  $\mu$ M for CD experiments. The protein concentration was estimated by a spectrometer using a molar extinction coefficient at 274 nm of 5960 M<sup>–1</sup> cm<sup>–1</sup>.<sup>52</sup> Peptides were dissolved in a 20 mM phosphate buffer in water at pH 7.4, obtaining a final concentration ranging from 400 to 500  $\mu$ M for NMR experiments and 100  $\mu$ M for CD experiments. The desired concentration of Ag<sup>I</sup> ions was achieved by using a stock solution of 20 mM AgNO<sub>3</sub> (Sigma Chemical Co.) in D<sub>2</sub>O. For copper(I) complexes, a 10 mM [Cu(CH<sub>3</sub>CN)<sub>4</sub>]BF<sub>4</sub> stock solution was prepared in a 20 mM phosphate buffer in D<sub>2</sub>O at pH 7.4 (containing 5% v/v CH<sub>3</sub>CN). 1.0 mM AA was added to both full-length protein and peptides just before use in order to avoid any possible oxidation of Cu<sup>I</sup> to Cu<sup>II</sup>. All of the solutions were degassed with water-saturated N<sub>2</sub> just before use and kept under an inert N<sub>2</sub> atmosphere. 3-(Trimethylsilyl)-[2,2,3,3-*d*<sub>4</sub>]propanesulfonate, sodium salt, was used as the internal reference standard for NMR measurements.

CD spectra were acquired on a Jasco J-815 spectropolarimeter at 288 K. A 0.1 cm cell path length was used for data between 180 and 260 nm, with a 1 nm sampling interval. Four scans were collected for every sample with a scan speed of 100 nm min<sup>–1</sup> and a bandwidth of 1 nm. Baseline spectra were subtracted from each spectrum, and data were smoothed with the Savitzky–Golay method.<sup>53</sup> Data were processed using an Origin 5.0 spreadsheet/graph package. The direct CD measurements ( $\theta$ , in millidegrees) were converted to mean residue molar ellipticity, using the relationship mean residue  $\Delta\epsilon = \theta / (33000cl \times \text{number of residues})$ , where *c* and *l* refer to the molar concentration and cell path length, respectively.

NMR spectra were acquired at 278 and 288 K using Bruker Advance spectrometers operating at proton frequencies of 600 and 900 MHz, with the last one equipped with a cryoprobe. NMR spectra were processed with XwinNMR 3.6 and TopSpin 2.0 software and analyzed with the program Cara.<sup>54</sup> Suppression of the residual water signal was achieved either by presaturation or by excitation sculpting,<sup>55</sup> using a selective 2-ms-long square pulse on water. The proton resonance assignment of the peptides was obtained by 2D <sup>1</sup>H–<sup>1</sup>H COSY, TOCSY, and NOESY experiments. The metal-interaction studies were performed by acquiring 2D <sup>1</sup>H–<sup>15</sup>N HSQC and <sup>1</sup>H–<sup>13</sup>C HSQC and 3D <sup>15</sup>N NOESY HSQC.

**Structure Determination.** NOE cross peaks in 2D <sup>1</sup>H–<sup>1</sup>H NOESY spectra acquired on  $\alpha$ S<sub>1–15</sub>–Ag<sup>I</sup> and  $\alpha$ S<sub>113–130</sub>–Ag<sup>I</sup> complexes at 278 K were integrated with the Cara program and were converted



**Figure 1.** NMR analysis of  $\text{Ag}^{\text{I}}$  binding to full-length  $\alpha\text{S}_{1-140}$ . (A) Overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $\alpha\text{S}_{1-140}$  in the presence of increasing amounts of  $\text{Ag}^{\text{I}}$ : 0 equiv (black), 0.125 equiv (blue), 0.250 equiv (gray), 0.375 equiv (magenta), 0.5 equiv (yellow), 0.65 equiv (green), 0.8 equiv (cyan), 1 equiv (light gray), 1.5 equiv (red), and 2 equiv (dark gray). (B and C) Differences in the mean weighted chemical shift displacements ( $\text{MW } ^1\text{H}$ - $^{15}\text{N } \Delta\text{CS}$ ) between free and  $\text{Ag}^{\text{I}}$ -complexed  $\alpha\text{S}_{1-140}$  at molar ratios of 1:1 (B) and 2:1 (C). The amide protons of Met-1 and Asp-2 were not detectable because of solvent exchange effects.

into upper internuclear distances with the routine *CALIBA* of the program package *DYANA*.<sup>56</sup> The intra- and interresidual constraints obtained were used to generate an ensemble of 30 structures by the standard protocol of simulated annealing in torsion angle space implemented in *DYANA* (using 10000 steps). To take into account the observed coordination behavior of silver, additional metal–proton distance constraints were imposed. No dihedral angle restraints and no hydrogen-bonding restraints were applied. The final structures were analyzed using the program *MOLMOL*.<sup>57</sup>

## RESULTS

**$\alpha\text{S}_{1-140}$ – $\text{Ag}^{\text{I}}$  Interaction.** The effects of  $\text{Cu}^{\text{I}}$  on NMR resonances of  $\alpha\text{S}$  were first evaluated by comparing the  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum of  $\alpha\text{S}_{1-140}$  in the absence or in the presence of 0.5 equiv of  $\text{Cu}^{\text{I}}$ . Met-1, Met-5, Met-116, and Met-127 overlapping cross peaks assigned to  $\text{H}\epsilon$ – $\text{C}\epsilon$  and  $\text{H}\gamma$ – $\text{C}\gamma$  were the ones most affected by the presence of the metal ion (Figure 1S in the Supporting Information). To be sure that the effects we observed did not occur from oxidation of the  $\alpha\text{S}$ – $\text{Cu}^{\text{I}}$  to  $\alpha\text{S}$ – $\text{Cu}^{\text{II}}$  complex, the  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum of  $\alpha\text{S}_{1-140}$  in the presence of 0.5 equiv of  $\text{Cu}^{\text{II}}$  was recorded as well. No effects were observed for  $\text{H}\epsilon$ – $\text{C}\epsilon$  and  $\text{H}\gamma$ – $\text{C}\gamma$  cross peaks of Met-1, Met-5, Met-116, and Met-127. On the contrary, the addition of  $\text{Ag}^{\text{I}}$  to  $\alpha\text{S}_{1-140}$  resulted in very similar  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra, supporting the suitability of  $\text{Ag}^{\text{I}}$  as a probe for  $\text{Cu}^{\text{I}}$  in the study of metal binding properties of  $\alpha\text{S}$ . In fact, also in this case, the cross peaks assigned to  $\text{H}\epsilon$ – $\text{C}\epsilon$  and  $\text{H}\gamma$ – $\text{C}\gamma$  of Met-1, Met-5, Met-116, and Met-127 were the most affected ones (Figure 1S in the Supporting Information). All of these findings confirm that the thioether groups of Met are involved in the copper(I) coordination sphere<sup>27,42</sup> and indicate that silver ions have binding properties very similar to those of the cuprous ion.

