Copper(I/II), α/β-synuclein, and amyloid-β : Menage à trois?

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ABSTRACT

Copper-binding to α-synuclein (aS) and amyloid-β (Ab) has been connected to Parkinson and Alzheimer disease, respectively, because Cu ions can modulate the peptide aggregation and these Cu-peptide complexes can catalyze the production of reactive oxygen species (ROS). In a significant part of AD brains, aggregation of aS and Ab are detected and it was proposed that Ab and aS interact with each other. Thus we investigated the potential interactions of Ab and aS via their binding of copper(I) and (II). Additionally, beta-synuclein (bS) was investigated, due to its additional methionine, a potential Cu(I) ligand. We found that: i) the peptides containing the Cu-binding domain Ab1-16, aS 1-15 and bS1-15 have similar affinities to Cu(II) or Cu(I). Ab1-16 was slightly stronger; ii) for Cu(I), the additional Met in bS1-15, increased the affinity slightly, iii) the exchange of Cu(I/II) between the two peptides is rapid (≤ ms) iv) a/bS1-15 and Ab1-16 form a ternary complex with Cu(II), v) Cu(I) likely promotes a transient ternary complex; vi) the different Cu(I/II) coordination of Ab1-16, aS1-15 and bS1-15 impact the capacity to produce ROS and to oxidize catechol vi) when Ab1-16, aS1-15 and Cu are present, the ROS production resembles more Ab1-16. The work gives insights into the coordination chemistry of these related peptides and the relevance of coordination differences, ternary complex and ROS production are discussed.

INTRODUCTION

Copper is an essential metal ion and is a catalytic center for several enzymes. Copper metabolism is tightly controlled by transporters and chaperons. Copper mismetabolism can be lethal as witnessed by the two genetic disorders concerning copper transporters, leading to copper overload (Wilson disease) or copper shortage (Menkes disease). An important mechanism for toxicity in the copper overload is the capability of free or loosely bound cooper to catalyze the production of reactive oxygen species (ROS).[1, 2]

Mismetabolism of copper has been reported in several neurodegenerative diseases, like Alzheimer's disease (AD),[3] Parkinson disease (PD),[4-6] prion disease,[7, 8] and amyotrophic lateral sclerosis.[9, 10] Often Cu is found to be bound to a disease specific amyloidogenic peptide or protein. Two of the most prominent are Cu bound to amyloid-beta (Ab) in AD and Cu bound to alpha-synuclein (aS) in PD. Cu-Ab and Cu-aS are able to catalyze the production of ROS in the presence of a physiological reducing agents (like ascorbate) and dioxygen, a reaction proposed to contribute to neurodegeneration.[11-13] Thus it was proposed that in AD and PD copper is misplaced, rather than a bulk overload or bulk shortage like in Wilson or Menkes diseases.[14]

Ab and aS form amyloid type aggregates under disease conditions through an autocatalytic mechanism of self-assembly. Ab forms so called amyloid plaques in AD, and aS forms Lewy bodies in PD. It is interesting to note that a significant part of AD brains exhibits also Lewy bodies formed by aS, in addition of amyloid plaques.[15] This Lewis body variant of AD exhibits a more rapid decline compared to the pure AD cases. It was proposed that Ab and aS interact with each other. Direct interactions might be possible, because in pathological state the two peptides can be colocalized. [16, 17] Both in vitro and in vivo experiments started to identify potential interaction mechanisms, which are not known yet.[16]

Ab and aS are both considered as intrinsically disordered proteins, meaning that in their isolated state they do not have a well defined 3D structure.[18] Even upon copper coordination (Cu(I) or Cu(II)), the peptides remain disordered.[19]

Cu-binding to monomeric Ab is relatively well characterized.[3] Several forms coexist at physiological pH. The most populated coordination sphere with the main ligands is depicted in Figure 1. In Ab, Cu(II) binds equatorially to the N-terminal amine (Asp1), the adjacent carboxyl group (Asp1), His6 and either His13 or 14 (Latter are in fast exchange). For aS, mostly the non-acetylated form was characterized and ther Cu(II) binds to the N-terminal amine, then to the amide (between Met1 and Asp2) and the carboxylate of the Asp2 side chain. The fourth ligand is either His50 or an external ligand.[4] This depends on pH and Cu stoichiometry. [20]

Cu(I) bind to two His in Ab in a linear geometry. The main two His are His13 and His14 but also His6 is implicated but to a lower extent.[3] In contrast, Cu(I) is bound to aS via the two sulfurs of Met1 and 5, and likely via carboxylate of Asp2 and a water in a tetrahedral geometry[4, 21-23]. The acetylated form of aS has the same coordination site.[24] Similarly to aS, bS is an intrinsically disordered protein found in the pre-synaptic nerve terminals. bS constitutes the majority of the synuclein content in healthy brain and it has an intrinsic ability to prevent aS aggregation and oligomerization. [25-28] Cu(II) binds to bS in the same way as to aS. [22] In contrast, there is a difference in Cu(I) coordination between aS and β-synuclein (bS). bS binds Cu(I) via Met10 instead of a water molecule (Figure 1), keeping Met1 and 5 as well as Asp2 as ligands in a tetrahedral geometry.[22] Considering that (i) Ab and aS are both intrinsically disordered peptides/proteins, (ii) both can bind Cu(II) and Cu(I), and (iii) they could directly interact under certain pathological conditions we decided to compare the Cu(II) and Cu(I)-binding of Ab and aS and to investigate their competition towards Cu(II) and Cu(I). Indeed, bearing in mind that both peptides can be colocalized and bind Cu, they could directly compete for Cu or exchange Cu. Although the affinity of Cu(II) for Ab and aS was extensively studied in the literature by several methods, and recently converged (about Kd of 10^{-10} M for Ab[29] and 10^{-9} M for non acetylated aS[30, 31],[32]), no direct competition was reported. In the case of Cu(I), the affinity of Ab is not consensual.[33, 34] and for aS only apparent binding constants (without taking into account the presence of buffer and reducing agent) are reported.[35] Also the impact of the additional Met in bS on the Cu(I) affinity is not known.

