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Review Article

Crossroads between copper ions and amyloid formation in Parkinson's disease

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Copper (Cu) ion dys-homeostasis and α-synclein amyloid deposits are two hallmarks of Parkinson's disease (PD). Here, I will discuss the connections between these features, with a major focus on the role of Cu in the α-synuclein (aS) amyloid formation process. The structurally disordered aS monomer can bind to both redox states of Cu (i.e., oxidized Cu(II) and reduced Cu(I)) with high affinity *in vitro.* Notably, the presence of Cu(II) (in absence of aS N-terminal acetylation) and Cu(I) (when in complex with the copper chaperone Atox1) modulate aS assembly into β-structured amyloids in opposite directions *in vitro*. *Albeit* the link to biological relevance is not fully unraveled, existing observations clearly emphasize the need for more knowledge on this interplay and its consequences to eventually combat destructive reactions that promote PD.

Parkinson's disease and copper metabolism

Parkinson's disease (PD), for which there is only symptomatic treatment [1,2], is the second most common neurodegenerative disorder after Alzheimer's disease. Assembly of the protein α-synuclein (aS) into oligomers and β-sheet-rich amyloid fibrils is linked to the molecular pathology of PD [3,4]. aS amyloids constitute the major content of pathological neuronal inclusions, Lewy bodies, found postmortem in the brain (*substantia nigra* region) of PD patients [5–7]. In accord, duplications, triplications, and point-mutations in the aS gene, enhancing concentration and aggregation propensity, are linked to familial PD cases [8]. At physiological conditions, the 140-residue aS can be detected in both an intrinsically unstructured form in the cytosol and a helical state on lipid membranes [9,10]. Despite the key role of aS in the onset and progression of PD, the functional role of this protein remains obscure, but appears related to synaptic vesicle trafficking, clustering, and membrane fusion (Figure 1) [11–13]. In agreement, aS is localized at presynaptic nerve terminals associated with synaptic vesicles [14–16]. The process of aS assembly to amyloid fibers is thought to result in toxic gain-of-function, in similarity to other neurodegenerative diseases where aggregation involves other amyloidogenic proteins. Amyloid formation of aS can be modulated by synthetic as well as naturally occurring molecules, for example, heavy metals, ring-fused 2-pyridones [17], other amyloidogenic proteins [18], bacterial proteins [19], chaperones [20], as well as by solution conditions, such as altered pH and oxidative stress.

Recent studies have suggested that metal ions play pivotal roles in PD and other neurodegenerative disorders [21–24] and imbalances of metal levels are strongly associated with many neurodegenerative diseases [25]. In fact, amyloid deposits, including Lewy bodies, are enriched in metal ions in addition to proteins. Cu ions, as well as other metal ions, are highly concentrated in the brain [26] and a gradual increase in brain metal ion content is thought to be a consequence of normal aging [27]. Cu is a redox active metal (cycling between reduced Cu(I) and oxidized Cu(II) states) that provides activity to several essential enzymes in humans [28]. In PD, Cu levels are found to be decreased in brain tissue (especially in the *substantia nigra* region which is the part most affected in PD) but increased in cerebrospinal fluid and blood [21,24]. The decrease in cellular Cu appears coupled to decreased expression of the Cu uptake protein, copper transporter 1 (Ctr1), which transports extracellular Cu into the cytoplasm. The