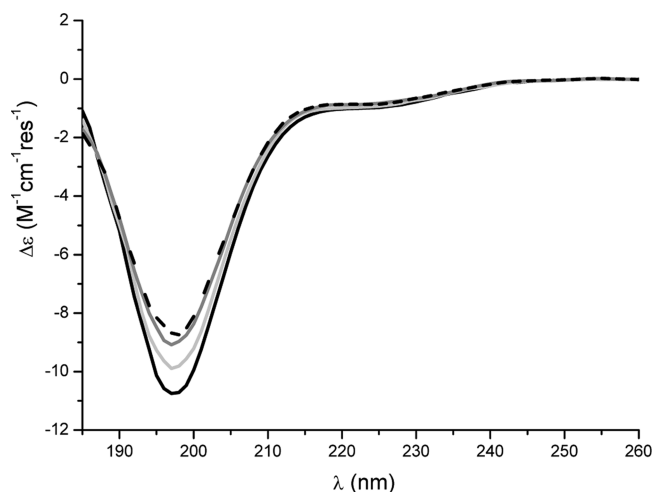
More detailed information on the  $\text{Ag}^{\text{I}}$  binding regions in the full-length protein was obtained by recording  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra at 288 K on uniformly  $^{15}\text{N}$ -enriched  $\alpha\text{S}$ , gradually increasing the amount of metal, and monitoring the metal-induced changes in the spectra. The addition of the diamagnetic

ion to the protein solutions at pH 7.4 caused selective shifts of both proton and nitrogen resonances of several residues (Figure 1). The largest effects were localized at both the N and C termini of the protein, with the major displacements observed for Val-3, Phe-4, Met-5, Asp-121, Asn-122, Tyr-125, and Met-127. His-50 and residues nearby (48–52) were affected as well, although to a lesser extent. However, no effects were observed on imidazolic protons of His-50 in 1D  $^1\text{H}$  spectra (Figure 2S in the Supporting Information), suggesting that this residue, and therefore region 48–52, is not involved in  $\text{Cu}^{\text{I}}$ / $\text{Ag}^{\text{I}}$  binding.

A 3D  $^{15}\text{N}$  NOESY HSQC spectrum was also recorded, in order to establish if any interplay between the identified  $\text{Ag}^{\text{I}}$  binding motifs in the full-length protein occurs. No NOE interactions between the N- and C-terminal regions were identified, suggesting the occurrence of two separate, independent metal binding sites, encompassing 1–5 and 116–127 regions, respectively. On the other hand, we identified a contiguous series of intraresidual  $\text{H}^{\text{N}}_i$ – $\text{H}^{\text{N}}_{i+1}$  NOE interactions in the C-terminal 119–130 region. Because no residual structure has been identified in the free monomeric state of  $\alpha\text{S}$  in water,<sup>58</sup> the appearance of NOE cross peaks in the C-terminal region of the protein is likely due to the binding of  $\text{Ag}^{\text{I}}$ , which induces structural rearrangements of that region.

These findings were also confirmed by far-UV CD spectroscopy. CD spectra were recorded on  $\alpha\text{S}$  solutions and on solutions just containing  $\text{AgNO}_3$  in order to exclude any interferences due to the use of nitrate salt. The spectra of both the free ligand and silver(I) complex showed a strong negative band at 198 nm (Figure 2), which is a diagnostic feature of random-coil conformations. However, the intensity of the random-coil band is progressively decreased as the metal concentration is increased (Figure 2), suggesting partial  $\text{Ag}^{\text{I}}$ -induced structuring of the main chain. The small extent of spectral modification confirms the unlikelihood of the occurrence of any interplay between the two different regions of the protein involved in  $\text{Ag}^{\text{I}}$  binding.





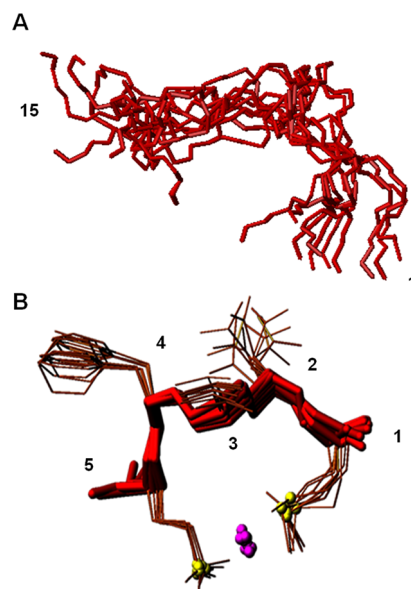
**Figure 2.** Far-UV CD spectra at 288 K of 10  $\mu\text{M}$  full-length  $\alpha\text{S}$  at pH 7.4. Spectra were recorded at different  $\text{Ag}^{\text{I}}$  concentrations: 0 equiv (black), 1 equiv (light gray), 2 equiv (dark gray), and 5 equiv (dashed black).

In order to obtain more detailed information on the specificity of the  $\text{Ag}^{\text{I}}$  binding to  $\alpha\text{S}$ , we investigated the metal binding features of two synthetic peptides,  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$ , encompassing the two regions of the full-length protein that were significantly affected by the presence of the metal ion.

