Hierarchical binding of copper\textsuperscript{II} to N-truncated Aβ\textsubscript{4–16} peptide\textsuperscript{†}

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N-Truncated Aβ\textsubscript{4–42} displays a high binding affinity with Cu\textsuperscript{II}. A mechanistic scheme of the interactions between Aβ\textsubscript{4–42} and Cu\textsuperscript{II} has been proposed using a fluorescence approach. The timescales of different conversion steps were determined. This kinetic mechanism indicates the potential synaptic functions of Aβ\textsubscript{4–42} during neurotransmission.

The amyloid-β (Aβ) peptides associated with Alzheimer’s Disease (AD) comprise a number of species. The “canonical” Aβ\textsubscript{1–42} and Aβ\textsubscript{1–40} peptides derived directly by proteolysis of the Amyloid Precursor Protein (APP) are complemented by N- and C-truncated species, yielded by a variety of brain proteases.\textsuperscript{1} Among them, the N-truncated Aβ\textsubscript{4–42} has been reported as particularly abundant in the hippocampus and cortex of sporadic AD patients, as well as in healthy controls.\textsuperscript{2,3} even exceeding Aβ\textsubscript{1–42} and Aβ\textsubscript{1–40}.\textsuperscript{4,5} Aβ\textsubscript{1–x} peptides can bind Cu\textsuperscript{II} using the N-terminus and H6, H13, and His14 residues.\textsuperscript{6–8} Hence, Aβ\textsubscript{1–16} has been adopted as a common model peptide in metal binding studies.\textsuperscript{9–11} The adventitious binding of Cu\textsuperscript{II} ions to Aβ\textsubscript{4–16} and the concomitant generation of reactive oxygen species (ROS) via the Cu\textsuperscript{II}/Cu\textsuperscript{I} redox pair has been proposed to be the molecular basis of oxidative stress and neuronal death in AD.\textsuperscript{12} On the other hand, Aβ\textsubscript{1–x} peptides bind a Cu\textsuperscript{II} ion more than three orders of magnitude more strongly (K\textsubscript{D} = 30 fM and 6.6 fM at pH 7.4 for Aβ\textsubscript{1–16} and Aβ\textsubscript{1–40} respectively), using their N-terminal ATCUN motif spanning the Phe4, Arg5 and His6 residues. These complexes are redox-inert and do not generate significant ROS. Cu\textsuperscript{II} ion transfer from Aβ\textsubscript{1–16} to Aβ\textsubscript{4–16} occurs upon adding the latter to the Cu\textsuperscript{II}Aβ\textsubscript{1–16} solution.\textsuperscript{13}

Significance to metallomics

N-Truncated Aβ\textsubscript{4–16} is abundant in both healthy and AD brains. Its Cu\textsuperscript{II} binding affinity is three orders of magnitude stronger than well-known Aβ\textsubscript{1–16} or Aβ\textsubscript{1–40}. Using a model peptide, Aβ\textsubscript{4–16}, we have elucidated the reaction mechanism of Cu\textsuperscript{II} with Aβ\textsubscript{1–16}, crucial to understand the physiological role and toxicity of Aβ peptides. The presence of two kinetic intermediates prior to the formation of the tight ATCUN complex has implications for the potential function of Aβ\textsubscript{4–42} as a Cu\textsuperscript{II} transporter during neurotransmission. The methodology used in this work may also stimulate the research of Cu\textsuperscript{II} interactions with other intrinsically disordered proteins (IDPs).

This reaction is quantitative, in agreement with the affinity difference, and fast, occurring within the sample preparation time ~s. Such a reaction suggested that Aβ\textsubscript{4–42} should prevail as a Cu\textsuperscript{II} binding Aβ species in the extracellular spaces of the brain. This finding gave rise to a hypothesis that Aβ\textsubscript{4–42} may have a physiological role as a synaptic Cu\textsuperscript{II} scavenger during neurotransmission.\textsuperscript{14} However, Cu\textsuperscript{II} release events in glutamatergic synapses may occur on a much faster, millisecond scale. Therefore, a thorough determination of association and dissociation rate constants for the participating species is necessary to help evaluate their relevance \textit{in vivo}. Such data have been obtained previously for Cu\textsuperscript{II}Aβ\textsubscript{1–x} complexes.\textsuperscript{15–17} Here, we studied the reaction mechanism for Cu\textsuperscript{II} binding to the model peptide Aβ\textsubscript{4–16} and found that the reaction follows a hierarchical fashion, going through two intermediate states and then reaching the final tight complex.

First, we studied the effect of N-truncation on the Cu\textsuperscript{II} binding kinetics. 20 nM Aβ labelled by HiLyte Fluor 488 on lysine 16 (FRHDSGYEVHHQK-HiLyte 488) was reacted with 400 nM Cu\textsuperscript{II} under various HEPES concentrations in order to obtain the HEPES-independent Cu\textsuperscript{II} binding rate constant (k\textsubscript{on}). The results are shown in Fig. 1a. The intercept of the fitted curve (Fig. 1b) was used to determine k\textsubscript{on}, which is $2.0(1) \times 10^{7}$ M\textsuperscript{-1} s\textsuperscript{-1}, 2.5 times slower than the value for Aβ\textsubscript{1–16}.\textsuperscript{17} k\textsubscript{off} was determined for the reaction of a Cu\textsuperscript{II} complex of unlabelled Aβ\textsubscript{4–16} with an excess of EDTA. The estimated value...
is $\sim 5 \times 10^{-5}$ s$^{-1}$, which divided by $k_{on}$ proposed here gives $K_d \sim 250$ FM. EDTA is a stronger Cu$^{II}$ chelator than A$\beta_{4-16}$, with a log $\beta$ of 18.7, which can be recalculated into a conditional constant $C_K$ of 16.0 at pH 7.5. $^{18}$ This value is sufficiently higher than that of Cu$^{II}$A$\beta_{4-16}$, 13.53, to assure full Cu$^{II}$ transfer, as demonstrated in Fig. 1c. The reaction was carried out for a range of EDTA/peptide ratios between 2 and 120. Pseudo-1st order kinetics for the Cu$^{II}$ transfer reaction was observed for all experiments. The non-linear response of $k_{off}$ to EDTA required the EDTA-independent $k_{off}$ value to be determined by the extrapolation of the empirical exponential fit to these data, as shown in Fig. 1d.