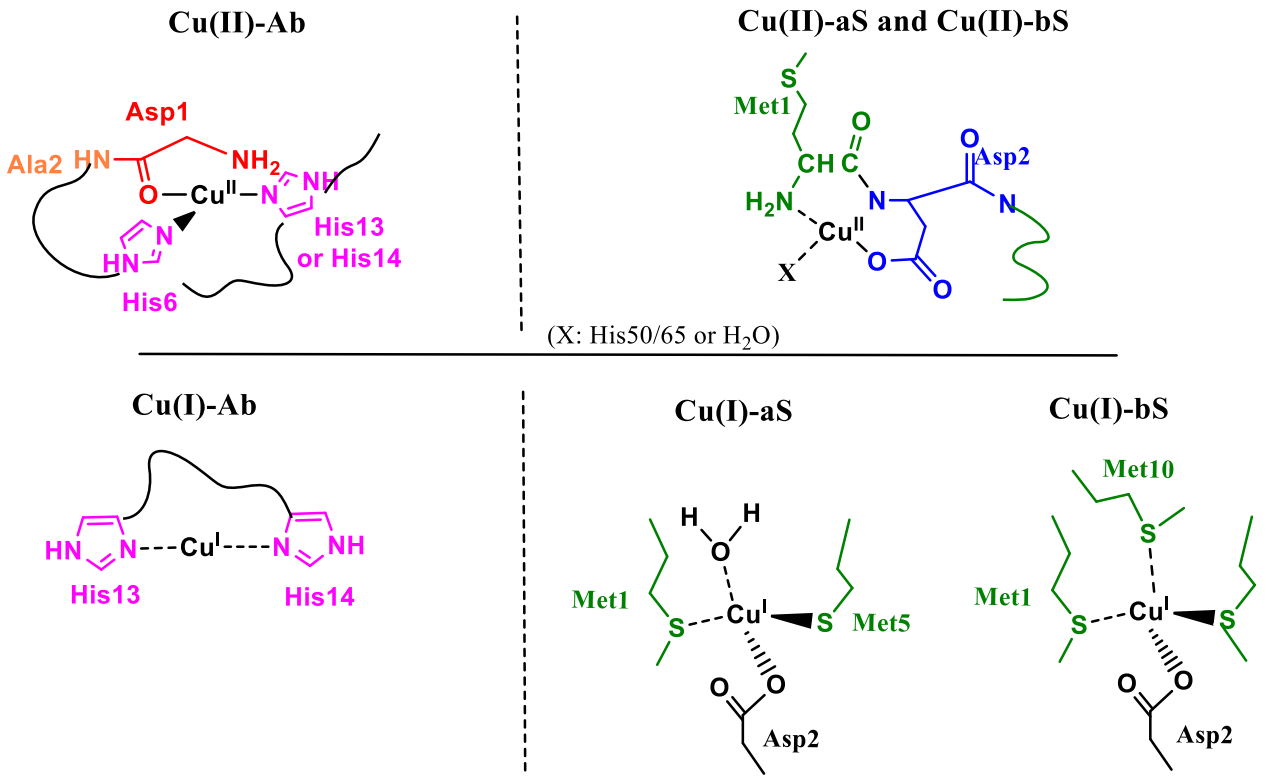

Figure 1: Models of the main coordination sites of Cu(II) and Cu(I) bound to Ab, aS and bS at neutral pH.

RESULTS

Affinity of Cu(I) and Cu(II) to aS1-15, bS1-15 and Ab1-16

Little is known about the Cu(I)-affinity of aS and bS and in particular if the additional Met in bS plays a role. Moreover, no comparison under the same conditions between aS (or bS) and Ab has been reported. In order to compare the binding affinity of Cu(I) to aS, bS and Ab and be able to obtain conditional dissociation constants, we used a competition assay of the metal-binding domains, i.e. aS1-15, bS1-15 and Ab1-16 with a relatively well characterized ligand BCA (bicinchoninic acid) that forms stable Cu(I)-BCA_2 complexes aerobically in aqueous solutions. The published absolute association constants differ by several orders of magnitude [33, 36, 37] and hence deducing absolute binding constants by competition is hampered. Nevertheless, BCA is very suited to determine relative binding constants between different competitors, like Ab1-16, aS1-15 and bS1-15 in the present case. BCA makes a 1:2 complex with Cu(I) and hence its conditional dissociation constant is strongly concentration dependent. In contrast, Ab1-16, aS1-15 and bS1-15 form 1:1 complexes and the dissociation constants are nearly independent on the concentration. Thus at the classical concentrations around 0.1 mM, BCA would retrieve all Cu(I) from the peptides and hence the K_d cannot be deduced. By lowering the concentration of all components, the equilibrium is shifted and Cu(I) is redistributed from BCA toward the peptides. As a consequence the absorption band used to determine the Cu(I)-BCA_2 concentration at about 555 nm drops as well. However, there is another more intense band (by a factor of 5) around 360 nm, that can be used instead.[38]

We titrated a solution of Cu(I)-BCA_2 at 1.8 µM with each peptide Ab1-16, aS1-15 and bS1-15 (An excess of BCA was used to ensure complete formation of Cu(I)-BCA_2). The peptide addition caused the gradual reduction of the bands at 520 nm and 360 nm, in line with the withdrawal of Cu(I) from BCA by the peptide. The example of bS1-15 is shown in Fig. 2 (for Ab1-16 and aS1-15 see Figure S1). As it can be seen from Figure 2, at 10 equivalents of bS1-15, the intensity of the characteristic bands of Cu(I)-BCA_2 dropped by about a half, indicating that about half of the Cu(I) is bound to bS. A similar behaviour was also observed for aS1-15 and Ab1-16.

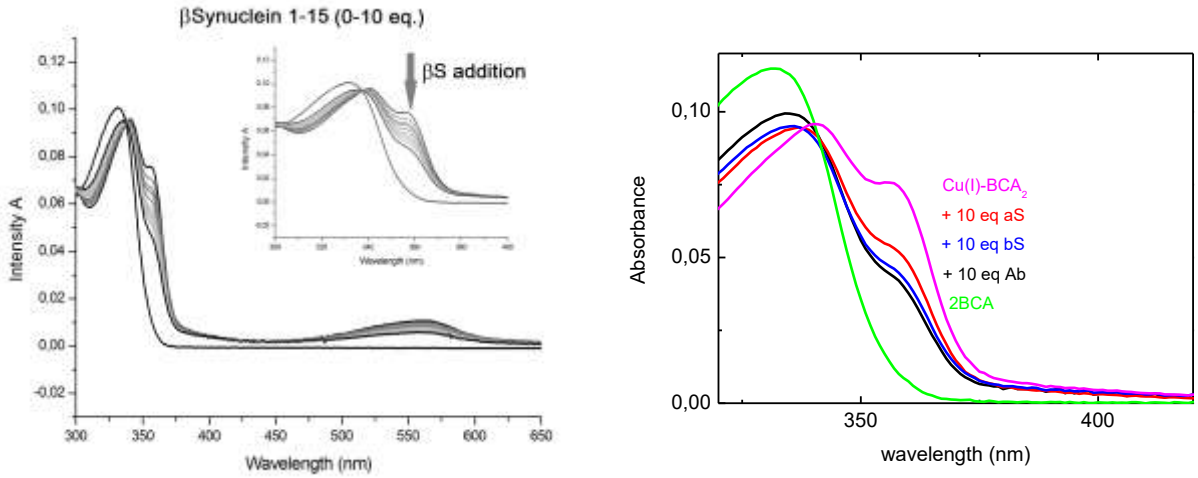

Figure 2: (left) titration of bS into a complex of Cu(I)-BCA (inset, magnification around 350 nm). right: comparison of addition of 10 equivalents of Ab1-16, bS1-15 and bS1-15 to Cu(I)-BCA. Conditions: BCA = 5.0 uM, Cu(I) = 1.80 uM, Peptides = from 1.8 to 18uM, Dithionite = 0.1 mM, Hepes buffer = 50 mM, pH 7.25 ± 0.05, 298 K

Figure 2 shows the comparison of the spectra of Cu(I)-BCA_2 complex recorded in presence of each investigated peptide. For all three peptides (Ab1-16, bS1-15 and aS1-15) the charge transfer bands of Cu(I)-BCA dropped significantly, between about 30 – 50%. This first indicates that the affinities of these peptides do not span much more than an order of magnitude. However, significantly reproducible differences were obtained and allowed to establish the following order of Cu(I) affinity: Ab1-16> bS1-15 > aS1-15, with a relative affinity of about 2.2 : 1.9 : 1 (For details see Mat. & Meth. section). Due to the incertitude of the absolute values for Cu(I)-binding to BCA, no absolute values can be given, but the dissociation constant is likely in or close to the nM range. This results clearly indicated that the additional Met in bS1-15 increases the affinity of Cu(I) compared to aS1-15.