**$\alpha\text{S}_{1-15}$ – $\text{Ag}^{\text{I}}$  Complex.** The details of  $\text{Ag}^{\text{I}}$  binding to  $\alpha\text{S}_{1-15}$  were explored at single-residue resolution using 1D and 2D  $^1\text{H}$  NMR spectroscopy.

The NMR investigation was performed on 400  $\mu\text{M}$  peptide samples at pH 7.4 in a 20 mM phosphate buffer at 288 K. The addition of silver ion to the peptide solution caused selective proton chemical shift displacements similar to those observed for the  $\text{Cu}^{\text{I}}$ – $\alpha\text{S}_{1-6}$  system<sup>42</sup> (Figure 3S in the Supporting Information). The largest effects were observed for Met-1 and Met-5 and residues nearby (Val-3, Phe-4, and Met-5), confirming the involvement of Met thioether groups in the metal binding.<sup>42</sup> On the contrary, no chemical shift displacements were detected for residues 7–15, indicating that these protons are distant from the metal binding site. Further analysis of  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectra showed substantial  $^{13}\text{C}$  and  $^1\text{H}$  downfield shifting of both Met-1 and Met-5  $\epsilon$ - $\text{CH}_3$ ,  $\gamma$ - $\text{CH}_2$ , and  $\beta$ - $\text{CH}_2$  groups and smaller effects on Met-1  $\text{C}\alpha$ – $\text{H}\alpha$ , Asp-2  $\text{C}\alpha$ – $\text{H}\alpha$  and  $\text{C}\beta$ – $\text{H}\beta$ , Val-3  $\text{C}\alpha$ – $\text{H}\alpha$ ,  $\text{C}\beta$ – $\text{H}\beta$ , and  $\text{C}\gamma$ – $\text{H}\gamma$ , and Phe-4  $\text{C}\beta$ – $\text{H}\beta$  cross peaks (Figure 4S in the Supporting Information). These findings strongly support Met-1 and Met-5 residues as the silver binding region with the Met  $\delta$ -sulfur atoms bound to  $\text{Ag}^{\text{I}}$  and confirm that previously found for  $\text{Cu}^{\text{I}}$ – $\alpha\text{S}_{1-6}$  systems.<sup>42</sup>

This information, together with the  $^1\text{H}$ – $^1\text{H}$  distances obtained from the NOESY spectrum recorded at 278 K, was successively employed as restraints for the structure determination of the  $\alpha\text{S}_{1-15}$ – $\text{Ag}^{\text{I}}$  complex, by using the DYANA program (see the experimental section for details). The obtained first 10 structures are shown in Figure 3. When fitted on all of the backbone residues, the absence of any preferred conformation is easily observed (Figure 3A), as confirmed by the high values of root-mean-square deviation (RMSD) for the backbone and heavy atoms, respectively  $3.54 \pm 1.05$  and  $4.89 \pm 1.30$  Å. On the contrary, following the fitting on the silver binding domain, 1–5 residues (Figure 3B), the structures assume a well-defined arrangement and the calculated RMSD



**Figure 3.** Superimposition of the first 10 structures obtained for the  $\alpha\text{S}_{1-15}$ – $\text{Ag}^{\text{I}}$  complex. The structures are fitted on all of the backbone residues (A) and on the 1–5 backbone residues (B). The sulfur-donor atoms of Met-1 and Met-5 are shown as yellow spheres. The silver ion is shown as a magenta sphere.

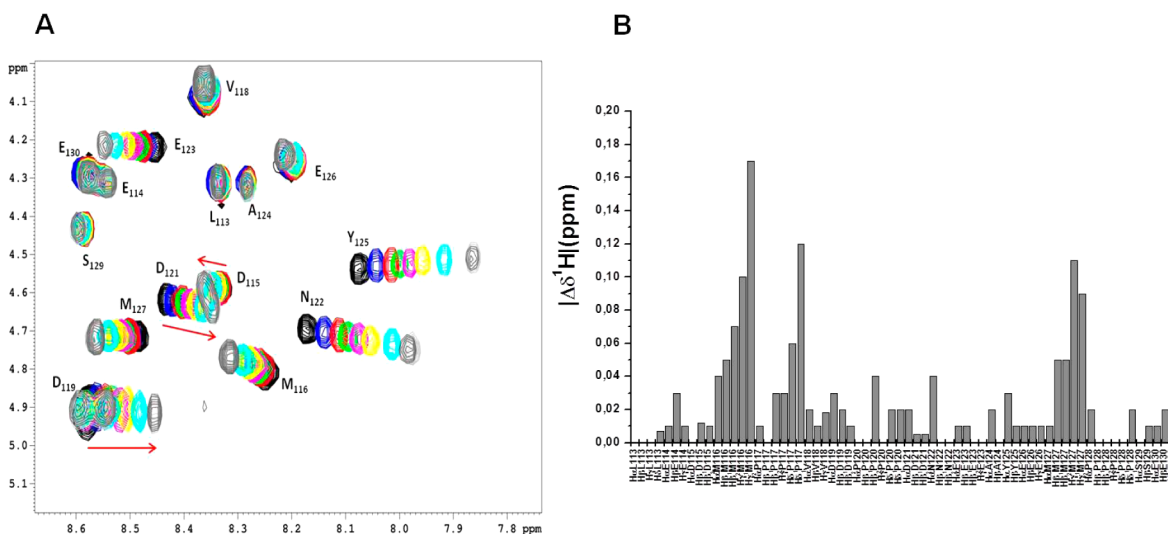
values are  $0.39 \pm 0.18$  and  $1.03 \pm 0.26$  Å, respectively, for the backbone and heavy atoms.

The absence of any preferred secondary structure is confirmed also by CD spectra (Figure 5S in the Supporting Information). Both the free ligand and silver(I) complex showed the intense negative band at 198 nm representative of random-coil conformation.<sup>59</sup> Increasing the amount of metal did not further affect the spectrum.

**$\alpha\text{S}_{113-130}$ – $\text{Ag}^{\text{I}}$  Complex.**  $\text{Ag}^{\text{I}}$  binding to the  $\alpha\text{S}_{113-130}$  fragment was explored by means of 1D and 2D  $^1\text{H}$  NMR and CD spectroscopy.