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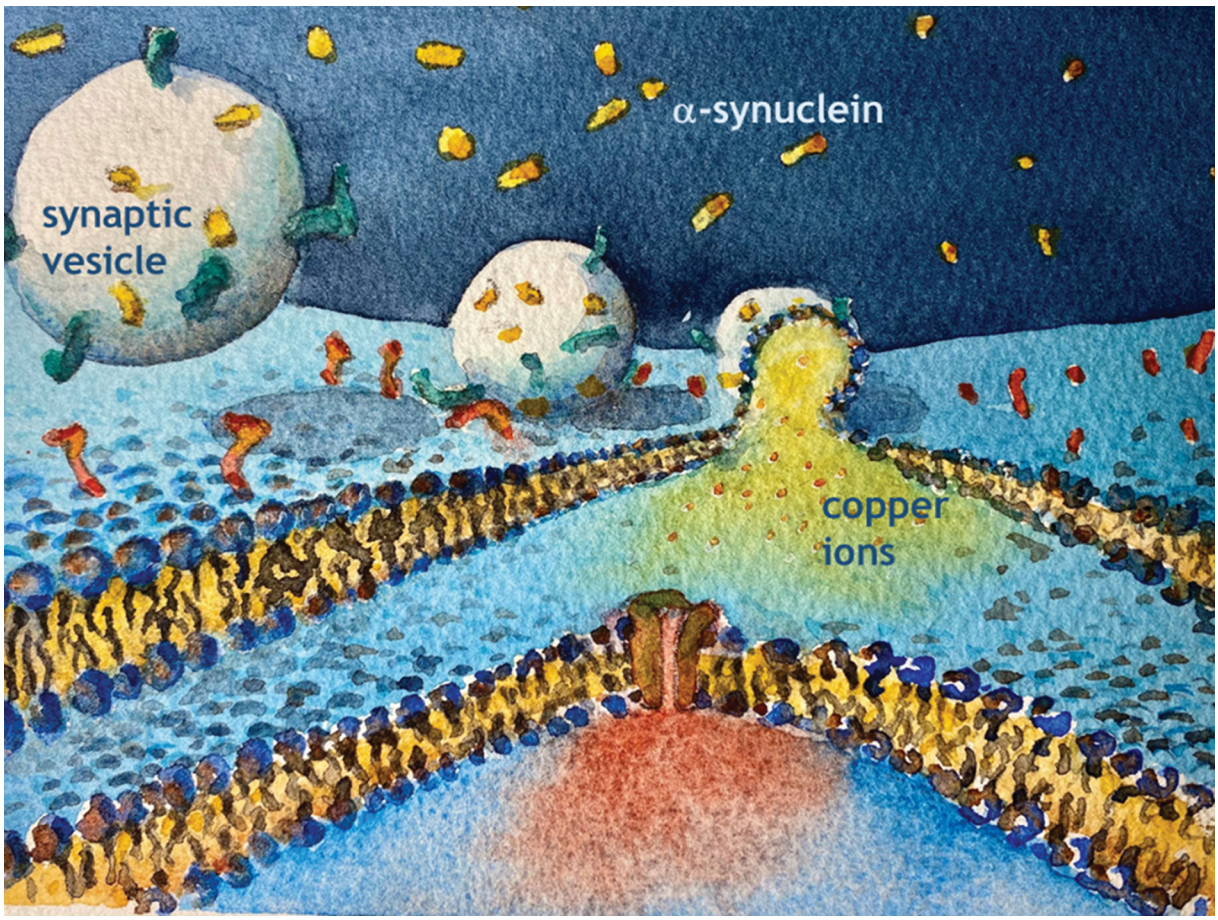

Figure 1. Artistic drawing of synaptic vesicles fusing with the plasma membrane at the presynapse

Copper (Cu) ions are used as signaling molecules in the brain and are released from vesicles at the synapse. Neuronal signaling processes, including the roles of aS, Cu, and their putative interaction, are important to study for insights into the origin and progression of PD. *Picture painted by my colleague, professor Fredrik Höök, Chalmers University of Technology.*

cytoplasm is a reducing environment and Cu ions are therefore transported to their destinations in the Cu(I) form [29]. Notably, there are no free Cu ions in cells under physiological conditions; instead, the metal ion is always protein-bound. In the cytoplasm, the Cu(I) chaperone Atox1 [30] transports Cu from Ctr1 to P_{1B}-type ATPases (ATP7A and ATP7B) in the Golgi, for loading of Cu-dependent enzymes [31], via direct Cu-bridged protein–protein interactions. Over 50 different proteins in human cells are thought to bind Cu but how Cu is delivered to those proteins (and their Cu-dependent functions) is elucidated for only a fraction [32].