Table 1: Cu(II) affinity (at pH 7.4)

peptide	K_d (nM)	K_a 10^9	refs.
MD-aS(31-56)	0.19	5.32	[32]
aS(1-17)	0.94	1.06	[39]
Ab(1-16)	0.207	4.83	[40]

Affinity data for Cu(II) for Ab and aS are shown in Table 1. They are based on potentiometric studies reported in the literature. From this data, which were obtained by the very same methodology, it appears that Ab1-16 has about a fivefold higher Cu(II) affinity than aS at pH 7.4, when only the N-terminal site is occupied and a similar affinity when His50 is also involved in the Cu(II) binding. We have preliminary data by UV-Vis titration using a new spectrometric dye confirming the about 5 fold difference. A full manuscript describing the usefulness of this dye is currently under preparation in our group.

Direct competition aS/bS1-15 with Ab1-16 for Cu(II):

Cu(II) affinity of aS, bS and Ab (full length and metal-binding domains) has been studied, however, direct competition experiments are lacking. Thus we investigated the direct competition between Ab1-16 and aS/bS1-15 towards Cu(II) by EPR (Figure 4, Table 2).

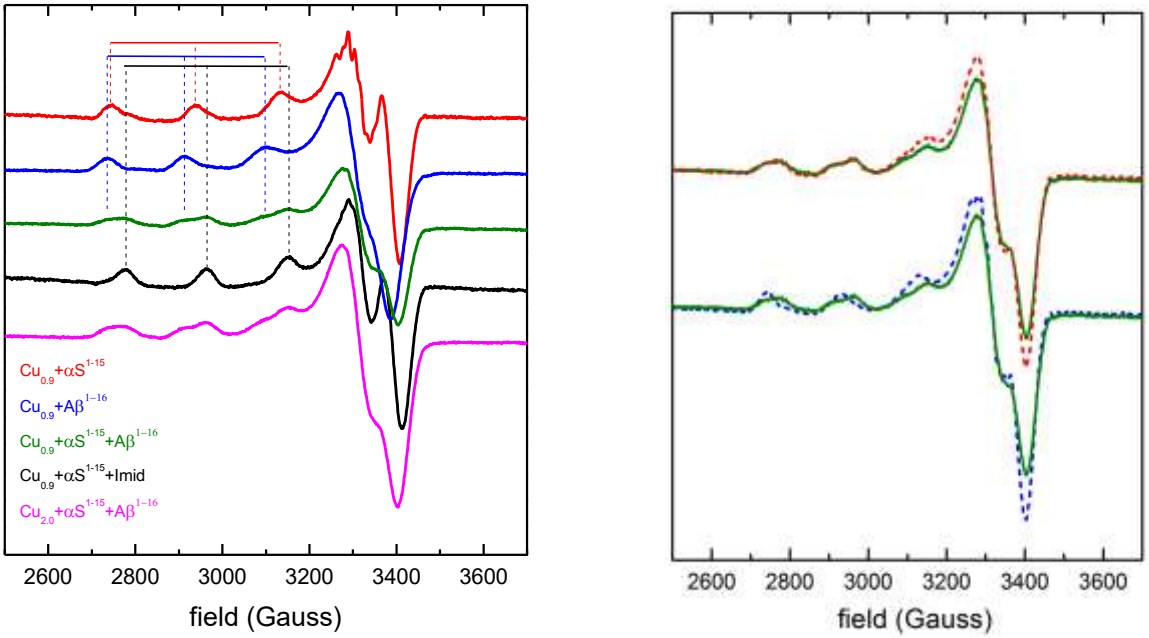

Figure 4: (left): ^{65}Cu(II) EPR spectra of Cu(II)-aS1-15 (0.9:1; red), Cu(II)-Ab1-16 (0.9:1; blue), Cu(II) +Ab1-16 + aS1-15 (0.9:1:1; green), Cu(II)-bS1-15 + imidazole (0.9:1:1; (black) and Cu(II) + Ab1-16 + aS1-15 (2:1:1; magenta). (right): Cu(II) + Ab1-16 + aS1-15 (0.9:1:1; green) and the linear combination (0.5 + 0.5) of Cu-Ab and Cu-aS (dotted blue) and Cu-Ab and Cu-aS + imidazole (dotted red). Conditions: Peptides concentrations were of 0.4 mM in 50 mM Hepes buffer pH 7.25 ± 0.05 and 5% of glycerol.

Table 2: EPR parameters

sample	$g_{//}$	$A_{//}$ (10^{-4} cm^{-1})
Cu(II)-aS1-15	2.24(3)	190 ± 5
Cu(II)-Ab1-16	2.26(2)	178± 5
Cu(II)-aS1-15 + imidazole	2.22(6)	179 ± 5
Cu(II) + aS1-15 + Ab1-16	~2.22(9) ~2.26(4)	~185 ± 5 ~182 ± 5

The EPR spectra of Cu(II)-aS1-15 (red), Cu(II)-Ab1-16 (blue) and the mixture of Cu(II) with Ab1-16 and aS1-15 (green) are shown. Cu(II)-Ab1-16 and Cu(II)-aS1-15 show different EPR spectra dominated by one species (Table 2). The difference is best seen on the third hyperfine peak from the left of the three resolved components of g parallel (indicated by the red and blue grate respectively). In contrast the first peak is localized at about the same g-value. The spectrum obtained for Cu, aS1-15, Ab1-16 at 0.9:1:1 ration is shown in green (Fig. 4). Two dominating species can be easily recognized. One species resembles the spectrum of Cu-Ab1-16 (blue grate), but the other does not resemble the Cu-aS1-15 spectrum. In fact this spectrum cannot be explained by a linear combination of the two spectra of Cu-aS1-15and Cu-Ab1-16

(Figure 4 right, blue dotted trace), but it rather indicates the formation of a different species, which might be a ternary complex with Cu(II) bound simultaneously to aS and Ab peptides. As observed from Figure 4, the second species of Cu-aS1-15-Ab1-16 resembles the spectrum of aS1-15 plus imidazole (black grate), in which an imidazole replaces the water in the coordination sphere of Cu(II)-bS1-15 (Figure 1).[20] Finally, as expected , the spectrum of Cu-aS1-15-Ab1-16 can be reproduced by a linear combination of Cu(II)-Ab1-16 and Cu(II)-aS1-15 with imidazole, at a ratio of 0.5 : 0:5 (Figure 4 right, red dotted line). This strongly supports that a ternary complex is formed, where both aS1-15 and Ab1-16 residues participate to the Cu(II) coordination sphere (Figure 5, bottom). One of the two non-coordinated His of Ab1-16 (i.e. His13 or 14, Figure 5 top left) is able to displace H_2O from the coordination sphere of Cu(II)-aS1-15 (Figure 5 top right). Thus, the coordination sphere of Ab1-16 does not change, in line with the EPR.