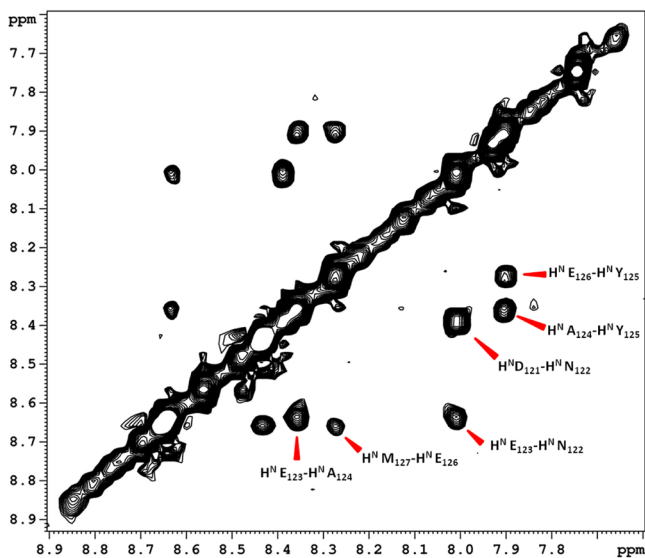
NMR spectra were recorded at 288 K on 400  $\mu\text{M}$  peptide samples at pH 7.4 in a 20 mM phosphate buffer. The addition of  $\text{Ag}^{\text{I}}$  to the peptide solution caused selective proton chemical shift displacements in  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra. The most affected amide protons belong to Asn-122 and Tyr-125 (Figure 4A). Amide protons of Met-116, Met-127, Asp-115, Asp-121, and Glu-123 were affected as well, although to a lesser extent. However, analysis of the aliphatic region (Figure 4B) showed that the major displacements occur for  $\text{H}\beta$  and  $\text{H}\gamma$  protons of Met-116 and Met-127, indicating their proximity to the metal ion, while only small chemical shift variations were observed for the side-chain protons of Val-118, Asp-119, Asn-122, and Tyr-125.

$^1\text{H}$ – $^{13}\text{C}$  HSQC spectra were additionally recorded to evaluate the effect of silver binding to  $\alpha\text{S}_{113-130}$ . Among all of the observed resonances, the  $\text{C}\epsilon$ – $\text{H}\epsilon$  cross peaks assigned to Met-116 and Met-127 exhibited the most significant chemical shift variations (Figure 6S in the Supporting Information). Cross peaks assigned to  $\text{C}\gamma$ – $\text{H}\gamma$  and  $\text{C}\beta$ – $\text{H}\beta$  of methionine residues were shifted as well, whereas no changes were observed on signals belonging to the other residues of the sequence, except for  $\text{C}\beta$ – $\text{H}\beta$  cross peaks assigned to proline residues, likely because of the structural rearrangement of the peptide backbone. All of the collected findings strongly indicate the  $\delta$ -sulfur atoms of Met-116 and Met-127 side chains as the silver binding donors.



**Figure 4.** (A) Overlaid  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra amide region of  $\alpha\text{S}_{113-130}$  in the presence of increasing amounts of  $\text{AgNO}_3$ : 0 equiv (black), 0.125 equiv (blue), 0.2 equiv (red), 0.3 equiv (green), 0.6 equiv (magenta), 0.8 equiv (yellow), 1 equiv (cyan), 1.5 equiv (light gray), and 3 equiv (dark gray). (B) Chemical shift displacements of the aliphatic region of  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra recorded in the absence and presence of 1 equiv of  $\text{Ag}^+$ .

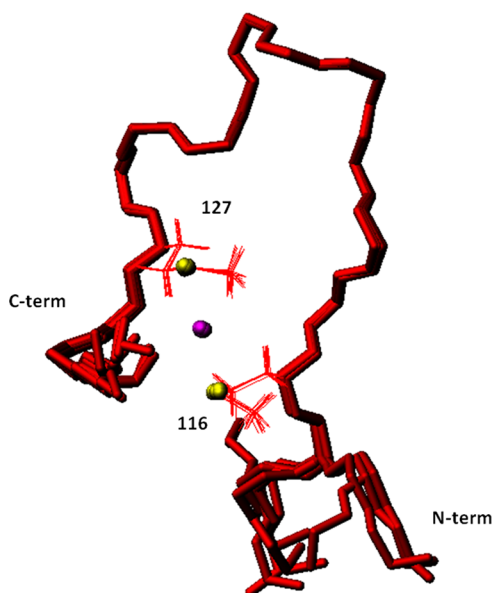
$^1\text{H}$ – $^1\text{H}$  NOESY experiments at 278 K were performed on the  $\alpha\text{S}_{113-130}$ – $\text{Ag}^+$  complex in order to obtain structural information. The spectrum obtained in the presence of 1 equiv of  $\text{AgNO}_3$  showed several dipolar correlations, which were converted into a set of distance constraints. Interestingly, the spectrum showed the occurrence of interresidual  $\text{H}^{\text{N}}_i$ – $\text{H}^{\text{N}}_{i+1}$  NOE interactions in the region encompassing residues 121–126 (Figure 5), resembling the behavior observed for the



**Figure 5.**  $^1\text{H}$ – $^1\text{H}$  NOESY spectrum of 400  $\mu\text{M}$   $\alpha\text{S}_{113-130}$  recorded in the presence of 1 equiv of  $\text{AgNO}_3$  at 278 K and pH 7.4.

full-length protein. This observation can also explain the large chemical shift variation observed for the amide protons of these residues. The distance values obtained from NOESY, together with the binding distances between the silver ion and Met-116 and Met-127  $\delta$ -sulfur atoms, were used as constraints for determining the structure of the metal complex, by using the DYANA program. The first 10 structures obtained are reported in Figure 6, which shows the occurrence of a bent backbone in

the region encompassing the two methionine residues and the metal ion.