In neuronal cells, ATP7A has been found to relocate to presynaptic nerve terminals where it loads synaptic vesicles with Cu [26,33]. Like neurotransmitters, Cu ions are players at synapses (Figure 1), modulating neuronal excitability via binding to receptors such as gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors [26,33]. Thus, not only is Cu important for specific enzymatic functions, emerging data show that these ions also participate in cell–cell signaling [26,33]. Cu released in the synaptic cleft can transiently reach concentrations over 100 μM and such ions are thought to be in the Cu(II) form. Thus, if Cu-loaded vesicles are disrupted before fusion with the plasma membrane, Cu(II) ions could be released inside cells. In addition, because oxidative stress is another hallmark of PD, Cu(I) ions may transiently be oxidized in disturbed brain cells. Considering the above, it may be of functional as well as pathological relevance that aS is annotated in UniProt (www.uniprot.org) as Cu-binding, and the protein has been found to bind both Cu(II) and Cu(I) *in vitro*. Thus, intracellular or extracellular aS may encounter both Cu(I) and Cu(II) *in vivo* (although *in vivo* interactions not directly proven yet).

Here, I will take a biochemical approach and describe the current understanding of roles of Cu in aS amyloid formation (and thus PD). As will be clear upon reading, there are many outstanding questions. Part of the reason I write this text is to emphasize the need for additional research both *in vitro* and *in vivo*.