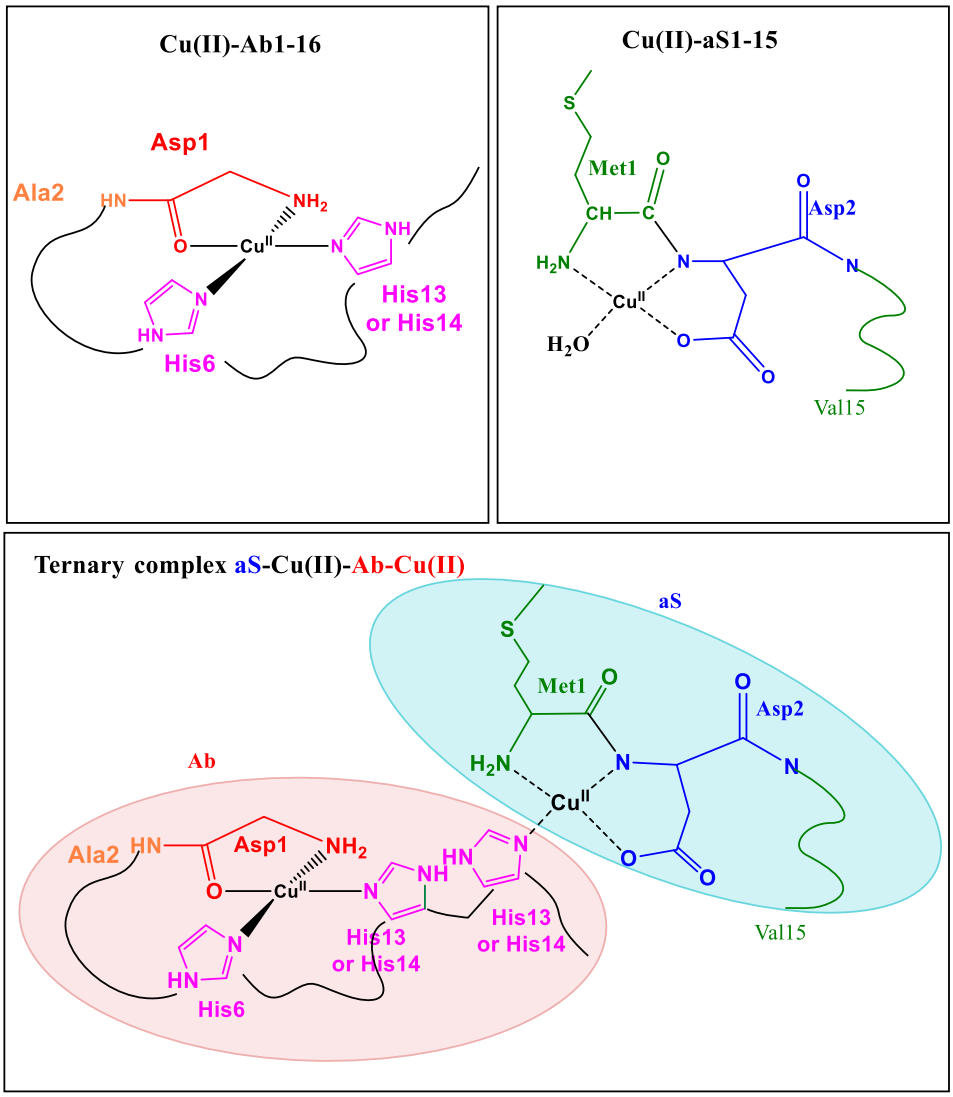

Figure 5: Structural models of Cu(II)-Ab1-16 (main component at pH 7.4), Cu(II)-aS1-15 and the ternary complex aS-Cu(II)-Ab.

A prediction of this model would be that adding a second equivalent of Cu(II), i.e. complexes formed by Cu(II) : Ab1-16 : aS1-15 at a ratio of 2:1:1 yields the same binding mode. Indeed, as shown in Figure 4 (left, magenta), adding a second equivalent of Cu(II) to Ab1-16-Cu-aS1-15 does not change the features of the EPR spectrum but increases the intensity by a factor of two. Again this spectrum can be obtained by a linear combination of the spectra Cu(II)-Ab1-16 and Cu(II)-aS1-15 plus imidazole at a ratio of 1:1 (not shown).

The fact that Ab1-16-Cu(II)-aS1-15 can be simulated by an equal contribution in the linear combination of Cu-Ab1-16 and Cu-aS1-15 + imidazole, indicates that the two binding modes have about the same apparent

affinity. However, the presence of a ternary complex does not allow a conclusion about the relative affinity of Cu-bS1-15 (without imidazole) compared to Cu-Ab.

Direct competition aS/bS1-15 with Ab1-16 for Cu(I):

In order to confirm the relative affinity between aS/bS1-15 and Ab1-16 obtained by competition with BCA (see above), direct competition experiments were conducted via NMR. Cu(I)-binding to aS1-15 and bS1-15 leads to a large shift of the terminal S-CH_3 of the Met. Ab1-16 does not contain Met, so the Met signals can be clearly attributed (Figure 6, asterisk). Figure 6 shows the NMR resonances of S-CH_3 groups of aS1-15(left) and bS1-15 (right). Addition of Cu(I) leads to an upfield shift of about 0.1-0.2 ppm (from 2.1 ppm to 2.2-2.3 ppm, for details see Table S1)). Subsequent addition of Ab1-16 (1 eq.) leads to a large, but not total, back shift, in line with the stronger affinity of Ab1-16 compared to aS/bS1-15. Moreover, addition of Ab1-16 leads to a lower back shift of bS1-15 compared to aS1-15, in line with the lower affinity of aS1-15 compared to bS1-15. Note that the same shifts were obtained with the opposite order of addition, i.e. when bS1-15 or aS1-15 was added to Cu(I)-Ab. Moreover, the intermediate shifts when Ab1-16, Cu(I) and aS/bS1-15 are present (compared to apo-aS/bS1-15 and Cu(I)-aS/bS1-15), shows that the Cu(I)-binding is in fast exchange, at least faster than the NMR time scale, i.e. ms or faster. That means that Cu(I) is transferred rapidly from Ab1-16 to a/bS1-15 and vice versa.