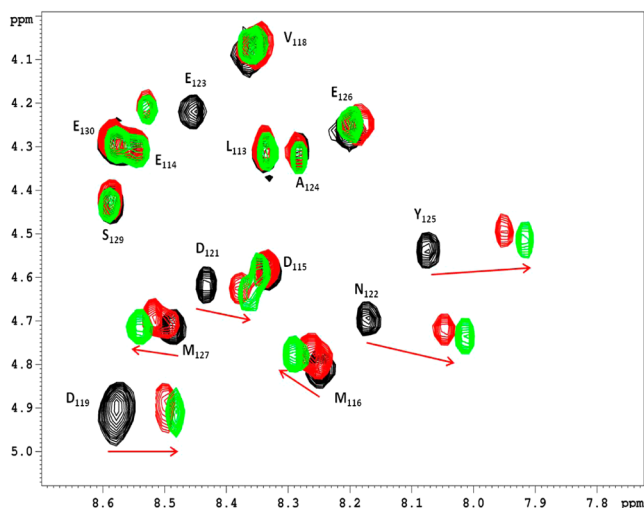


**Figure 6.** Superimposition of the first 10 structures obtained for the  $\alpha\text{S}_{113-130}$ – $\text{Ag}^+$  complex. The structures are fitted on 116–127 backbone residues. The sulfur-donor atoms of Met-116 and Met-127 are shown as yellow spheres. The silver ion is shown as a magenta sphere.

Far-UV CD spectroscopy confirmed the results obtained with NMR. The spectrum of the free  $\alpha\text{S}_{113-130}$  at 288 K is characteristic of a random-coil peptide, showing a strong negative band at 198 nm (Figure 7S in the Supporting Information). Upon  $\text{Ag}^+$  addition, the intensity of the random-coil band is reduced and a weak negative band at 220 nm appears.

Finally, we monitored the interaction of  $\alpha\text{S}_{113-130}$  with  $\text{Cu}^+$  in order to directly compare the  $\text{Ag}^+$  and  $\text{Cu}^+$  behaviors.  $^1\text{H}$ – $^1\text{H}$  TOCSY and  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectra of  $\alpha\text{S}_{113-130}$  in the presence of 1 equiv of  $\text{Cu}^+$  showed very similar chemical shift

displacements (Figure 7), suggesting the occurrence of similar metal–peptide interactions. The  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectrum



**Figure 7.** Overlaid  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra amide proton region of  $\alpha\text{S}_{113-130}$  in the absence (black) and presence of 1 equiv of  $\text{Cu}^{\text{I}}$  (red) and 1 equiv of  $\text{Ag}^{\text{I}}$  (green).

suggested, as in the case of  $\text{Ag}^{\text{I}}$ , the binding of  $\text{Cu}^{\text{I}}$  to the  $\delta$ -sulfur atom on the methionine residue side chain, with the cross peaks assigned to  $\varepsilon$ - $\text{CH}_3$  groups of Met-116 and Met-127 being the most shifted, together with  $\beta$ - and  $\gamma$ - $\text{CH}_2$  methionine group cross peaks (data not shown).

**Comparison of the Binding Affinities.** In order to evaluate and compare the binding affinity of  $\text{Cu}^{\text{I}}/\text{Ag}^{\text{I}}$  sites in  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$ , we additionally investigated silver coordination to a binary system containing both peptides. The fingerprint regions of  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra of such a solution were compared with the corresponding ones of  $\alpha\text{S}_{1-15}$  or  $\alpha\text{S}_{113-130}$  in both the presence and absence of  $\text{Ag}^{\text{I}}$ . Figure 8A shows that the NH–H $\alpha$  correlations of the two apo  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  peptides (displayed in green and magenta, respectively) are well superimposed on the corresponding ones in the binary system (black contours), indicating that no interaction between peptides occurs under these conditions. Furthermore, we found that the addition of 1.0 equiv of  $\text{Ag}^{\text{I}}$  to solutions containing both  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  (black contours, Figure 8B) resulted in chemical shift variations very similar to those detected for each single peptide in the presence of 0.5 equiv of  $\text{Ag}^{\text{I}}$  (green and magenta contours for  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$ , respectively). Such data indicate that  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  possess very similar binding affinities and suggest that  $\text{Ag}^{\text{I}}$  ions are equally distributed among the two binding sites. In fact, as shown in Figure 8C, further addition of silver to the binary system (1.6 equiv of  $\text{Ag}^{\text{I}}$ ) resulted in effects almost identical with the ones detected for  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  with 0.8 equiv of  $\text{Ag}^{\text{I}}$ . Finally, we compared the NH chemical shift variations detected for the binary solution with those found for the same residues in the full-length protein (Figure 9). Nicely, the recorded effects are very similar, strongly supporting the indication that the two binding sites might compete for copper/silver binding in the full-length  $\alpha\text{S}$  as well.

The apparent  $K_{\text{d}}$  constant of silver association at both binding sites was further evaluated by analysis of NMR and CD spectra, obtained upon titrations of both  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  fragments with  $\text{Ag}^{\text{I}}$ . Chemical shift variations of selected proton

resonances in the amide region of the  $^1\text{H}$ – $^1\text{H}$  TOCSY spectra recorded with increasing concentrations of metal ion were measured and used to build affinity binding curves for the three systems. For  $\alpha\text{S}_{1-15}$  and  $\alpha\text{S}_{113-130}$  fragments, data were fit to a model incorporating one  $\text{Ag}^{\text{I}}$  ion per molecule (with dissociation constant  $K_{\text{d}}$ ) by using the program DynaFit.<sup>60</sup> Changes in the chemical shift values of amide resonances of Val-3, Phe-4, and Met-5 were used to evaluate the affinity toward  $\text{Ag}^{\text{I}}$  of the N-terminal binding site  $\alpha\text{S}_{1-15}$ , whereas Met-116 and Met-127 amide resonances were used to evaluate the affinity of  $\alpha\text{S}_{113-130}$  toward the  $\text{Ag}^{\text{I}}$  ion (Figure 8S in the Supporting Information). The obtained results gave an apparent  $K_{\text{d}}$  value in the micromolar range and confirmed the 1:1 stoichiometry for both fragments, further supporting similar binding affinities.

