Coordination and redox properties of copper interaction with α-synuclein

Coordination and Redox Properties of Copper Interaction with α-Synuclein

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Abstract

Parkinson's Disease (PD) is a severe neurodegenerative disorder affecting movements. After Alzheimer's disease, is the most common form of neurodegeneration. PD is characterized by the loss of neurons producing dopamine and by the presence of protein aggregates in the brain, known as Lewy body. The main constituent of Lewy body is the misfolded form of α-synuclein (αSyn), able to form oligomers and fibrils. In addition to protein aggregation, brain damage induced by oxidative stress is also a frequent phenomenon in PD. αSyn is able to bind Copper ions in both Cu(II) and Cu(I) oxidation states. The metal binding is also maintained when αSyn interacts with membranes. Interestingly, copper binding to αSyn has strong impact either in protein misfolding or in free radical formation, such to provide a link between protein aggregation and oxidative damage. In this review the role of copper and αSyn in PD is discussed with a particular emphasis to elucidate (i) the interaction between copper and αSyn; (ii) the reactivity and (iii) potential toxicity associated with copper-αSyn complexes.

1. Introduction

Copper, similarly to other transition metal ions, is an essential element for all living organisms. It is required for many cellular functions, such as metabolic processes and organ functions in humans. Human body has a sophisticated machinery that makes copper available when needed and, at the same time, eliminates copper when in excess. This complicated regulation, known as copper homeostasis, is ensured by a network of proteins and other biological molecules which take care of copper absorption, transport, distribution, storage and excretion [1-6]. Any failure of the homeostatic pathways leads to copper excess or deficiency in the body, which in turn, causes very serious diseases [7-10].

Copper has deleterious effects for neurodegenerative diseases as well [7, 8, 11-16]. The main effects are mediated by its redox properties and its ability to generate free radicals. In addition, copper is able to bind to proteins involved with neurodegeneration promoting their misfolding [17-19].

After Alzheimer's disease (AD), Parkinson's disease (PD) is the second most common form of neurodegeneration. As AD, PD is a progressive disorder characterized by damage and death of neurons. Similarly to AD, PD is correlated with high levels of oxidative stress, with the production of reactive oxygen species (ROS) and mitochondrial dysfunction [20-23]. Although possessing several similarities with AD, PD has different mechanisms, symptoms and treatments. In fact, the *substantia nigra* is the brain regions initially affected by PD, while the hippocampus and the entorhinal cortex are the areas primarily injured in AD. This explains the difference in observed symptoms, like movement and coordination impairments in PD and learning and memory impairments in AD. As for AD, no cure is available for PD so far.

The scientific community has put a lot of efforts for understanding the mechanisms associated with the disease onset and progression. In fact, their comprehension is crucial for a

rational drug design of new therapeutic agents. In this review the role of copper and α-synuclein (αSyn) in PD is discussed with a particular emphasis to elucidate (i) the interaction between copper and αSyn; (ii) the reactivity and (iii) potential toxicity associated with copper-αSyn complexes.

2. The synucleins and Parkinson's disease

PD is a devastating progressive neurodegenerative disease whose major clinical symptoms are tremor, movement impairments, postural instability, gait difficulty, and rigidity. It is characterized by the loss of dopaminergic neurons [24, 25] and by the presence of intracellular proteinaceous inclusions (Lewy bodies) mainly consisting of aggregated forms of the amyloidogenic protein α-synuclein (αSyn) [26-28].

αSyn is a 140 amino-acid protein, expressed within the central nervous system and concentrated at the presynaptic terminals of neuronal cells [29-32], where it can be found free in the cytosol or associated to synaptic vesicles or the mitochondrial membrane [33, 34]. Despite the evidence about the involvement of αSyn in neurodegeneration, its real function remains unknown. Three domains are identified along the protein sequence (Figure 1): (i) the amphipathic N-terminus (encompassing residues 1-60) which contains seven imperfect amino acid repeats involved in the interaction of the protein with lipid membranes and detergent micelles; (ii) the highly hydrophobic non amyloidogenic component (NAC, residues 61-95), that is responsible for protein-protein interaction during the aggregation process, and (iii) the acidic C-terminal region (residues 96-140), rich in Glu and Asp residues.

1 MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV
41 GSKTKEGVVH GVATVAEKTK EQVTNVGGAV VTGVTAVAQK
81 TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP
121 DNEAYEMPS EEGYQDYEPEA

Figure 1. Primary sequence of αSyn. The three different domains are shown with different colours. Underlined amino acids represent copper anchoring sites.

αSyn is a non structured protein and it belongs to the class of "intrinsically disordered proteins" (IDP) [35, 36]. However, its N-terminal 100 residues region has high affinity for negatively charged lipids, and it undergoes a random coil to α-helix conformational transition upon interaction with lipid membranes and detergent micelles *in vivo* and *in vitro* (Figure 2) [37, 38].

The interaction of αSyn with membranes is involved in its physiological function *in vivo*, as well as in its misfolding and aggregation processes that is thought to be involved in the pathogenesis of Parkinson's disease [39-48]. It has been observed that αSyn modulates presynaptic pool size and neurotransmitter release [49-53]. It has been recently suggested in fact that αSyn might act as a chaperone, promoting the rapid assembly of the SNARE complex involved in the neurotransmitter release from presynaptic vesicles [54]. All these functions are mediated by the interaction of αSyn with synaptic vesicles.