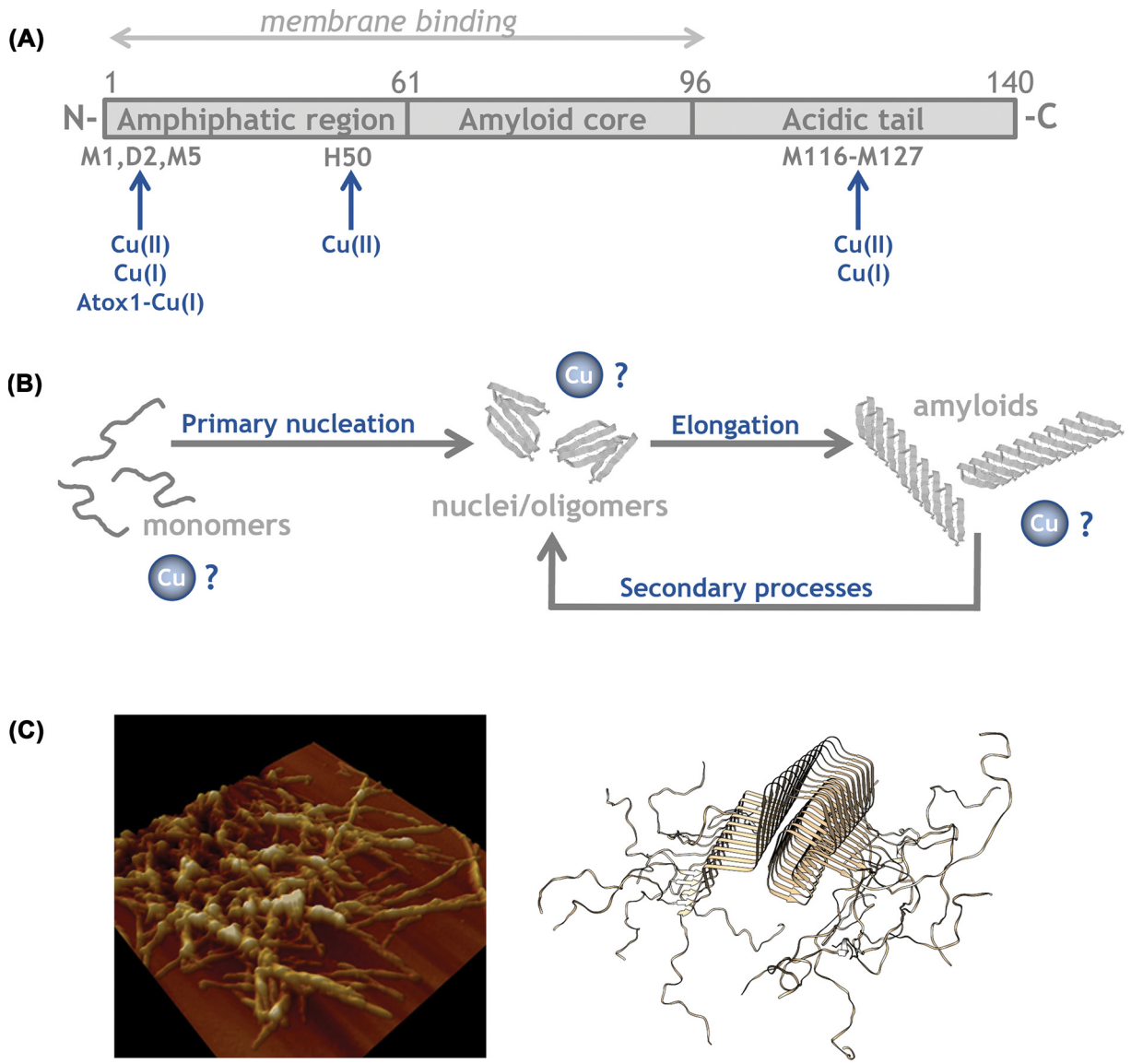

Figure 2. Scheme of aS sequence, its amyloid formation mechanism, and amyloid structures

(**A**) The primary sequence of aS, with its three parts labeled along with Cu(II), Cu(I), and Atox1-Cu(I) interaction sites. The lipid–vesicle interaction part of aS is also indicated. Typically, in cells, the N-terminus is acetylated. (**B**) Amyloid formation involves primary nucleation, fiber elongation, and secondary processes (such as secondary nucleation and fiber fragmentation). Cu may affect all these steps. It is not known if Cu(II)/Cu(I) can bind to oligomers or preformed fibers directly. It is also not known where (if) the Cu ions end up in the final amyloid structure when added to aggregating monomers. There may also be off-pathway reactions (not indicated in figure). (**C**) Amyloid fibers visualized by atomic force microscopy (left) and an example of a high-resolution structure of an aS amyloid (pdb: 2N0A), right.

Cu binding to aS monomers

In vitro, both Cu(I) and Cu(II) bind to aS [34,35] and structural features, binding sites, and affinities for these interactions have been the focus of many spectroscopic studies [34–38]. Whereas the high affinity Cu(II) site involves backbone nitrogen atoms in residues Met1 and Asp2, and at some conditions His50, the high-affinity Cu(I) site involves side chain sulfurs of Met1 and Met5 [39,40]. Notably, all these Cu-binding sites are in the N-terminal part of aS, which is also the binding region for lipid vesicles (Figure 2A). The affinities, falling in low-μM or nM range, together with various coordination geometries have been comprehensively summarized in, e.g., [41]. In addition,

Essays in Biochemistry (2022) **66** 977–986
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and perhaps related to initiation of aggregation, electron paramagnetic resonance data have demonstrated a binding mode in which Cu(II) bridges between two aS peptides [42].

It was recently reported that, when bound to aS, Cu could reversibly cycle between oxidized and reduced forms with a biologically relevant redox potential [43–45]. Subsequent work showed that Cu-bound aS was able to act as a ferri-reductase using Cu as a catalytic center to reduce Fe ions [46,47]. Since the co-ordination sphere for Cu(II) and Cu(I) are markedly different (only Asp2 as common ligand), one would expect a high reorganization energy for such transitions. Still, redox activity of Cu-bound aS could increase cellular oxidative stress, which may cause tyrosine cross-links [48] and oxidation of, e.g., the neurotransmitter dopamine [24]. With respect to the latter, it is intriguing that dopaminergic neurons (mostly found in *substantia nigra*) have the highest susceptibility to degeneration/death in PD. Still, Cu redox activity was found to be reduced when bound to aS as compared with free Cu, and therefore the complex was proposed to scavenge oxygen radicals [49]. In addition to the N-terminal high-affinity Cu sites, there are also low-affinity binding sites for both redox states of Cu in the C-terminal part of aS [50]. Also, other metal ions can interact (weakly, mM affinities) with negatively charged residues in aS's C-terminal part.