## DISCUSSION

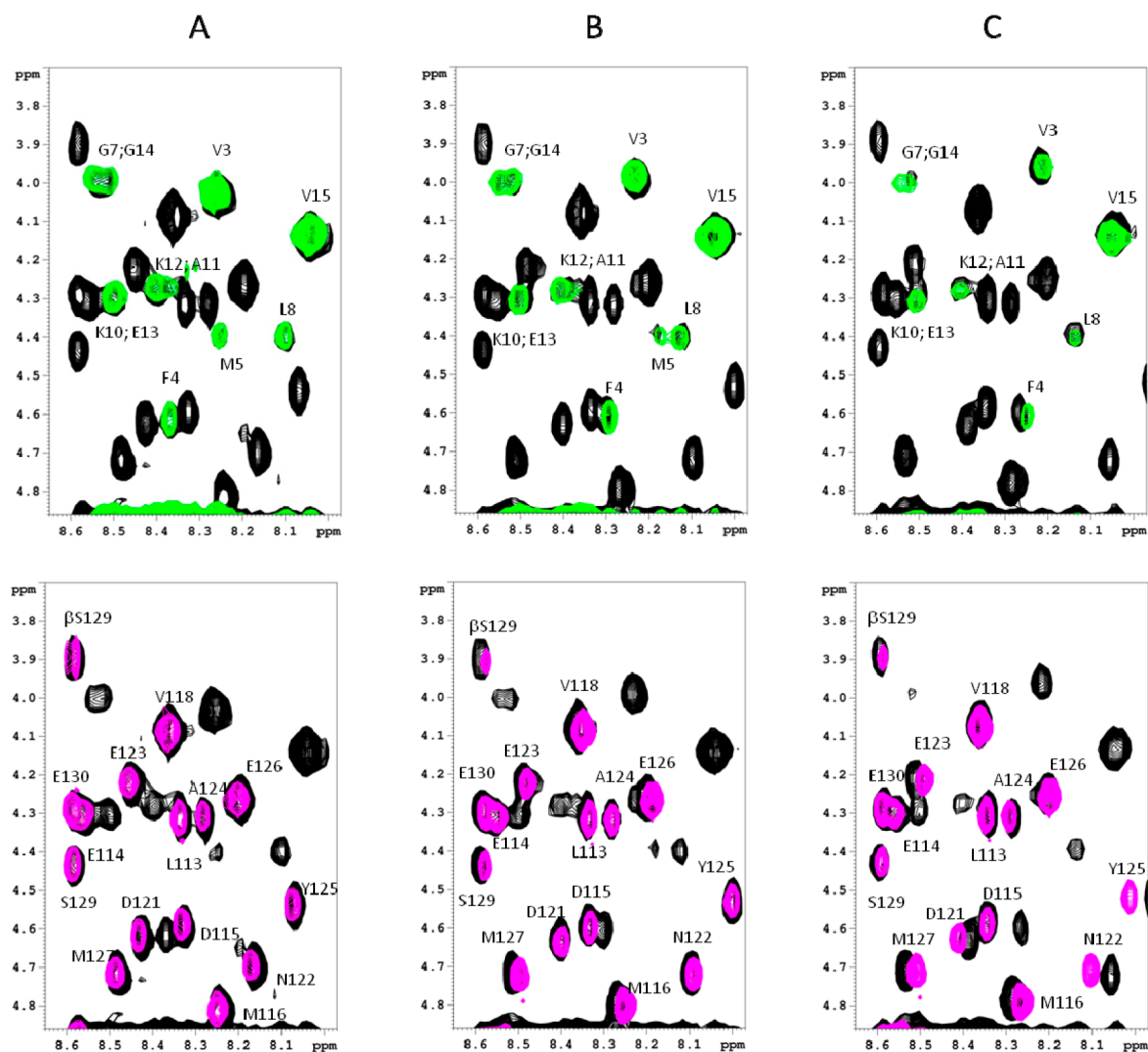
The interaction of  $\alpha\text{S}$  with metal ions, and in particular with copper, is widely considered to play a critical role in the etiology of PD.

$\text{Cu}^{\text{II}}$  binding to  $\alpha\text{S}$  has been shown either to induce aggregation and precipitation of  $\alpha\text{S}$  or to catalyze the production of  $\text{H}_2\text{O}_2$  and other ROS, which are involved in oxidative stress and resulting in damage and loss of dopaminergic cells in PD brain.

Recently, it has been shown that the redox potential of  $\alpha\text{S}-\text{Cu}^{\text{II}}/\alpha\text{S}-\text{Cu}^{\text{I}}$  (0.018 V vs  $\text{Ag}/\text{AgCl}$ ) is higher than that of various highly abundant cellular reductants such as AA, NADH, GSH, etc.;<sup>24</sup> therefore redox reactions involving a  $\alpha\text{S}-\text{Cu}^{\text{II}}/\alpha\text{S}-\text{Cu}^{\text{I}}$  couple are likely to occur, and these may be responsible for the depletion of GSH in the *substantia nigra* of PD patients, considered to be strong evidence of the oxidative stress hypothesis.<sup>1,61</sup> Moreover, it has been demonstrated that, even though a  $\alpha\text{S}-\text{Cu}^{\text{II}}/\alpha\text{S}-\text{Cu}^{\text{I}}$  redox pair cannot directly oxidize dopamine, it can generate  $\text{H}_2\text{O}_2$  (as described in eqs 1 and 2), which, on the contrary, does.<sup>61,62</sup>