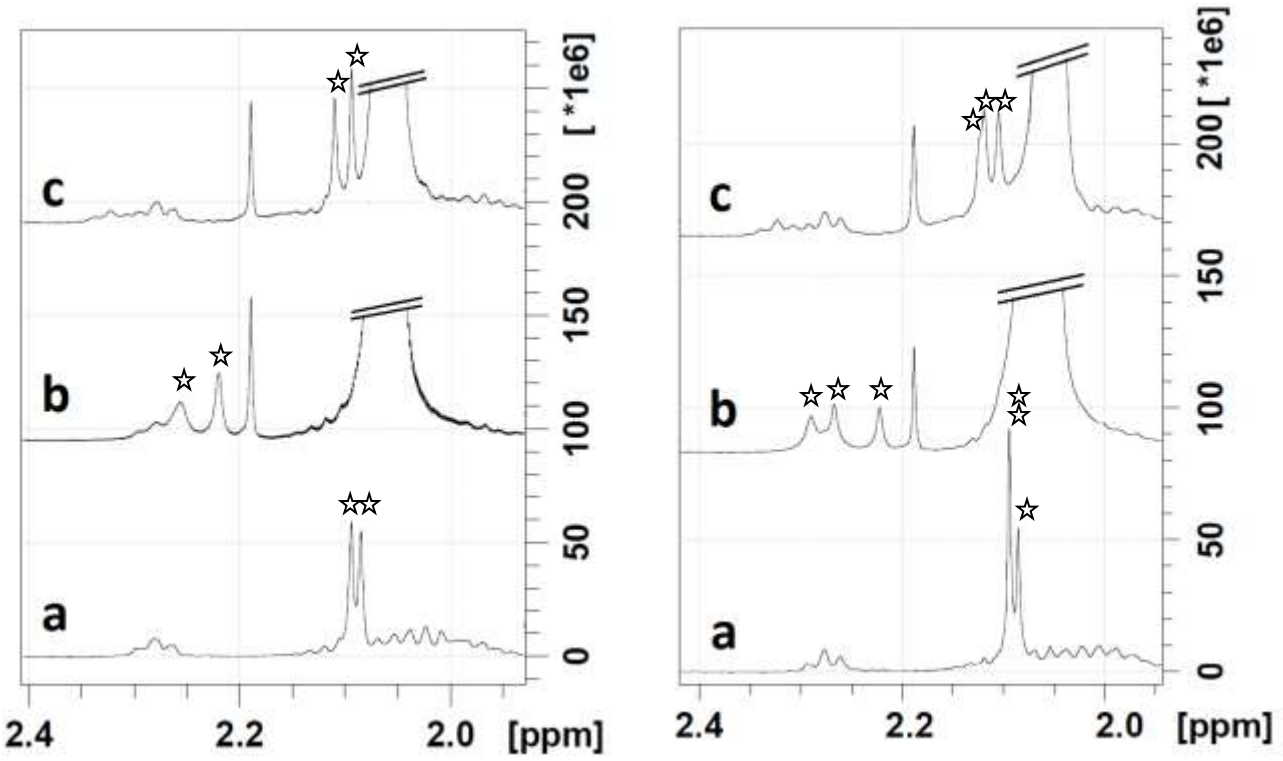

Figure 6: 1H-NMR of aS1-15(left) and bS1-15 (right) (asterisk indicate the CH_3-S of the methionines). a) aS1-15and bS1-15 without Cu(I); b) upon addition of 1 equivalent of Cu(I), i.e. Cu(I)-aS/bS1-15; c) upon addition of 1 equivalent of Ab, i.e. Cu(I)-aS/bS1-15 + Ab. Conditions: Peptides (aS/bS1-15)= 0.4 mM, Ab1-16= 0.4 mM, Cu(I) =0.36 mM, Ascorbic Acid = 3 mM, PBS buffer = 40 mM, pH 7.25 ± 0.05, 25 C.

These conclusions are also supported by the effect seen for the resonances of H-C_ε of the three His (Figures S2). His, occur only in Ab1-16 and are the main Cu(I) ligands. In the presence of a/bS1-15, Cu(I) and Ab1-16, the chemical shift of the three H-C_ε are much closer to the one of Cu(I)-Ab1-16 than Ab1-16, in line with a stronger affinity of Ab1-16 for Cu(I). However, in contrast to the resonances of CH_3-S of the methionines,

the chemical shift of the His is quite sensitive to slight changes of the pH. Thus, the chemical shifts on CH_3-S of the methionines (Figure 6) are more reliable.

When the relative affinities were calculated based on the chemical shifts, Ab1-16 showed an about 30 times stronger affinity than bS1-15 and aS1-15 (for calculation see Table S1). Although the tendencies are the same as for the affinity measurements with BCA, quantitatively this result does not fit well, as with BCA a factor of about 2 was calculated (see above). It is well possible that this is due to the formation of ternary complexes, as it was shown above for Cu(II). In summary, the NMR data support on the one hand the results of the BCA test, i.e. that Ab is slightly stronger than a/bS, but indicates also that a transient ternary Ab-Cu(I)-a/bS complex can be formed.

ROS production of Cu-complexes of aS, bS1-15 and Ab

The catalytic activity of Cu bound to aS, bS and Ab has been proposed to contribute to the oxidative stress observed in AD and PD. In vitro evaluation of the capability of ROS production of Cu-peptide is often evaluated by measuring the oxidation rate of ascorbate in the presence of dioxygen. Ascorbate is present at high concentration intra and extracellularly in the brain and its oxidation yield ROS like H_2O_2 and HO°.

In order to see if the different coordination spheres of aS1-15, bS1-15 and Ab1-16 have an impact on the ROS production and to evaluate the case when bS1-15 and Ab1-16 are present we measured the time dependence of ascorbate consumption.

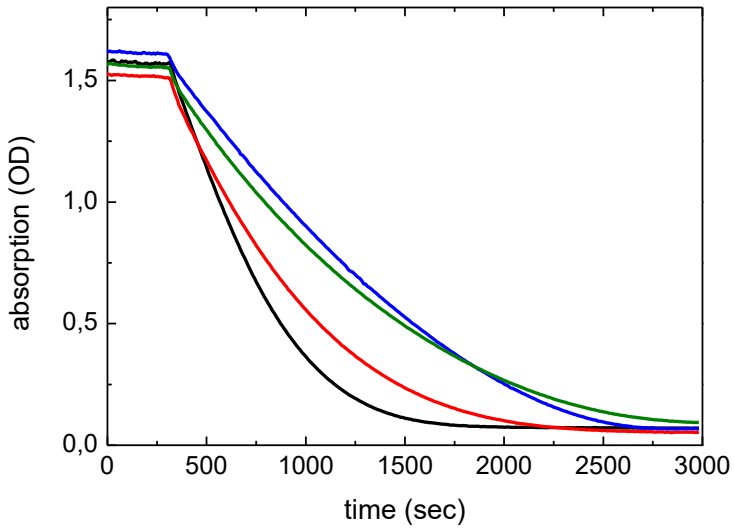

Figure 7: Traces of ascorbate consumption measured by absorption at 265 nm of Cu-aS1-15 (red), Cu-bS1-15 (black), Cu-Ab1-16 (blue) and Cu +Ab1-16 + aS1-15 (green). The reaction was triggered by the addition of Cu after 300 seconds. Conditions: Ascorbate 100 µM, Peptide 12uM, Cu(II) 10uM, Hepes 50mM pH 7.25 ± 0.05.

Figure 7 shows the kinetic traces at 265 nm, where ascorbate absorbs. At the beginning Cu is not present and the absorption of ascorbate at 265 nm is quite stable, indicating only little ascorbate oxidation occurs without Cu addition. When Cu is added at around 300 seconds, the slope increased rapidly as expected and in line with the literature for the Cu catalyzed ascorbate oxidation.[41, 42] Cu(II)-Ab1-16 shows the lowest inclination, hence has the lowest catalytic activity. The traces for aS1-15 and bS1-15 have a steeper slope upon Cu addition suggesting a higher catalytic activity. There is a tendency that bS1-15 appears to be more active than aS1-15. In the presence of both peptides, bS1-15 and Ab1-16, and Cu (ratio 1:1:0.83), the slope was similar to Ab1-16. This is in line with the slightly stronger affinity of Ab1-16 towards Cu(I) and Cu(II) than

aS1-15, and hence the ascorbate oxidation is dominated by the Cu-Ab1-16. The decline of the curve occurs at longer times than those in the presence of either Ab1-16 or aS1-15 alone, but the difference is too small to be considered significant.

More information about the redox reactivity of the Cu-peptide complexes can be obtained from oxidation experiments of catechol substrates mimicking dopamine. A detailed analysis of such reactivity is available for Cu-Ab1-16 and Cu-aS1-15 from our previous studies. [43, 44] We thus investigated the oxidation of 4-methylcatechol by copper(II) in the presence of both peptides to see if the formation of a ternary complex Ab1-16-Cu^{II}-aS1-15 has an observable influence on the reactivity of Cu-Ab1-16, which on the basis of the affinity should be the dominant species in solution. As shown in Figure 8, the kinetic trace of the catalytic oxidation of 4-methylcatechol in the presence of the chromophoric reagent 3-methyl-2-benzothiazolinone hydrazone (MBTH) is monophasic when promoted by copper(II) salt (green trace), whereas in the presence of Ab1-16 (blue trace) or aS1-15 (red trace) the reaction profile is biphasic, as previously observed.[43, 44] The first stoichiometric step, which is concluded within the first five seconds, was attributed to copper(II) reductoin. The second, linear phase of the plot corresponds to catalytic turnover and features a slower reaction for Cu-aS1-15 compared to both Cu-Ab1-16 and Cu-Ab1-16-aS1-15. According to our previous investigations, [43, 44] the slow step of the catalytic cycle involves the formation of a Cu^{I}/O_2 intermediate. The rate of the reaction observed for the ternary system Cu-Ab1-16-aS1-15 is intermediate between those of the two binary complexes, but again more similar to that of Ab1-16. This behavior can be accounted for by two different situations: *i*) a ternary complex is formed with different reactivity compared to the binary complexes; *ii*) during turnover copper can be coordinated by both peptides with prevalence for Ab1-16. The latter scenario is possible, considering that copper(I) is the redox state which is mostly accumulated during turnover and that copper(I) has higher affinity for Ab1-16 than aS1-15.