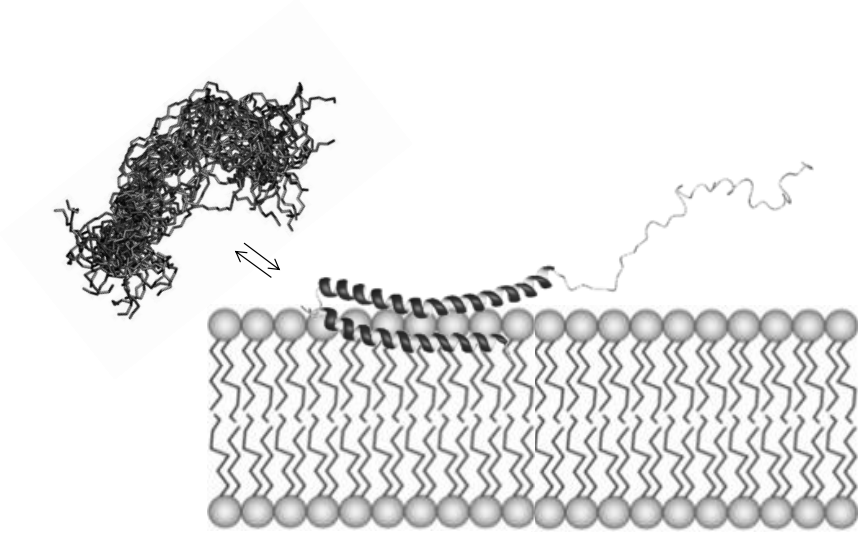

Figure 2. Schematic representation of structural transitions of αSyn after interacting with lipid membrane. αSyn cartoon showing the α-helix is derived from αSyn structure deposited in RCSC protein data bank PDB 1D 1XQ8 [38].

Soluble and insoluble fractions of Lewy bodies, extracted from brain tissues, contain αSyn (i) acetylated at the N-terminal group and (ii) phosphorylated at Ser129 [55, 56]. Only few years ago, Selkoe and coworkers pointed out that acetylation of αSyn is common in mammals [57]. From that point, extensive research has been conducted to evaluate the impact of N-terminal acetylation on

specific protein characteristics, such thermal stability, conformation, aggregation propensities, membrane interaction and metal binding [58-65]. The majority of the data support that acetylated αSyn (Ac-αSyn) has increased helical folding propensity, membrane binding affinity and resistance to aggregation.

αSyn has the propensity to misfold and aggregate [66] and, as stated above, αSyn oligomerization is considered a key event in the development of PD [67]. The formation of oligomers, fibrils and large aggregates is dependent on several factors, including protein overexpression, changes in pH, oxidative stress, interaction with dopamine and metal binding [68]. Protein oligomerization and fibrillation are strongly promoted by copper coordination to αSyn [18, 19]. On the contrary, Ac-αSyn-Cu(II) interaction results in minor oligomerization enhancement [62].

3. Copper–α-Synuclein Interaction

3.1 Cu(II) binding

The scientific community began to be interested in understanding Cu(II) binding to αSyn since 1999 when Paik et al. showed that protein oligomerization is induced by copper(II) ions [18]. Asp and Glu residues, abundantly present at the C terminus of αSyn, were identified as Cu(II) binding donors and a 59 μM dissociation constant was measured [18].From that point, a lot of investigations have been carried out to identify the metal binding properties of αSyn, as it is described in a number of recent reviews [13, 69, 70]. Several evidences have pointed out that the N-terminal region contains the highest affinity Cu(II) binding site, while the C-terminus acts as a weaker metal site [71-73]. Cu(II)- αSyn interaction at the N-terminus involves the amine and imidazole groups of Met1 and His50, respectively, which might behave as simultaneous (Figure 3A) or independent (Figure 3B) metal anchoring points. These different coordination modes were deduced by looking at the Cu(II) induced line broadening of NMR signals of various αSyn constructs. In the case of wild type αSyn, the most relevant effects were found at the N- and C- termini and His50 region. Conversely, two

diverse constructs, built to impair His50 binding, showed no consensus (Figure 3). The first one, where His50 is blocked by diethyl pyrocarbonate (DEPC) retains the paramagnetic effects only at the C-terminus (Figure 3A), while the other, having His50 substituted by Ala, still shows line broadening at the N-terminus (Figure 3B). These different behaviours might depend on (i) experimental conditions, such as pH and temperature, and (ii) the possibility that DEPC modifications occur on N-terminus as well, as previously demonstrated [72].