When considering Cu binding to aS, one must take into account that aS can undergo several post-translational modifications (such as acetylation, phosphorylation) *in vivo* [50]. In fact, most aS peptides in humans are N-terminally acetylated [51] and residues in the C-terminus may be phosphorylated [52]. It was reported that N-terminal acetylation of aS abolishes (or, drastically reduces) Cu(II) binding at the high-affinity N-terminal-binding site [39,53], but Cu(I) binding is preserved [54]. Residue-specific NMR characterization of Cu(I) interaction with acetylated and nonacetylated aS showed that the metal-induced structural change was of a larger magnitude when Cu(I) interacted with the acetylated form [54]. Since most aS is N-terminally acetylated, and Cu in the cytoplasm is in the Cu(I) form, one may argue that Cu(II) interactions with aS are biologically irrelevant. However, as noted above, Cu(II) may be transiently present inside cells, and it is released as Cu(II) at the synapse; to that, a small fraction of aS *in vivo* may not be acetylated. Cu(II) could interact with such nonacetylated aS, inside cells or extracellularly, thereby triggering formation of amyloid seeds that then could recruit acetylated aS for further aggregation. It is also possible that weak Cu(II) binding in the C-terminus of aS affects aggregation [52,55] at certain local or transient *in vivo* conditions.

Effects of Cu on aS amyloid formation

Amyloid formation is a complicated and heterogeneous process that involves going from nm-sized unstructured monomers to μm-sized cross-β-structured amyloid fibers. Primary nucleation, resulting in nuclei or oligomers, is followed by elongation (i.e., monomer additions) to amyloid fibers. Amyloid fibers may then engage in secondary processes, including fiber fragmentation and secondary nucleation on the amyloid surface, and there may also be various off-pathway reactions throughout these reactions (Figure 2B,C). The nucleation process is slow and results in a lag time when monitoring the kinetic process in test tubes via fluorescence from Thioflavin-T (ThT), a commonly used probe molecule that emits upon binding to amyloids. The lag time is followed by a rapid increase in ThT fluorescence, indicative of amyloid fiber elongation, until, at later times, a stationary state is reached where the ThT fluorescence, and thus the amyloid amount, remains constant. Typically, for 50 μM aS, at shaking conditions with a glass bead, the lag time is around 15 h (pH 7, 37°C). Without shaking and glass bead, aS aggregation takes more than a week to begin (pH 7) [56].

Early *in vitro* studies of aS amyloid formation showed that many di- and tri-valent metal ions (e.g., Al(III), Cd(II), Fe(III), Co(II), Cu(II)) accelerate the reaction when added in mM concentrations, with Cu(II) being the metal ion with the largest accelerating effect [57,58]. Subsequent studies investigated the effects of Cu(II) at more biologically relevant concentrations, i.e., μM range, on aS amyloid formation kinetics and, again, acceleration of amyloid formation (i.e., reduction in the lag time) was observed [59]. Somewhat surprising, inspection of the literature revealed only a few subsequent *in vitro* studies in which the effects of metal ions on aS aggregation have been systematically studied as a function of metal concentration, solution conditions, and aS variants. It has been shown, as expected due to lack of strong binding, that Cu(II) has no effect on amyloid formation of N-terminally acetylated aS [60,61]. Also the introduction of the H50Q substitution (a disease-causing mutation that involves a Cu(II)-binding residue) into nonacetylated aS reduced the magnitude of Cu(II)-induced acceleration of aS amyloid formation [61,62].