In order to enhance understanding of the role, if any, played by  $\alpha\text{S}$  in oxidative stress, it is essential to characterize first the  $\text{Cu}^{\text{I}}/\text{Cu}^{\text{II}}$  binding mode to  $\alpha\text{S}$ . So far, the majority of the studies have focused on the investigation of  $\text{Cu}^{\text{II}}$  interaction with  $\alpha\text{S}$ . Two metal binding sites have been identified and well characterized: one at the N termini of the protein and the second one centered at His-50 (see ref 27 for a review). On the contrary, very few investigations have been carried out on the interaction of  $\alpha\text{S}$  with  $\text{Cu}^{\text{I}}$ . Recently,  $\text{Cu}^{\text{I}}$  binding to full-length  $\alpha\text{S}$  at pH 6.5 was investigated by Binolfi and co-workers.<sup>42</sup> The study has identified and characterized one  $\text{Cu}^{\text{I}}$  binding site located at the N-terminal region of the protein, with the  $\delta$ -sulfur atoms of Met-1 and Met-5 indicated as the  $\text{Cu}^{\text{I}}$  donor group. The NMR-derived dissociation constant  $K_{\text{d}}$  is in the micromolar range, in full agreement with values from previous reports on  $\text{Cu}^{\text{II}}$  binding to proteins and peptides involving Met residues as primary metal-anchoring ligands.<sup>63,64</sup> Moreover, such association values indicate the existence of a finite probability to have  $\text{Cu}^{\text{I}}$  bound to  $\alpha\text{S}$ . The formed complex, also at very low concentration, might have catalytic activity, amplifying the toxic effects associated with ROS formation.

In this work, NMR and CD spectroscopies were used in order to characterize all of the possible  $\text{Cu}^{\text{I}}$  binding regions in  $\alpha\text{S}$ . In addition to  $\text{Cu}^{\text{I}}$ , we have used  $\text{Ag}^{\text{I}}$  as a probe for  $\text{Cu}^{\text{I}}$  binding to the protein.  $\text{Ag}^{\text{I}}$  is a diamagnetic, NMR-active metal ion, and it is therefore amenable to NMR studies. Because it is redox-inactive,  $\text{Ag}^{\text{I}}$  is often used to investigate  $\text{Cu}^{\text{I}}$  sites. The



**Figure 8.** Overlaid  $^1\text{H}$ - $^1\text{H}$  TOCSY fingerprint regions of a  $\alpha\text{S}_{1-15}$ - $\alpha\text{S}_{113-130}$  binary system (black) and of  $\alpha\text{S}_{1-15}$  (green) or  $\alpha\text{S}_{113-130}$  (magenta): (A) apo solution; (B and C) different  $\text{Ag}^{\text{I}}$  addition. For a  $\alpha\text{S}_{1-15}$ - $\alpha\text{S}_{113-130}$  binary system (black contours), additions of 1.0 and 1.6 equiv of  $\text{Ag}^{\text{I}}$  are shown in parts B and C, respectively. For  $\alpha\text{S}_{1-15}$  (green contours) or  $\alpha\text{S}_{113-130}$  (magenta contours), additions of 0.5 and 0.8 equiv of  $\text{Ag}^{\text{I}}$  are shown in parts B and C, respectively.

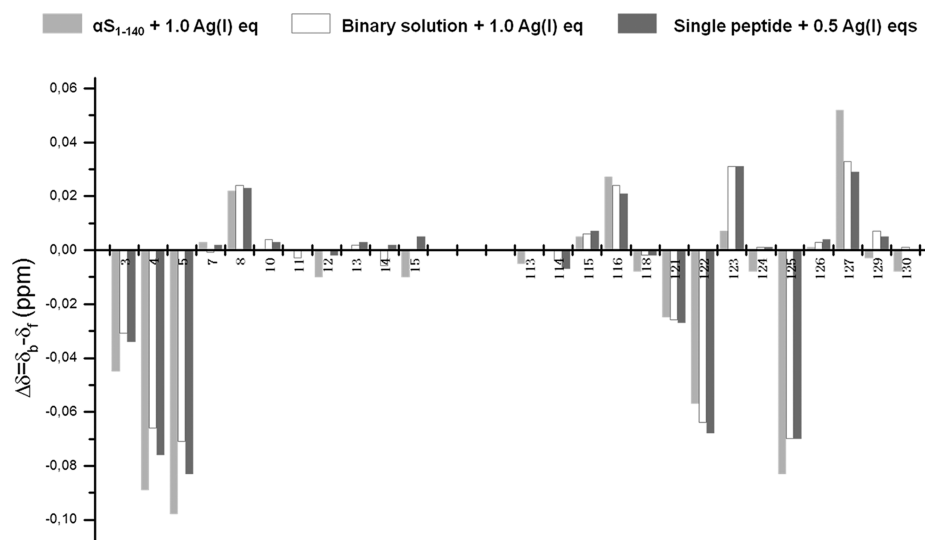
two ions have, in fact, identical d-shell electron configurations ( $d^{10}$ ) and oxidation states, and although  $\text{Ag}^{\text{I}}$  is larger than  $\text{Cu}^{\text{I}}$ , in many cases it adopts complexes similar to those formed by  $\text{Cu}^{\text{I}}$ . The fact that, unlike  $\text{Cu}^{\text{I}}$ , silver it is not susceptible to oxidation has led to its use as a probe of  $\text{Cu}^{\text{I}}$  sites in proteins such as metallothionein, in which it displays a high affinity for cysteine-rich sites<sup>65–67</sup> or in methionine-rich sites.<sup>49</sup>

The chemical shift variations of amide groups recorded for the full-length  $\alpha\text{S}$  protein (Figure 1) after the addition of  $\text{Ag}^{\text{I}}$  are very similar to the ones previously measured for  $\text{Cu}^{\text{I}}$ ,<sup>42</sup> strongly indicating analogue coordination environments for both metal ions. Furthermore, the effects detected on  $\alpha\text{S}_{1-15}$  upon  $\text{Ag}^{\text{I}}$  addition (Figures 3S and 4S in the Supporting Information) were comparable to the ones reported for the  $\alpha\text{S}_{1-6}$ - $\text{Cu}^{\text{I}}$  complex, where the Met-1 and Met-5 thioether groups coordinate to  $\text{Cu}^{\text{I}}$ .<sup>42</sup>