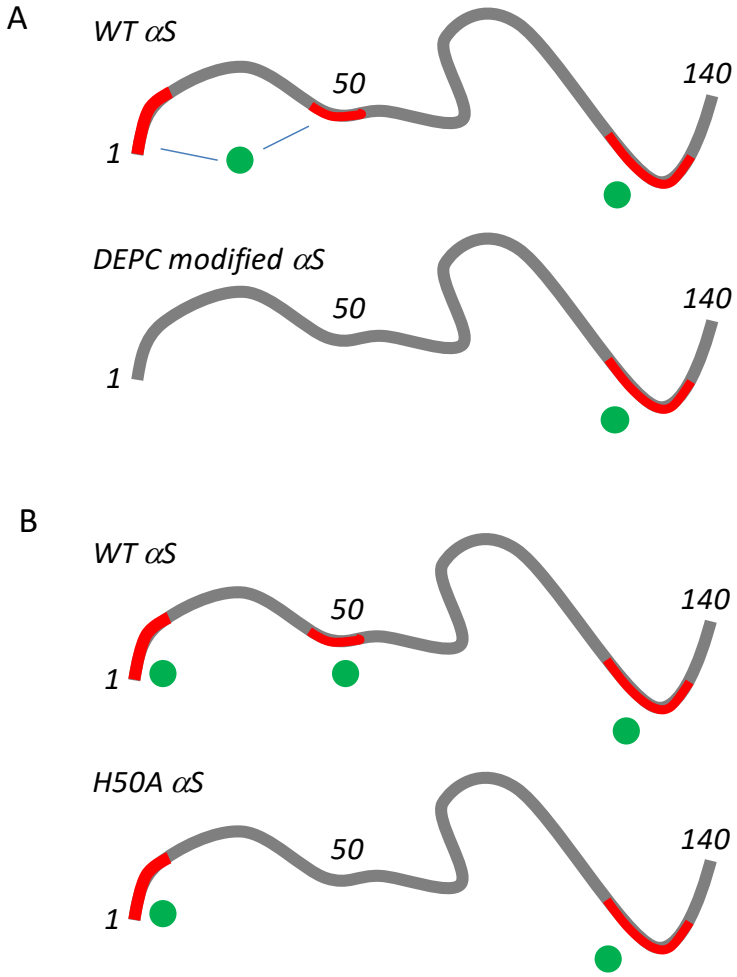

Figure 3. Proposed scheme of copper(II) binding region in αSyn according to the NMR paramagnetic effects measured on backbone signals (red regions). The comparison between A. wt αSyn and DEPC modified αSyn or B. wt αSyn and H50A αSyn are consistent with different binding domains.

ITC experiments also support the presence of two independent Cu(II) binding sites [73], αSyn1-9 (MDVFMKGLS) and αSyn48-52 (VAHGV) regions were identified as the strongest and independent metal binding domains (Figure 3B). The corresponding association constants (K_A) are

5.9×10^5 M^{-1} and 7.5×10^4 M^{-1}, respectively [73]. On the other hand, following ITC experiments on full length protein are consistent with stronger Cu(II) association, being the K_D values 0.11 ± 0.01 μM and 35.0 ± 4.0 μM for the the N-terminal and His50 binding sites, respectively [74].

Potentiometric and spectroscopic analysis performed on model peptides encompassing αSyn N-terminal residues, αSyn1-17, αSyn1-28 and αSyn1-39, provided the first proof of the Cu(II) coordination sphere [75]. The most abundant species present at physiological pH is a 2N2O complex, where Cu(II) is bound to the amino terminal group of Met1, backbone amide nitrogen and carboxylate of Asp2, and a water molecule (Figure 4A). The complex is very stable, compared to other copper(II) complexes with 2N donor ligands, because of the involvement of Asp2 carboxylate and the formation of two adjacent five- and six-membered chelate rings. This binding mode is fully retained in all three peptides, independently of the length of the primary sequence. αSyn1-6 sequence is the minimal copper(II) binding unit, as demonstrated by the structural model derived from NMR, CD and UV-Vis investigations [74].

In the perspective to better understand the role played by His50 in copper binding, a strong contribution is derived from investigations of Cu(II) coordination to peptide models containing both M1-D2- and -H50- residues [76]. The ligand was designed by starting from αSyn31-59 sequence preceded by M29-D30 residues. At physiological pH, the 3N1O species is the predominant one. As shown in Figure 4B, His imidazole replaces the water molecule present in the coordination sphere of Cu(II) complexes [76].

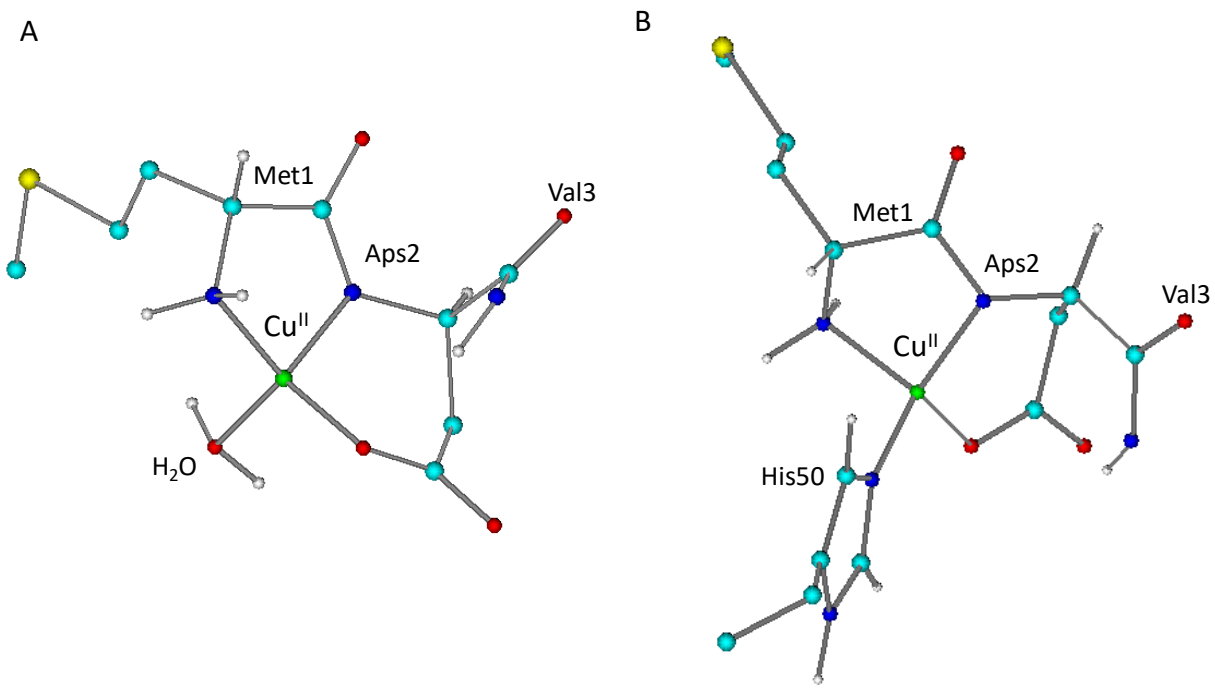

Figure 4. Representation of the coordination sphere of Cu(II)-αSyn complexes. A. {2N2O} and B. {3N1O} binding modes.