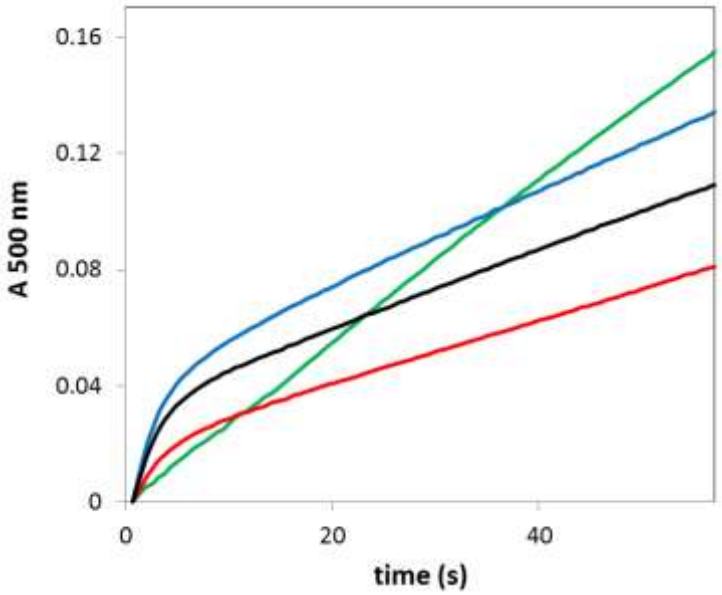

Figure 8: Kinetic traces at 500 nm in the initial phase of the oxidation of 4-methylcatechol (2 mM), in the presence of MBTH (2 mM), by Cu^{II} (25 μM) (green trace), [Cu-Ab1-16] (25 μM) (blue trace), [Cu-aS1-15] (25 μM) (red trace), and [Cu-Ab1-16-aS1-15] (25 μM) (black trace) in 50 mM Hepes buffer at pH 7.4.

DISCUSSION

The results above reported about the relative Cu(I)- and Cu(II)-affinities of aS1-15, bS1-15 and Ab1-16 deduced from either direct competition or by an external ligand under similar conditions. Although, individual values for Cu(I/II)-Ab1-16 and Cu(I/II)-bS1-15 have been reported in the literature, it is often difficult to directly compare values from different methods and under different conditions, in particular when the differences are small, like for Ab1-16 and a/bS1-15. Moreover, the effect on Cu(I)-binding of an additional Met found in bS1-15 compared to aS1-15 has not been evaluated yet.

For Cu(II) an about 5 times stronger affinity of Ab1-16 compared to a/bS1-15 at pH 7.4 has been reported by potentiometric measurements under the same conditions from the same group (Table 1). Our preliminary results corroborated this difference. A comparison by a direct competition was not possible, due to the formation of a ternary complex. For Cu(II)-Ab, recent measurements converged to a Kd at pH 7.4 of Cu(II) for Ab1-16 of about 10^{-10} M (in the absence or corrected for Cu-interaction with buffer).[29, 45] There was a small dependence on the length of the peptide, i.e. Ab1-40 was about 2.5 times stronger than Ab1-16. Most of the reports on aS1-15 are in about the same range, i.e. 10^{-9} to 10^{-10} M, again with a slightly stronger affinity of the full-length protein than aS1-15, most likely due to the binding of His50.[30-32]

From the coordination chemistry point of view it is interesting to note that the two quite different equatorial binding modes have a very similar affinity at pH 7.4. Although in both cases Cu(II) is bound to the N-terminal amine (Asp1 or Met1, respectively), in the case of Ab1-16 the main coordination consists of the backbone carbonyl (Asp1) and two His (Figures 1, 5). This binding mode involves one entropically favoured 5 membered chelate ring (Cu(II) bound to N-terminal amine and carbonyl of Asp1. On the other hand, for Cu(II)-a/bS1-15, apart from the N-terminal amine (Met1), Cu(II) is bound to the deprotonated amide N^- of Asp2 and the COO^- of the Asp2 side chain. The fourth ligand is not provided by the peptide, and is likely H_2O (Figure 5). For Cu(II)-aS, two chelate rings are formed (a 5 and a 6 membered), which are entropically favoured and hence compensate the large enthalpic penalty to deprotonate an amide. A similar site is formed in peptides with the N-terminal sequence Xxx-His (the canonical peptide is GHK), with the only difference that the Nδ of His is at the place of the carboxylate side chain of Asp2. As imidazole is a stronger ligand than carboxylate for Cu(II), the affinity of GHK is about 3 orders of magnitude higher (10^{-13}).[46]

The relative affinity of Cu(I) to aS1-15, bS1-15and Ab1-16 were determined by a competition assay with an external ligand BCA. Direct competition was monitored by NMR, that showed the same tendency, but did not agree quantitatively with the results of BCA. A possible explanation for this discrepancy is the formation of a ternary complex, very likely transient due to the fast exchange (≤ ms) of Cu(I) between the peptides. The relative affinities determined via competition with BCA suggested a stronger affinity of bS1-15 compared to aS1-15. This can be assigned to the presence of a third Met in bS1-15 (at position 10) compared to a Lys in aS1-15. Indeed, spectroscopic studies showed that all 3 Met are involved in Cu(I) coordination of bS1-15. [47] The affinity increases only by about a factor of 1.9. Compared to Ab1-16, bS1-15 and aS1-15 have a lower affinity for Cu(I), but the all three peptide are approximately in the same order of magnitude. The reports on Cu(I) affinity of Ab1-16 is still controversial but likely it is in the span of 10^{-7} to 10^{-11} M [33, 34] for the affinity at pH 7.4. For Cu(I) binding to aS, only apparent binding affinities were reported, i.e. which do not consider the competition with the buffer or other present compounds. These apparent affinities of Cu(I) for truncated or full length aS were in the lower µM range.[21, 23, 35] Thus lower affinities were obtained compared to the estimated Cu(I)-affinities here.

Ab1-16 binds Cu(I) by two His in a linear geometry. In contrast, aS1-15 and bS1-15 bind Cu(I) in a tetrahedral geometry. Ligands in Cu(I)-bS1-15 are Met1, Asp2, Met5 and likely H_2O, and ligands in Cu(I)-bS1-15are Met1, Asp2, Met5, and Met10 (see Figure 1). Despite this different coordination spheres the affinities are in the same order of magnitude. His has been reported to be a stronger ligand for Cu(I) than Met at pH 7.4.[48] Indeed Ab1-16 with two His ligands has a stronger affinity than aS1-15 or bS1-15 with 2 or 3 Met, respectively. The difference of His versus Met in the present case is small when compared to the results of Rubino et al. [48]. This might be due to the additional 2 ligands in the tetrahedral coordination of aS/bS, compared to the diagonal Cu(I)-Ab1-16.

Biological implications: Globally, Ab, bS and aS have a similar affinity towards Cu(I) and Cu(II), which are moderate compared to other metalloproteins.[19] Thus these peptides are likely only able to bind copper at particular copper-rich places (like certain synapses/neurons) and under condition with perturbed copper homeostasis and the free or loosely bound copper concentration increases. Such conditions have been reported in AD [49] and might be also the case in PD.[50] There are several reports in the literature indicating that Ab and bS can be colocalized and even interact under disease conditions, in particular in the Lewis-body variant of AD.[16] Thus the concomitant presence of Cu, Ab and bS might be possible.