In a recent study from my lab, we confirmed the previously observed divergent effects of Cu(II) on acetylated and nonacetylated aS and added the new observation that A53T aS (another disease-causing mutation, not involving a Cu(II)-binding residue) amyloid formation kinetics was not affected by Cu(II) additions regardless of acetylation status [63]. Still, Cu(II) binds to nonacetylated A53T aS like it does to the wild-type protein based on visible circular dichroism data. Combined with several additional experiments, we concluded that the reason for no kinetic effect

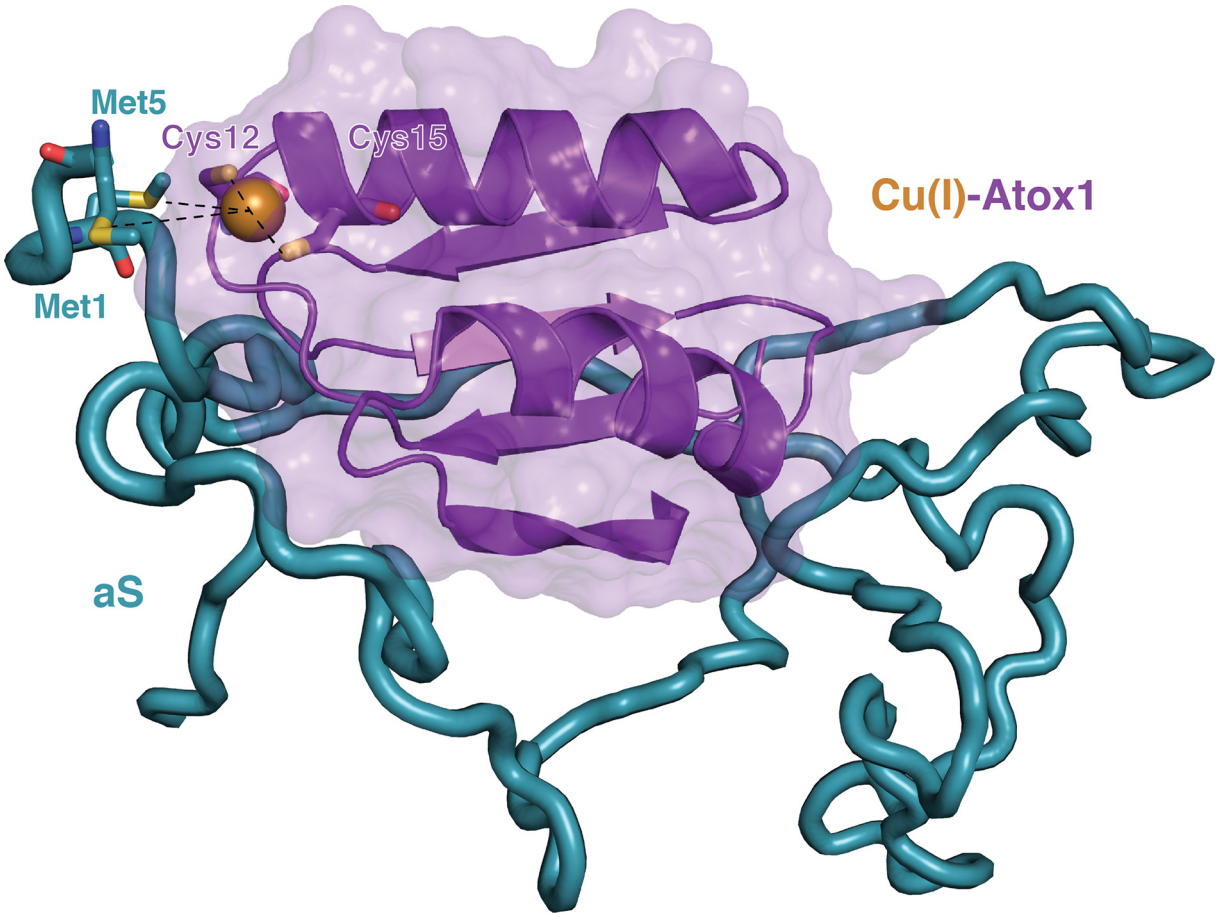

Figure 3. Ternary complex Atox1-Cu(I)-aS

Cartoon of the complex formed between aS (green) and Cu(I)-loaded Atox1 (purple, with Cu ion as gold sphere) as deduced from solution NMR experiments. Cu(I)-binding residues shown in stick (Met1, Met5 in aS; Cys12, Cys15, in Atox1). The affinity is in the low micromolar range and thus this complex may form in cells. *Illustration prepared by Björn Burmann, Gothenburg University.*

upon Cu(II) binding to nonacetylated A53T aS is because this variant exhibits an intrinsically faster aggregation speed. The faster aggregation speed of A53T aS as compared with wild-type aS has been linked to less long-range contacts in the mutant's monomeric state [64]. Thus, Cu(II) binding to wild-type aS may change the monomer conformation toward that of A53T aS, while the variant is already in a more extended conformation. That Cu(II) binding destabilizes long-range (aggregation blocking) interactions between the N- and the C-termini in wild-type aS has been suggested before [62]. There have been no *in vitro* studies of effects of Cu(II) on amyloid fiber elongation (i.e., using amyloid seeds, so nucleation is bypassed) or secondary processes specifically.

Notably, the effect of Cu(I) binding on αS amyloid formation *in vitro* has not been reported. This is because it is hard to perform traditional aS aggregation experiments over several days at conditions where one would keep Cu(I) in the reduced state throughout. To get around this, we recently investigated the effect of Cu(I)-loaded Atox1 on aS amyloid formation. Once formed, Cu(I)-loaded Atox1 is stable in nonreducing conditions [65]. Atox1, as well as other Cu transport proteins, are expressed in most neuronal cells [57,66].

Protein-mediated Cu effects on aS amyloid formation