The role played by His50 in Cu(II) binding was further evaluated by monitoring tryptophan fluorescence quenching upon metal interaction. To address this issue Phe4, Tyr39, Phe94 and Tyr125 were individually substituted by Trp in WT and H50S αSyn [77, 78]. Among all these constructs, fluorescence quenching is observed on F4W αSyn and F4W/H50S αSyn only, thus leading the authors to exclude any specific role of His50 in Cu(II) binding to the N-terminal region. However, it is important to point out that, compared to W4, which is very close to the N-terminus binding site, W39 and W94 are 11 and 44 residues far from His50, respectively. Therefore, by taking into account that no specific structural rearrangements of αSyn are observed upon Cu(II) coordination, these residues could be very far from the metal center, thus possibly explaining the unchanged Trp fluorescence on Y39W and F94W αSyn.

The involvement of His50 in the N-terminal copper binding site of αSyn is clearly demonstrated by EPR and ESEEM spectra which strongly support imidazole participation to the metal coordination sphere (Figure 4B) [79, 80]. In addition to those two binding modes, there is

evidence for an independent Cu(II) binding site located in the proximity of His50 region as well [80, 81]. This domain is much less effective in copper binding and it includes a 3N1O species. The three nitrogen donors are from His50 imidazole nitrogen and His50 and Val49 main chain nitrogens, while the oxygen ligand is from a bound water molecule [81].

His50 binding to Cu(II) is strongly dependent on pH, and it is completely lost at acidic pH values as observed with model peptides encompassing the N-terminal and His50 regions. Interestingly, these two anchoring points are able to form intermolecular species thus promoting aggregation and oligomerization [82]. Very similar conclusions are derived from investigations with a larger model peptide, αSyn1-56, supporting that copper(II), once anchored to either the N-terminus or His moieties acts as a bridge for two, three different protein molecules, illustrating how Cu(II) promote oligomerization and how close chain dimers or trimers are formed (Figure 5) [83].

His50 coordination to Cu(II) is completely lost in membrane bound αSyn, where copper binding is only at the N-terminus (Figure 4A) [84]. This behaviour is due to the fact that His50, being embedded in the α-helix structure, is less flexible and prone to reach the protein N-terminus. This is confirmed by the fact that TFE induced α helical conformations of wt αSyn and αSynS1-19 adducts as well, retain the Cu(II)-binding site at the N-terminus only [85].

As reported *above*, recent evidence revealed the existence of N-terminal acetylation of αSyn (Ac-αSyn) [57-64]. This post-translational modification completely removes the Cu(II) binding ability of the N-terminal region making all the data obtained previously less relevant from the biological point of view. In fact, as expected, no Cu(II) coordination anchoring points are available at the N-terminus, when the amino group of Met1 is acetylated. However, CD, ESI-MS and NMR investigations reveal that Ac-αSyn still interacts with Cu(II). The metal binding affinity is much lower and His50 and C-terminal regions are the preferred binding domains [61].

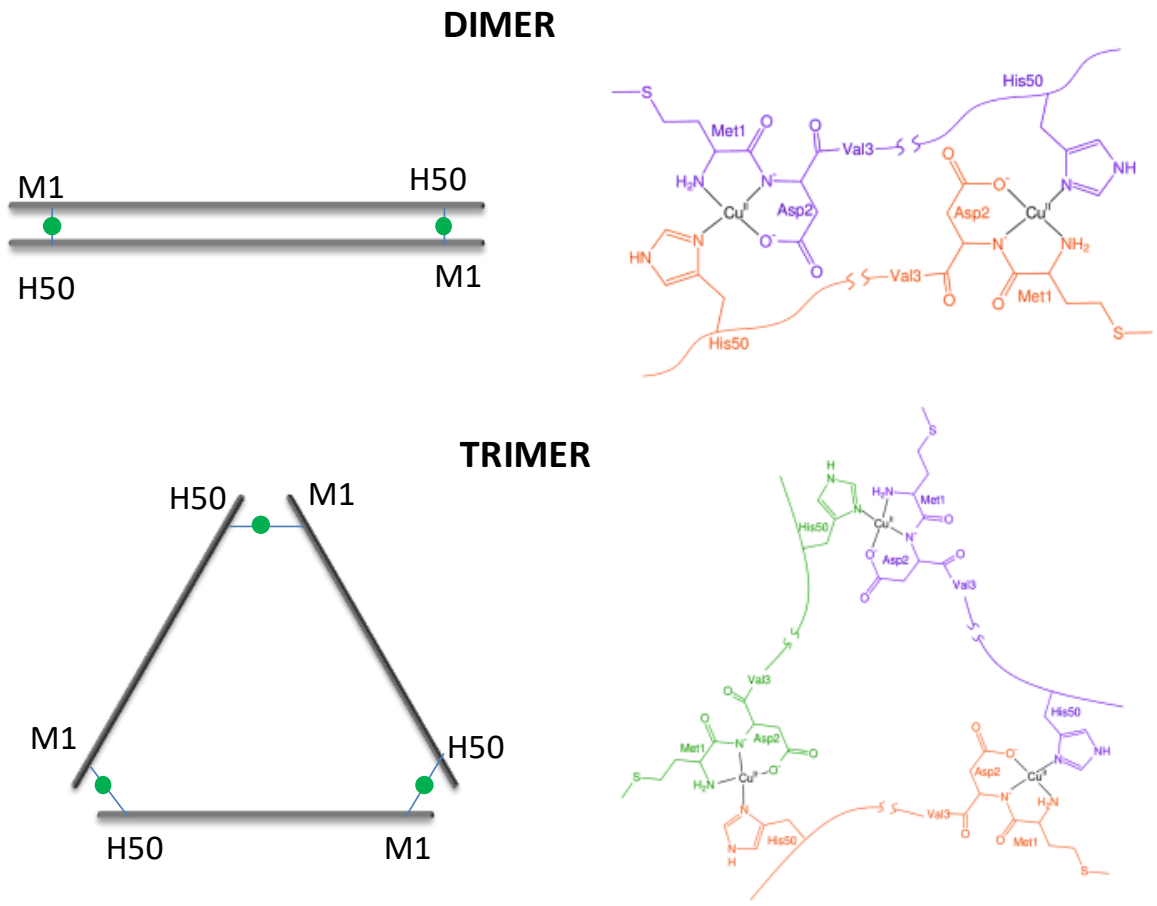

Figure 5. Illustration of αSyn dimer and trimer arrangement mediated by Cu(II) interaction. Adapted from figure 4 of reference 83.