The present study used truncated peptide containing the principle metal-binding domains. These truncated peptides do not form amyloid fibrils, so they are widely used as models for the metal-binding in the monomeric state. Ab1-16 contains all the ligands for the first Cu(I) and Cu(II)-binding site. In contrast, in aS the His at position 50 can bind to Cu(II) bound to the N-terminal site (Figure 1) depending on pH. [20] This means that the formation of the ternary complex of Cu(II)-aS with His of Ab would be in competition with the binding of the internal His50. Since the His50 binding is not very strong, Ab binding and hence formation of a ternary complex should still be possible. Another, feature to consider is the N-terminal acetylated forms of aS. This form reduces the Cu(II) affinity, and hence Cu(II) distribution between Ab and aS would be even more pushed toward Ab.[51]

It is important to note that the presence of His50 and the N-terminal acetylation does not have any impact on the Cu(I) affinity and hence on the Cu(I) distribution between Ab and a/bS as neither His 50 nor the N-terminal amine is involved as a ligand in the highest affinity Cu(I) binding site aS.

Although the affinities can be modulated (see above), our data indicate that for monomeric Ab, aS and bS, Cu in either redox state can be preferentially bound by one peptide but not exclusively. There is always a distribution between the peptides. Moreover, Cu(I/II) state can be rapidly exchanged (<sec) between these peptides and hence distribution can be rapidly reached. Of course these considerations are limited to the monomeric state and can change in the aggregated state. An appealing finding in terms of interaction of the triad Cu, Ab and a/bS is the ternary complex. Indeed, Cu could be suggested as a factor inducing a direct interaction of Ab with aS. Although this might be an interesting feature, we do not know the stability and hence if such a complex is formed at lower concentrations. There are two scenarios in which such a ternary complex might be relevant: i) the aggregation of Ab and aS is an autocatalytic process and hence a slight change in the nucleation can have a dramatic effect. Thus even a weak and transient formation of a Ab-Cu-aS complex might have an impact on the aggregation, ii) the interaction of Ab and aS could not only occur via Cu-bridging coordination, in case there are also peptide-peptide interactions, the overall affinity could be considerably increased. Such Cu-induced peptide-peptide interactions could be even stronger in an aggregated state that is more structured and has a higher surface for interaction.

Despite the differences in affinities between Ab1-16 and aS1-15 for Cu(I/II) are small, there was a non negligible impact on the oxidation of ascorbate and the pseudo-catecholoxidase activity. The capability to oxidize ascorbate and hence produce ROS is more than twice as high for aS1-15 or bS1-15 compared to Ab1-16. Thus Cu-aS/bS is potentially more dangerous than Ab concerning the ROS production. However, if both peptides, aS/bS1-15 and Ab1-15, are present, ROS production is similar to Cu-Ab1-16 alone and is lower compared to aS/bS1-15 alone, in line with the stronger affinity of Ab1-16. Also in the case of the pseudo-catecholoxidase activity, the catalytic rate observed in the presence of both peptides (Ab1-16 and aS1-15) resembles more the one of Cu-Ab1-16, although for this reaction Cu-Ab is more reactive than Cu-aS.[43, 44] Thus in case of the colocalization of aS, Ab and Cu, the question to which of the two peptides Cu is bound, becomes a relevant question in terms of oxidative stress. However, one has to keep in mind that redox active state of Cu-Ab and Cu-aS is different from the two resting states Cu(I) and Cu(II) (shown in figure 1) [52, 53] and ternary complexes [54] might play an important

CONCLUSIONS

Despite their very different coordination sphere between Ab and a/bS in the case of Cu(II) and between Ab, aS and bS in the case of Cu(I), they have quite similar conditional affinities. The additional methionine in bS is a ligand to Cu(I) and increases slightly the affinity, but has no effect of Cu(II)-binding in line with the fact

that Met10 is not a ligand to Cu(II). Moreover, Cu(I) and Cu(II) are relatively labile and ms or faster exchange between the peptide can occur. The different coordination spheres have an impact on the capacity to catalyze of ROS production, and hence depending where the Cu is bound to, aS or Ab, the ROS production will change. Moreover, a ternary complex can be formed between Ab and a/bS in the presence of Cu(II) and likely Cu(I). This gives the possibility to have a direct, Cu mediate interaction of the two peptides Ab and aS, which might have an important impact on the aggregation behavior.

MATERIALS AND METHODS

Peptide and reagents.

Peptides: Ab1-16 (sequence DAEFRHDSGYEVHHQK) was purchased from GeneCust (Dudelange, Luxembourg) with purity grade > 98%. Stock solutions of the peptides were prepared by dissolving the powder in milliQ water (resulting pH ~ 2) and stored at -20C. Peptide concentration was then determined by UV-visible absorption of Tyr10 considered as free tyrosine (at pH 2, (ε_{276}-ε_{296}) = 1410 $M^{-1}cm^{-1}$).

αS(1-15) (MDVFMKGLSKAKEGV−NH2) and βS(1-15) (MDVFMKGLSMAKEGV−NH2) peptides were synthesized on solid phase using Fmoc chemistry. 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 semi-linear gradient of 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in CH_3CN 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.

αS(1-15) and βS(1-15) lyophilized peptides were weighed and dissolved in pure water to obtain three high concentration stock solutions. The stock solution concentration of both αS(1-15) and βS(1-15) was titrated by UV-Vis absorption monitoring the phenylalanine absorption peak at 258 nm (typical of Phe-containing peptides) with a molar extinction coefficient of 195 $M^{-1}cm^{-1}$, obtaining a 1.80 mM and 2.57 mM stock solution for αS(1-15) and βS(1-15) respectively. The obtained stock solutions were frozen and stored at -20°C.Dithionate stock solution was used for UV competition analysis in order to avoid the oxidation of Cu(I) into Cu(II). Stock solution (0.2 M) were daily freshly prepared in pure water.

Ascorbate stock solution (0.1 M) for NMR analysis and UV-ROS analysis were daily freshly prepared weighting 19.8 mg of powder and dissolving it in 1 ml of pure water. The concentration of ascorbic acid was also estimated during ROS analysis considering the molar extinction coefficient of 14500 $M^{-1}cm^{-1}$ at 265 nm (0.1 mM solution correspond to an absorbance of 1.45)

All other reagents were purchased from Sigma Aldrich.

Electron Paramagnetic Resonance (EPR).

EPR data were recorded using an Elexsys E 500 Bruker spectrometer, operating at a microwave frequency of approximately 9.5 GHz. All spectra were recorded using a microwave power of 20 mW across a sweep width of 150 mT (centered at 310 mT) with a modulation amplitude of 0.5 mT. Experiments were carried out at 120 K using a liquid nitrogen cryostat. pH was controlled after sample measurements (7.25 ± 0.05). For pH-dependent measurements no buffer was used, pH was controlled after EPR measurements.

EPR parameters were directly read on the spectrum, with the $g_\parallel$ values deduced from the $B_\parallel$ value according to $g_\parallel = (h\nu) / \mu_B B_\parallel$) where $\mu_B = 9.274\ 10^{-24}$ $J.T^{-1}$, $h = 6.626\ 10^{-34}$ J.s. and υ is the recording frequency. $B_\parallel$ is defined as the mean value of B_2 and B_3, the positions of the second and third hyperfine line respectively. $A_\parallel$ value is the difference between B_3 and B_2, and is given in 10^{-4} cm^{-1} according to $A_\parallel = 10^5\ g_\parallel\ \mu_B\ A'_\parallel / hc$ with $A_\parallel$ in 10^{-4} cm^{-1} and $A'_\parallel$ in mT, where $c = 2.9979\ 10^{10}$ $cm.s^{-1}$. The experiments were performed using ^{65}Cu

but the values given in Table 1 and in the text were recalculated for the most abundant ^{63}Cu isotope, to allow a more direct comparison with data obtained with mixture of $^{63}Cu/^{65}Cu$ in their natural abundance.