The absence of significant conformational changes of  $\alpha\text{S}$  upon  $\text{Ag}^{\text{I}}$  binding, shown by invariance of the far-UV CD spectra (Figure 2), together with the absence of any NOE interaction between the N- and C-terminal regions of  $\alpha\text{S}$ , suggests the unlikelihood of any interplay between the two sites

in metal binding such as to allow the use of two model peptides, each containing a single metal binding site, for characterization of the metal coordination sphere at the molecular level. A series of 1D and 2D homo- and heteronuclear experiments recorded in the presence of increasing amounts of metal revealed a 1:1 stoichiometry for  $\text{Ag}^{\text{I}}/\text{Cu}^{\text{I}}$  binding to both the N-terminal ( $\alpha\text{S}_{1-15}$ ) and C-terminal ( $\alpha\text{S}_{113-130}$ ) fragments. The effects observed for the two fragments were fully in agreement with those observed for the full-length protein, supporting the suitability of the two peptides as models for copper/silver binding sites in  $\alpha\text{S}$  (Figure 9). A very similar silver/copper binding mode was detected for the two sites. In both cases, coordination of  $\text{Ag}^{\text{I}}$  by  $\delta$ -sulfur atoms of methionine residues was widely supported by the changes detected in the NMR spectra, with the major chemical shift variations observed for methionine amide and side-chain protons. The significant  $\text{H}^{\text{N}}$  chemical shift modifications detected for Val-3 and Phe-4 at the N terminus or Asn-122 and Tyr-125 at the C terminus were attributed to the sensitivity of amide protons to very local effects, such as backbone torsion angle modification, steric clash occurrence, etc., rather than to





**Figure 9.** Chemical shift variations of amide protons due to Ag<sup>I</sup> binding in the full-length protein (light gray) in the αS<sub>1-15</sub>–αS<sub>113-130</sub> binary system (white) and in αS<sub>1-15</sub> and αS<sub>113-130</sub> fragments (gray).

electronic effects due to metal coordination. The absence of chemical shift variations of the side-chain protons of these residues likely confirms our assumption. In particular, for the N-terminal binding site, the participation of Met-1 NH<sub>2</sub> group in copper coordination was hypothesized,<sup>27</sup> but the same chemical shift variation experienced by both Met-1 and Met-5 Hα (Figure 3S in the Supporting Information) allows us to exclude this possibility. Furthermore, the obtained 3D NMR structure of the Ag<sup>I</sup>–αS<sub>1-15</sub> and Ag<sup>I</sup>–αS<sub>113-130</sub> complexes (Figures 3 and 6) supported interaction of the Ag<sup>I</sup> ion with the sole methionine sulfur atoms because no other side chains are found in the proximity of the metal ion in any of the 30 structures generated.

Even though in both sites Cu<sup>I</sup> and Ag<sup>I</sup> experience the same coordination environment, more detailed NMR and CD analysis of the single fragments pointed out some differences in terms of metal-induced conformational effects. On the one hand, no changes in the native random-coil backbone conformation are visible for the N-terminal fragment, as shown either by the similarity between the CD spectra recorded on both the apo-αS<sub>1-15</sub> and the Ag<sup>I</sup>–αS<sub>1-15</sub> complex (Figure 5S in the Supporting Information) or by the absence of any nontrivial NOEs in the <sup>1</sup>H–<sup>1</sup>H NOESY spectrum, which result in a 3D structure with no preferred conformations. On the other hand, the C-terminal αS<sub>113-130</sub> site undergoes large structural modification upon Ag<sup>I</sup> binding. Both CD and NOESY spectra clearly show modification of the backbone conformation, with a decrease of the random-coil content (Figure 7S in the Supporting Information) and the appearance of sequential interresidual H<sup>N</sup><sub>*i*</sub>–H<sup>N</sup><sub>*i*+1</sub> NOEs in the region encompassing residues 121–127 (Figure 5). The obtained 3D NMR structure confirmed the occurrence of a bent backbone, stabilized by the formation of a hydrogen bond between Ala-124 H<sup>N</sup> and Asp-121 CO and between Met-127 H<sup>N</sup> and Asp-123 CO, typically found in β-turn conformations, observed in almost all of the 30 generated structures. Nicely, the NOE patterns observed for the C-terminal complex resemble the ones observed for the full-length αS<sub>1-140</sub>, where interresidual H<sup>N</sup><sub>*i*</sub>–H<sup>N</sup><sub>*i*+1</sub> NOEs appear as well, although in a more extended region (encompassing residues 118–130).

Besides the different structural effects induced by Ag<sup>I</sup> binding to the N- and C-terminal fragments, competition studies carried out on ternary systems containing both N- and C-terminal fragments and Ag<sup>I</sup> showed that the metal binds to both sites with very similar affinities. The NMR- and CD-based estimation of the dissociation constants gave apparent *K*<sub>d</sub> values in the 10<sup>–5</sup>–10<sup>–6</sup> M range for both silver binding sites. Moreover, a comparison between the effects recorded on the ternary system and the ones recorded on the full-length αS showed very similar proton chemical shift displacements (Figure 9), supporting the hypothesis that the two binding sites may compete for copper/silver binding. Finally, the two metal binding domains, located far away from the NAC region involved in protein fibrilization, are consistent with similar copper binding behaviors in fibrils, which might indeed have redox activity similar to that of the monomer system.

## CONCLUSIONS

In this work, the structural details of Cu<sup>I</sup>/Ag<sup>I</sup> binding to αS were investigated by CD and NMR spectroscopy. A comparison between the metal-induced effects monitored on the full-length protein and on two *ad hoc* designed model peptides indicated the existence of two separate Cu<sup>I</sup>/Ag<sup>I</sup> binding domains encompassing the 1–5 and 116–127 regions, respectively. Both domains exhibit similar metal binding affinities and comparable metal coordination spheres, characterized by two Met sulfur atoms likely in a linear geometry, as shown in previously investigated two-coordinated copper(I) model complexes.<sup>68</sup> However, further investigations would be necessary to determine the exact metal geometry. NMR structural characterization of the two binding sites showed that metal interaction at the N termini does not yield any particular structural rearrangements; on the contrary, the binding to Met-116 and Met-127 induces a β-turn backbone conformation stabilized by Ala-124 H<sup>N</sup>–Asp-121 CO and Met-127 H<sup>N</sup>–Asp-123 CO hydrogen bonds.

## ■ ASSOCIATED CONTENT

## ■ Supporting Information

NMR and CD spectra, chemical shift variations, binding curves. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

The authors declare no competing financial interest.

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