3.2 Cu(I) binding

Compared to the plethora of studies with Cu(II), less data are available for the interaction between αSyn and Cu(I). The first evidence of Cu(I) binding to αSyn identifies the N-terminus as the major binding site for Cu(I) as well [86]. The binding donors are identified as the sulphur atoms of Met1 and Met5 thioether group, with an affinity in the micromolar range. Subsequent analysis point out that Cu(I) association can also occur at the C-terminus, where two additional thioether groups from Met116 and Met127 are able to bind the cuprous ion with similar binding affinity [87]. The two metal binding sites contain the –M(X)$_n$M– motif which is well known for its ability to coordinate Cu(I) and Ag(I) ions, which is often used as probe for Cu(I) binding [88, 89]. The structural characterization of the two Cu(I) complexes, obtained by using model peptides reveals specific conformational

rearrangements, especially at the C-terminal region, where the hydrogen bonds between Ala124 HN and Asp121 CO, and between Met127 HN and Asp123 CO, stabilize a β-turn conformation [87]. The key role played by Met residues in Cu(I) coordination is further underlined by investigations on Met/Ile substituted αSyn complexes. A strong correlation between the number of Met groups coordinating the metal ion and Cu(I)- αSyn affinity is evident [90]. This behaviour is also clear from the analysis of Cu(I) interaction with α-synuclein (αSyn) [91].

The N-terminal regions of αSyn and βSyn are highly conserved, bearing just six point mutations, K10M, A27T, G31E, K45R, H50Q and T54S. Met at position 10 in βSyn provides a new thioether ligand for Cu(I) and increased metal binding affinity [91, 92]. In addition to the key role played by Met side chain in Cu(I) binding, recent EXAFS studies indicate that Asp2 carboxylate is also coordinated to Cu(I) in both αSyn and βSyn [91]. The structure of the Cu(I)-βSyn1-15 complex is shown in Figure 6. It is derived from the NOEs data measured for the peptide βSyn1-15 in presence of Ag(I) [91].

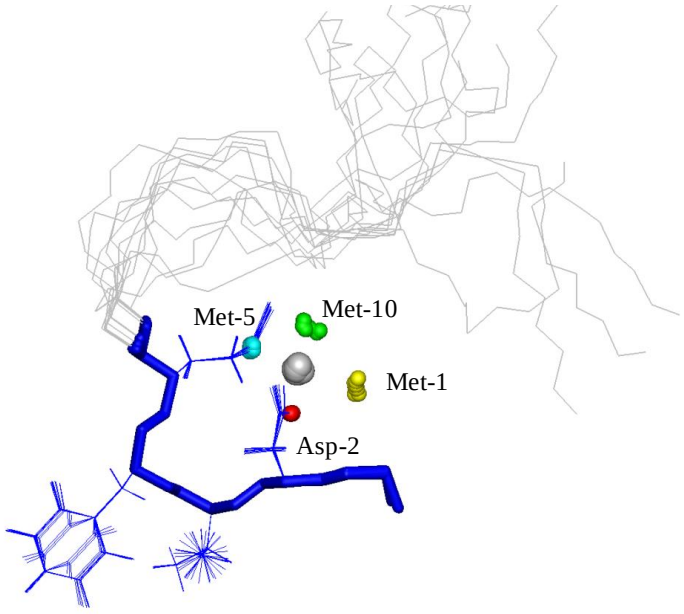

Figure 6. Superimposition of the first 15 structures obtained for the Ag(I)-βSyn1-15 complex.The structures are fitted on the 1-5 backbone residues with RMSD values for the backbone atoms 0.14 ± 0.08 Å. The sulfur donor atoms of Met-1, Met-5 and Met-10 are shown as yellow, cyan and green spheres, respectively. The carboxylic oxygen atoms of Asp-2 are shown as red spheres and the silver ion is shown as a grey sphere. Figure was created with MOLMOL 2K.1.0. Figure adapted from reference 91.

It is worth mentioning that Asp2 carboxylate is the only common donor atom of Cu(II) and Cu(I) coordination spheres. It may be speculated that the redox cyclying between Cu(II)-αSyn and Cu(I)-αSyn is combined with a high reorganization energy due to the marked difference in the two coordination spheres. It is also possible that redox reaction is mediated by in-between states, as it occurs for Cu(II)-Aβ and Cu(I)-Aβ switch [93].

Finally, very recent investigations on Ac-αSyn indicate that Cu(I) binding is conserved in the acetylated protein as well. Contrary to what happens for Cu(II), acetylation of the amino group does not affect Cu(I) binding abilities. As for αSyn, Met1 and Met5 thioethers are identified as the copper(I) donor groups, with affinity in the micromolar range [94]. Interestingly, Cu(I) interaction with Ac-αSyn induces α helical rearrangement at the N-terminal region, which, on the contrary is not observed for amino free αSyn [94].

4. Reactivity of copper-α-synuclein complex

Copper concentration in living organisms is regulated by a sophisticated system of storage and transport proteins [95]. However, an imbalance of copper homeostasis is observed in neurodegenerative and prion diseases [96, 97]. This aspect is particularly important for Parkinson's disease in which copper may strongly affect the aetiology of the disease due to its possible interaction with αSyn, as described in the previous chapters of this review.