To test if the Cu chaperone Atox1 could deliver Cu(I) to aS and, thereby, affect amyloid formation, we added Cu(I)-loaded Atox1 to aS aggregation reactions *in vitro*. We found that, instead of Cu(I) transfer, a Cu-dependent complex formed which blocked aS from forming amyloids [67]. Further biochemical characterization revealed micromolar affinity between the two proteins and interacting residues in both proteins were identified by NMR [68]. As expected from the Cu(I) dependence, the interaction sites involved each protein's Cu(I)-binding site: the N-terminal

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part up to residue 24 in aS and residues 11–16, which include C12 and C15 that coordinate Cu(I), in Atox1 along with additional residues elsewhere in both proteins (Figure 3). This complex resembles the protein–protein complexes that act as intermediates during normal Cu(I) transport in which the Cu(I) ion is bridged by residues in both proteins [69]. Importantly, the Atox1-aS interaction was not abolished by N-terminal acetylation of aS. Although only indirect evidence of physical interaction *in vivo*, we demonstrated Cu-dependent proximity (i.e., within 40 nm of each other) of Atox1 and aS in neuronal cells [68]. Based on the results, we imagined that metal-dependent chaperoning, as exemplified by Cu(I)-Atox1, is another cellular mechanism, in addition to the protein chaperone network, that protects and controls the proteome. Interactions between Atox1 and aS may hold back aS amyloid formation at normal conditions, whereas at PD conditions, with reduced Cu content in cells, the complex is abolished and aS is free to aggregate. However, our work does not exclude that *in vivo*, Atox1 instead delivers Cu(I) to aS, and aS uses the metal for a yet unknown function.

The Atox1 interaction appeared specific as we found no aS interaction with another human Cu chaperone, the copper chaperone for superoxide dismutase 1 (CCS). CCS loads Cu(I) to superoxide dismutase 1 (SOD1), a protein which harbors antioxidant activity and is also the amyloidogenic protein in amyotrophic lateral sclerosis. Interestingly, it has been shown that Lewy bodies in PD patients also contain SOD1 [70]. More recent cell studies (co-immunoprecipitation and immunohistochemistry) revealed an interaction between aS and SOD1; it also appeared that both proteins could affect the aggregation of the other protein [71]. However, no *in vitro* experiments with purified proteins have been pursued and the role of Cu in these interactions has not been investigated.