Cu(II) competition and ternary species: The peptides concentration was of 200 μM with 0.9 equivalents of Copper II ($CuSO_4$ salt) to a final concentration of 180 μM of the Cu(II)-peptide complex. pH was stabilized with 50 mM Hepes buffer and then controlled after sample measurements (7.25 ± 0.05). The formation of the aS/bS/Ab-Cu(II) complex were monitored by EPR. To the aS/bS-Cu(II) solution 1.0 equiv. of Ab was added in the EPR tube and then mixed and monitored by EPR. A further equivalent of Cu(II) was finally added in solution (final concentration of Cu(II) 360μM). Same method was managed adding 1.0 equiv. of aS/bS to the Ab16-Cu(II) solution. 5% of Glycerol was considered during the sample preparation and dilution to avoid crystallization of the solvent.

NMR: NMR spectra were acquired at 278 K using a Bruker Avance 500 spectrometer operating at proton frequency of 500 MHz. NMR spectra were processed and analyzed with the TopSpin 3.1 software. Suppression of residual water signal was achieved either by presaturation or by excitation sculpting[55], using a selective 2 ms long square pulse on water. Proton resonances assignment of the peptides is described in literature [22, 56] for αS/βS 1-15 and for Aβ1-16, respectively].

Cu(I)-competition analysis: Peptide concentration for NMR competition were of 0.4 mM (stock solutions previously dosed by UV) of both aS/bS1-15 and Ab1-16. All the peptides were dissolved in deuterated phosphate buffer (50 mM) at pH of 7.25 ± 0.05. The peptide solutions were degassed inside the NMR tube (5mm) for half hour with Argon flux. After degassing the ^1H NMR spectra were acquired for monitoring the apo forms of a/bS1-15 and Ab1-16. The tubes containing the samples were degassed again, then, 0.9 equiv. of Cu(I) were added from a CH_3CN solution of $[Cu(CH_3CN)_4]BF_4$ salt (0.1 M). To avoid the oxidation of copper, 6 μL of a 2M solution of Ascorbic Acid (dissolved in D_2O) was used (final concentration of 3 mM). The Cu(I)- α/bS1-15/Ab1-16 complexes formation were monitored by recording NMR ^1H spectra. Finally, the Cu(I)-competitor (Ab in the case of a/bS1-15-Cu(I) complexes or a/bS in the case of Aβ-Cu(I) complex) was added from a deuterated and degassed stock solution of the peptide to the NMR tube with an Hamilton syringe trough a septum. The competition between the complexes was monitored by ^1H NMR spectra. No broadening effects were observed during the analysis, confirming the absence of Cu(II) in solution.

The affinity ratio of Ab compared to a/bS for Cu(I) was calculated by :

K_{Ab} / K_{aS} = [Cu-Ab] x [a/bS] / [Ab] x [Cu-a/bS]

(for more details see SI)

UV-Vis: UV-Vis spectra were recorded on an Agilent 8453 spectrometer at 25°C in 1 cm path length quartz cuvette.

ROS production estimation via ascorbate consumption: The catalytic activity of aS/bS/Ab- Cu(I)/(II) was estimated through monitoring the consumption of ascorbate by measuring the decreasing of the UV band at 265nm (molar extinction coefficient of 14500 $M^{-1}cm^{-1}$) characteristic of non-oxidized Ascorbate. In the ascorbate solution (0.1 mM) inside the cuvette, with a pH of 7.25 ± 0.05 stabilized with Hepes buffer 50 mM, after 300 seconds, 10μM of Cu(II) (from 0.92 M $CuSO_4$ stock solution previously dosed by UV) and 12 μM of peptides (aS, bS or Ab from previously dosed stock solutions) were added, monitoring for 3600 seconds the decreasing of the UV ascorbate band at 265 nm.

Cu(I)-competition using BCA: BCA (bicinchoninic acid) forms stable 2:1 complexes in aerobic conditions with Cu(I), undergoing to the formation of 2 isolated charge transfer bands (357 nm and 562 nm with a molar extinction coefficient of 42640 $cm^{-1} M^{-1}$ and of 6590 $cm^{-1} M^{-1}$, respectively) in the UV spectrum ([38] and references therein). BCA solution (5.0 μM) in Hepes buffer 5mM at pH 7.25 ± 0.05 was prepared directly in the cuvette. Cu(I) was added to the BCA solution from a 20mM stock solution ($[Cu(CH_3CN)_4]BF_4$ dissolved in CH_3CN) in order to obtain a 1.8 μM Cu(I)-BCA_2 final concentration (an excess of BCA was

considered in order to preclude the possibility of free copper). To avoid the oxidation of Cu(I) into Cu(II) an excess of dithionite was added in solution (1.0 mM final concentration) from a concentrated stock solution (0.1M). Aliquots of αS/βS1-15 and Aβ1-16 were finally added in the solution inside the cuvette from concentrated stock solutions (around 2.0mM for all the peptides, previously described), the titration up to 10equiv. was performed in respect to the Cu(I) concentration, monitoring the decrease of the Cu(I)-BCA_2 CT bands. The αS/βS1-15 and Aβ1-16 final concentration started from 1.8 (1.0 equiv.) up to 18 μM (10.0 equiv). The recorded relative absorbance values were used to estimate the K_A of the peptide-Cu(I) complexes.

The following equation was used to calculate the ratio between the affinity of the peptide and the competitor BCA from Figure 2 at following total concentration [BCA] = 5 μM; [Cu(II)] = 1.8 μM; [peptide] = 18 μM. [Cu-BCA_2] concentrations were deduced from the absorption at 360 nm.

$K_{Pep} / K_{BCA} = [BCA]^2 \times [Cu\text{-}Pep] / [Cu\text{-}BCA_2] \times [Pep]$

with [BCA] +[Cu-BCA] = 5μM; [Pep] + [Cu-Pep] = 18μM;[Cu-Pep] +[Cu-BCA] = 1.8 μM

Catalytic oxidation of 4-methylcatechol (4-MC), and phenol, in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH), copper(II), [Cu^{II}–Ab1-16], [Cu^{II}-aS1-15] and [Cu^{II}-Ab1-16-aS1-15] complexes: Kinetic experiments were performed on an Agilent 8453 spectrophotometer and monitored between 190 and 1100 nm using an optical cell with 1 cm path length. The reactants were mixed under magnetic stirring in a thermostated cell at 25.0 ± 0.5 °C.
4-MC oxidation experiments with copper(II) nitrate, [Cu–Ab1-16], [Cu-aS1-15] and [Cu-Ab1-16-aS1-15] (25 μM for copper(II) and 50 μM for peptide total concentration) were carried out by reacting equimolar concentrations of 4-MC and MBTH (2 mM) in 50 mM Hepes buffer at pH 7.4. The formation of the red adduct between the quinone product and MBTH was monitored at 500 nm. Phenol oxidation experiments were performed in the same experimental conditions, except copper(II) and peptide concentration that were kept at 5 μM and 10 μM, respectively.
Blank experiments of oxidation of phenol and 4-MC under the same conditions, but in the absence of copper(II) salt or Cu^{II}-peptide complexes, showed that autoxidation of the substrate was negligible during the time of monitoring the experiments. All measurements were performed in duplicate.

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