Besides assessing the structural features and the binding affinity it is important to evaluate the reactivity associated to the complexes that copper can form with αSyn in order to clarify their possible role in cell damage. In particular, the copper-αSyn interaction may influence the following processes, which are strongly interrelated: (i) to modulate the intrinsic redox reactivity of copper which can lead to the production of ROS, (ii) to promote the oxidation of external substrates present in the cell, (iii) to induce relevant post-translational modifications in αSyn itself.

The following reaction schemes are useful to address some general aspects of the redox reactivity of copper in this context.

- *Formation of ROS:*

$Cu^{2+} + A_{red} \rightarrow Cu^{+} + A_{ox}$

$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-}$

$2\ O_2^{-} + 2H^{+} \rightarrow O_2 + H_2O_2$

$H_2O_2 + Cu^{+} \rightarrow OH^{-} + OH^{\bullet} + Cu^{2+}$

where A_{red} is a reducing species present in the biological solution, such as ascorbate.

- *Pseudo-catecholase activity:*

$Cu^{2+} + CatH_2 \rightarrow Cu^{+} + sQ^{\bullet} + H^{+}$

$Cu^{+} + O_2 \rightleftarrows CuO_2$

$CuO_2 + CatH_2 \rightarrow Cu^{+} + sQ^{\bullet} + H^{+}$

$2\ sQ^{\bullet} + 2\ H^{+} \rightarrow CatH_2 + Q$

- *Monooxygenase activity:*

$Cu^{2+} + A_{red} \rightarrow Cu^{+} + A_{ox}$

$Cu^{+} + O_2 \rightleftarrows CuO_2$ or $2\ Cu^{+} + O_2 \rightleftarrows Cu_2O_2$

$Cu_2O_2 + SH \rightarrow 2\ Cu^{+} + SOH + H_2O$

where as before A_{red} is a reducing species and SH the substrate of the monooxygenase reaction.

- *Superoxide dismutase activity:*

$2\ O_2^{-} + 2H^{+} \rightarrow O_2 + H_2O_2$

The initial event occurring in reaction schemes 1-3 is the same, i.e. production of a reduced copper species, but the various mechanisms differ in the following reactivity of this species. In the ROS formation mechanism, the Cu(I) complex simply reacts with O_2 through an outer sphere

electron transfer, producing superoxide, which in turn will initiate a Fenton's reaction chain. In the other two reaction schemes, instead, formation of some copper-dioxygen complex is involved. Both CuO_2 and Cu_2O_2 types of dioxygen adducts are formed by mononuclear, non-coupled dinuclear, or dinuclear copper enzymes [98], and have been characterized in a number of synthetic mononuclear [99-101] and dinuclear copper complexes [102, 103]. Both types of copper-dioxygen adduct can promote oxidase and monooxygenase reactions, but the mononuclear CuO_2 adduct initially formed in the reacion between Cu(I) and O_2, is not a strong oxidant and is generally assumed to evolve toward more reactive copper(II)-hydroperoxo or copper(III)-oxo species [104].

In reaction scheme 2, we refer to pseudo-catecholase activity because genuine catecholase activity implies two-electron oxidation of the substrate and does not proceed through the formation of semiquinone radicals [105]. In fact, both catechol oxidases and biomimetic copper complexes exhibiting such reactivity contain dinuclear metal centres [105, 106]. This situation is impossible to reproduce in the copper-αSyn complex, and in any copper complex with other neuronal peptides, because the peptide does not contain two proximal binding sites to host a pair of copper ions. It should also be added that both pseudo-catecholase and pseudo-monooxygenase activities can be promoted by ROS species produced by reaction scheme 1, although in this case it is expected that the reaction on the substrate will occur with lack of regioselectivity. For copper-αSyn, the pseudo-catecholase activity is of special importance, in view of the strong connection between the protein and dopamine in dopaminergic neurons.

In general, the studies reported so far with copper-αSyn were performed using the protein (usually expressed), or its peptide fragments, containing a free primary amine group at the N-terminal, whereas recent evidence shows that *in vivo* αSyn is N-acetylated in mammals [56, 107]. As explained in the “copper-α-synuclein binding” paragraph, αSyn N-acetylation abolishes the high affinity copper(II) coordination site [61], but it maintains the copper(I) coordination set unchanged

[94]. This implies that what is currently known about the redox properties and reactivity of the Cu(II)/Cu(I)- αSyn complexes needs to be revised and extended to the biologically relevant Cu(II)/Cu(I)–Ac-αSyn system. However, as data on the latter are scarce, here we will mostly focus on the extensive literature accumulated for copper bound to non-acetylated αSyn.

Lee *et al.* investigated the redox properties of copper-αSyn complex showing that Cu(II) can be reduced to Cu(I) under anaerobic conditions, whereas, in the presence of O_2, reoxidation of Cu(I) is associated with the generation of ROS, which can promote dityrosine cross-linking [108].

Zhou *et al.* showed that oxidation of Cu(I)–αSyn complex by atmospheric oxygen leads to the formation of hydrogen peroxide, which exhibits a cytotoxic behaviour [109]. However, a further study of the same group suggests that the conversion of unstructured αSyn to α-helical conformation reduces the production of ROS [85].

Further studies indicated that Cu(II)–αSyn can promote dopamine oxidation, in the presence of the reductive dye 3-methyl-2-benzothiazolinone hydrazone (MBTH), although the contribution of free copper to this reactivity was not investigated [110]. This study also showed that the reduction Cu(II)–αSyn with ascorbate produces hydroxyl radicals. In general, reactive catechols like dopamine and its metabolites, can exacerbate the toxicity effects of metal ions through redox reactions [111]. In addition, dopamine quinone itself has been reported to accelerate and stabilize the formation of cytotoxic αSyn protofibrils [112, 113].