Conclusions and outlook

The amyloidogenic protein aS, playing a key role in PD, is annotated as a Cu-binding protein and binds Cu(II) as well as Cu(I) *in vitro*. Cu(II) ions also accelerate amyloid formation *in vitro* (in absence of N-terminal acetylation). The outstanding question without answer yet is the biological relevance of these metal–protein interactions. Does aS interactions with Cu play roles in aS function and/or in dysfunction *in vivo*? Is it too much or too little Cu, or the wrong redox state, that is problematic? Using a cell culture model, it was reported that Cu binding to aS in cells regulates aS cellular localization and increases aggregation as well as toxicity [72]. Another study showed that in the presence of Cu, aS amyloid fibers were more toxic to neuronal cells than in the absence of the metal [73]. Also, silencing Ctr1 expression, thus blocking Cu uptake, reduced aS aggregation in cells and reduced neuronal loss in mice [74]. On the other side, focusing on reduced Cu levels in PD patients' brains causing dysfunction, there has been a clinical trial on PD patients with the Cu(II) compound CuATSM (ATSM = diacetylbis(N[4]-methylthiosemicarbazonato)) that is supposed to deliver extra Cu to the brain [24]. Several synthetic compounds have been tested for their ability to deliver Cu to cells and restore metal homeostasis, but mechanisms and consequences are not clear yet [75,76].

Although the biological aspect is clearly important to investigate further, there are many biochemical and biophysical experiments that could add important insights. With today's ability to perform sensitive and quantitative kinetic aggregation studies *in vitro*, we can learn a lot more about how Cu (added at different time points, at different solution conditions, and with various aS variants) affects amyloid formation mechanisms using advanced analytical approaches as well as novel single-molecule techniques. Also, the amazing improvements in structural analysis of amyloid fibers that have been made in recent years, could be used to pinpoint Cu-binding sites in amyloids of aS variants and reveal if Cu-binding affects the resulting amyloid fold. It is also interesting to explore if the timing of Cu interaction (i.e., before, during, or after S amyloid formation) results in different binding sites and, if Cu-bound aS amyloids harbor catalytic activity (reported recently for amyloid-β amyloids [77]). With respect to possible functions, can aS interact with other Cu-transport or Cu-binding proteins (in addition to Atox1) in a Cu-dependent way? If so, is this related to synaptic signal transmission via Cu-filled synaptic vesicles? Clearly, future studies on this topic can constitute the careers of many scientists to come. And it should as we desperately need to find new ways to tackle PD and other disorders involving amyloids. The prevalence of these diseases is rapidly increasing due to population aging but we still lack cures.

Summary

- The protein aS, annotated as copper binding in UniProt, forms amyloids in Parkinson's disease.
- aS binds copper ions in *in vitro* and *in vivo* studies imply biological consequences of such interactions.

- Cu(II) accelerates aS amyloid formation *in vitro*, but N-terminal acetylation of aS (common modification in cells) abolishes this effect.
- Protein (Atox1)-mediated Cu(I) interaction blocks aS aggregation *in vitro*.
- Many more *in vivo* as well as *in vitro* biochemical studies are desired to reveal both the functional and dysfunctional significance of cross-reactivity between aS and Cu.

Competing Interests

The author declares that there are no competing interests associated with the manuscript.

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Abbreviations

aS, α-synuclein; CCS, copper chaperone for superoxide dismutase 1; Ctr1, copper transporter 1; Cu, Copper; GABA, gamma-aminobutyric acid; NMDA, N-methyl-D-aspartate; PD, Parkinson's disease; SOD1, superoxide dismutase 1; ThT , Thioflavin-T.

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Essays in Biochemistry (2022) **66** 977–986
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