This reactivity is important because there are several evidences that the amyloid aggregation process of αSyn is strongly affected by site-specific oxidation, dityrosine cross-linking and protein truncation [70, 114, 115]. The most important reaction is the oxidation of one of the four Met residues present in αSyn (Met1, Met5, Met116 and Met127), because this modification can inhibit amyloid fibril formation and promote the formation of stable αSyn oligomers [116-118]. The

oxidation of methionine residues also affects the membrane-binding properties of αSyn, by diminishing the affinity of αSyn to the membrane upon oxidation [119].

A study from Cappai *et al*., shows that the incubation of Cu(II) -αSyn with dopamine leads to methionine sulfoxidation [120]. Moreover, oxidation of Met1 and Met5 at the N-terminal portion of αSyn can be easily promoted by the presence of copper(II) and hydrogen peroxide [116, 121, 122] or copper(II) and a reductant such as ascorbate [86]. A recent NMR study also shows that air exposure of the reduced Cu^{I}-αSyn complex leads to rapid oxidation of methionine residues [123]. Another oxidation sensitive residue is His50, which is oxidized in the presence of Cu(II) and H_2O_2 [122].

Recently, our group analysed the reactivity of copper(II)–peptide complexes, containing the N-terminal portion of αSyn bearing the minimal copper coordination unit, in oxidative reactions of catechols and phenols [124]. However, the copper–αSyn complex exhibits no significant tyrosinase-like reactivity, since its ability to promote phenol monooxygenase and diphenol oxidase reactions is lower than that of free copper(II). On the other hand, the superoxide dismutase reactivity (reaction scheme 4) of copper–αSyn complex is comparable to that of free copper.

We therefore concluded that the structural rearrangement in the metal coordination sphere required in Cu(II)/Cu(I) cycling prevents the copper–αSyn complex to be a good catalyst in reactions that involve dioxygen coordination to copper(I). However, our study confirms that redox cycling of Cu^{2+}/Cu^{+} ions may cause concomitant modifications of αSyn through radical Fenton-like reactions.

An intriguing reactivity of Cu–αSyn is the interplay of this complex with iron homeostasis. Brown *et al.* proposed that αSyn can bind simultaneously copper(II) and iron(III), and that copper bound to the protein can act as an electron transfer centre between a donor such as NADH and an acceptor such as iron(III) [125, 126]. This ferrireductase reactivity could affect iron metabolism, which is also altered in PD pathogenesis.

5. Toxicity of copper-α-synuclein complex

The mechanism of αSyn toxicity is an actual challenge that is crucial for the elucidation of PD pathogenesis. Here, we intend to summarize the mechanisms that contribute to αSyn toxicity where the involvement of copper is demonstrated or hypothesized. As described in the previous paragraph, one of the most relevant relation between the formation of copper-α-synuclein and its toxicity is represented by post-translational modifications induced by metal-induced oxidative stress. This aspect is extremely important also because synucleinopathies and neurodegenerative diseases in general are associated with high levels of oxidative stress in the brain [115, 127, 128].

Methionine can be easily oxidised to sulfoxide *in vivo* by different oxidizing agents such as hydrogen peroxide, hypochlorite, chloramines and peroxynitrite. In the presence redox-active metal ions, the oxidation occurs in mild conditions, since it only requires e.g. copper in the cuprous state and molecular oxygen. However, the methionine sulfoxidation process is fine regulated under physiological conditions because in the cytosol several methionine sulfoxide reductases (Msr) are involved in the repair of methionine sulfoxidation (Figure 7) [115, 129]. Msr enzymes catalyse the reduction of oxidised methionines back to the sulfide form [130]. Two isoforms, Msr A and Msr B, are specific for reduction of the *(S)*-Met-SO and *(R)*-Met-SO enantiomers, respectively [131]. This has led to the hypothesis that αSyn may act as a catalytically regenerated oxidant scavenger in physiological conditions, thus performing an important protective role until this equilibrium is broken by an increase of oxidative stress.

On the other hand, further oxidation of methionine sulfoxide to sulfone leads to a final product that cannot be reduced by methionine sulfoxide reductases. This process is therefore irreversible within the cell and might have important consequences in the pathogenesis by contributing to the final state of the aggregation. *In vitro* experiments show that when αSyn is

incubated with reduced copper and oxygen the oxidation is limited to sulfoxide [123, 124], whereas the formation of sulfone is observed when also hydrogen peroxide is present [121, 122].

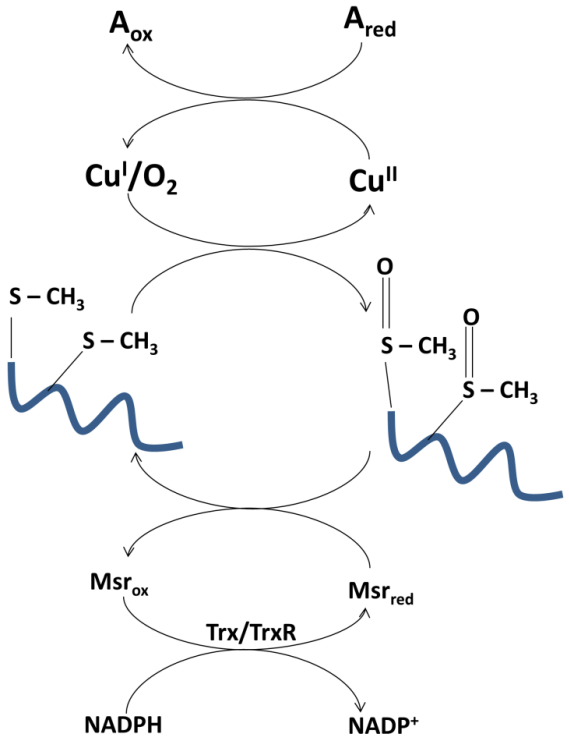

Figure 7. Schematic representation of the mechanism of oxidation and reduction of methionine residues in αSyn. In reducing environment copper can activate molecular oxygen to easily oxidize methinonine residues to methionine sulfoxide. The reduction of methionine sulfoxide is catalyzed by methionine sulfoxide reductase (Msr). This enzyme uses the thioredoxin reductase (TrxR)-thioredoxin (Trx) system for its enzymatic redox cycle, which is a NADPH/$NADP^+$ mediated process.

It is worth mentioning that other reactions are possible where copper ions can play a role. Nitration of tyrosine residues and dityrosine dimer formation are classical hallmarks of pathological conditions [132]. Human αSyn contains four tyrosine residues: one located in the N-terminal region at position 39 and three others in the C-terminal region, at positions 125, 133, and 136. An extensive literature confirms that the formation of nitrotyrosine has important consequences for αSyn toxicity [114, 115, 133-135]. In addition, another important post-translational modification involving tyrosine is the formation of dityrosine through aromatic ring coupling. Dityrosine formation has been observed upon oxidation of copper(I) and consequent formation of ROS (reaction scheme 1) [108].

How these nitrative and oxidative modifications influence the aggregation of αSyn to toxic oligomers is object of great debate and numerous studies have appeared [136], so that these aspects are outside the scope of the present review.

Phosphorylation of serine and tyrosine residues present in the C-terminal portion of αSyn is another relevant post-translational modification that has been extensively studied over the past years, albeit its neuroprotective *vs.* neurotoxic role is still object of debate [137]. The role of metal ions, and copper in particular, in this mechanism needs to be investigated more in detail, because phosphorylation of serine and tyrosine in the C-terminal seems to affect αSyn-metal interactions [138]. In particular, phosphorylation at Tyr-125 or Ser-129 residues increased the binding affinity of Cu(II), Pb(II), and Fe(II) to the protein, which provides evidence for the possible role of multiple interactions between α-synuclein and metal ions on the regulation of protein aggregation by its C-terminal.

A further issue is the involvement of copper into the interplay between αSyn and dopamine, because one of the physiological functions of αSyn is related to its involvement in dopamine metabolism and storage [139]. As explained in the previous chapter, we have shown that the oxidation of dopamine and other catechols is slower when the reaction is catalysed by copper-αSyn complex compared with free copper [124]. However, once the dopamine quinone is formed it leads to the formation of cytotoxic αSyn protofibrils [112, 113]. Also dopamine can bind to αSyn forming stable oligomers [140, 141], the toxicity of which is still debated [142-145].

Finally, another hypothesis regarding the relationship between copper homeostasis and PD pathogenesis is based on the evidence that total copper concentration in the pathogenic neurons affected by PD is decreased [146]. Some evidence suggests that a reduction of copper in neurons in PD can reduce the antioxidant defense related to superoxide dismutase (SOD1) [97]. These

observations might thus relate a loss of copper-dependent protective mechanisms to the neurodegenerative cascade.

6. Conclusions

Copper-αSyn interaction plays a crucial role in PD, because it influences various aspects of the pathophysiology of the protein, such as aggregation, accumulation, and induction of post-translational modifications.

Until recently, the effects of copper-αSyn interaction were studied in the frame of the high affinity binding of the Cu^{II} ion to the N-terminal portion of the protein. However, the finding that *in vivo* the αSyn terminal amino group is acetylated, dramatically decreasing the affinity for copper(II) [61], makes the model used so far of little use. On the other hand, the interest is now shifted to the interaction of the protein with Cu^{I}, as copper(I) coordination is not affected by N-terminal acetylation [94]. In particular, the reactivity of Cu^{I}-Ac-αSyn has to be better characterized.

Another general aspect of PD pathology that has received little attention so far is the spreading of αSyn oligomers in different brain areas. Protein propagation is common to other neurodegenerative diseases [147] and seems to involve an interplay between different amyloidogenic proteins. For instance, interaction between β-amyloid (Aβ) and αSyn [148] or between Aβ and prion protein [149] appear to be relevant for Alzheimer's disease. Since all the proteins involved are able to bind copper, the metal ion and the associated redox reactivity might play an important role also in protein-protein interactions. We have recently reported the study of a copper-mediated interaction with truncated Aβ 1-16 and αSyn1-15 peptides [92], that could be an important starting point for similar analysis with the full length proteins.

Finally, the role of membrane in the αSyn physiology and pathology needs to be further investigated. The structure of αSyn shifts from random coil to α-helix conformation upon interaction with lipid membranes and detergent micelles. More studies are therefore required to clarify this

"third" partner in the copper-αSyn relationship, because it certainly has an influence on the reactivity of copper-Ac-αSyn complexes.

Abbreviations

Parkinson's Disease (PD)

α-synuclein (αSyn)

Alzheimer's disease (AD)

reactive oxygen species (ROS)

non amyloidogenic component (NAC)

intrinsically disordered proteins (IDP)

acetylated αSyn (Ac-αSyn)

diethyl pyrocarbonate (DEPC)

Nuclear Magnetic Resonance (NMR)

Circular Dichroism (CD)

Ultraviolet-Visible (UV-Vis)

Electron Paramagnetic Resonance (EPR)

Electron spin echo envelope modulation (ESEEM)

trifluoroethanol (TFE)

electrospray ionisation mass spectrometry (ESI-MS)

extended X-ray absorption fine structure (EXAFS)

Nuclear Overhauser Effect (NOE)

3-methyl-2-benzothiazolinone hydrazone (MBTH)

methionine sulfoxide reductases (Msr)

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