To gain a glimpse of a possible reaction mechanism of Cu$^{II}$ binding to N-truncated A$\beta_{4-16}$, we performed binding experiments at a 1:1 mixing ratio of A$\beta$ to Cu$^{II}$ with increasing concentration. In such experiments, the effect of the second Cu$^{II}$ shown in Fig. 1d. extrapolation of the empirical exponential fit to these data, as demonstrated in Fig. 1c. The reaction was carried out for a range of EDTA/peptide ratios between 2 and 120. Pseudo-1st order kinetics for the Cu$^{II}$ transfer reaction was observed for all experiments. The non-linear response of $k_{off}$ to EDTA required the EDTA-independent $k_{off}$ value to be determined by the extrapolation of the empirical exponential fit to these data, as shown in Fig. 1d.

Next, a double mixing stopped flow technique was employed to further explore the potential intermediate complexes formed after the initial Cu$^{II}$ binding. This technique was successfully applied to probe the interconversion between component I and component II Cu$^{II}$ coordination species of A$\beta_{1-16}$ and A$\beta_{1-40}$. $^{17}$ 2 $\mu$M A$\beta_{4-16}$ and 2 $\mu$M Cu$^{II}$ were mixed in a delay loop and after various delay times the reaction was “frozen” by adding an excess of EDTA (Fig. 2b). Taking advantage of the disparities in reactivity of different Cu$^{II}$A$\beta_{4-16}$ species with EDTA, the time evolution of the population of individual species could be resolved and analyzed, enabling us to depict details of the binding process.

As shown in Fig. 2b, the amplitude of fluorescence recovery strongly depends on the delay time, indicating that a much more inert (less reactive towards EDTA) complex (‘‘dark’’ complex) formed after around 2 s. We equate this end complex, (A$\beta$–Cu$^{III}$), to the very stable ATCUN-type Cu$^{II}$A$\beta_{4-16}$ complex reported previously. $^{13}$ Furthermore, because the reaction rate is concentration independent after 2 s as mentioned above, we propose that a peptide conformational rearrangement process leading to this final complex must occur at around 2 s.

In order to describe the whole process of Cu$^{II}$ binding of N-truncated A$\beta_{4-16}$, we hypothesized a reaction scheme as shown in Fig. 3a. The individual amplitudes of the two phases in Fig. 2b were determined by a global fit, which were further fitted by the scheme with KinTek software to validate it (Fig. 3b). The amplitudes indicate the amounts of two intermediates, Species I and Species II, at different reaction process stages, and could be
fitted well by the predicted mechanism, with fitted rate con-
stants listed in Table 1. A corresponding free energy landscape
illustration of CuII binding with Aβ4–16 is shown in Fig. 3c.

Finally, the activation energy of the (Aβ–Cu)D complex was
determined to be 64(3) kJ mol⁻¹ (Fig. 4) by performing a series
of double mixing experiments at different temperatures (raw
data shown in Fig. S1, ESI†).

The chemical properties of ATCUN CuII complexes of Aβ4–x
peptides, such as high thermodynamic stability, absence of ROS
production due to their resistance to oxidation and reduction,
redundancy of copper to transfer to metallothionein-3 (MT3) and
easy sequestering of CuII from Aβ4–16, give rise to a concept that
Aβ4–x peptides (long-length Aβ4–42 and its C-truncated analogs)
may serve as guardians of synaptic function, by sequestering
excess CuII ions released during neurotransmission in glumatometric
pathways.14,19 The key unsolved issue is how these exchange-inert
complexes relay copper back to neurons to maintain the proper
copper cycling. Furthermore, CuII-free Aβ4–42 can be neurotoxic
by forming oligomeric species.20 Detailed knowledge on mechanisms of CuII
association with and dissociation from Aβ4–x peptides, represented here by Aβ4–16,
is thus crucial to understand the physiology and toxicity of these Aβ peptides.

The discovery of long-lived kinetic intermediates in the
formation of the ATCUN complex of Aβ4–16 is a game changer
in the above considerations. The lifetimes of Species I and
Species II complexes are comparable to the intervals between
pulses of neurotransmitter release in glutamatergic neuronal
pathways.21 Therefore, these complexes may well contribute to
the biological activity of Aβ4–42, and of putative short peptide
fragments generated by neprilysin cleavage, such as Aβ4–9.22,23

There is only one way in which four nitrogen ligands of the
ATCUN motif can be arranged around the CuII ion, and so it is
reasonable to assume that the intermediate species contain the
coordinatively unsaturated CuII. Such species have been implicated
in the reverse reaction of CuII dissociative transfer from
CuIIAβ4–16 to MT3, to explain the catalytic effect of glutamate,24
but it has not been observed directly. The Species I and in
particular the longer-lived Species II complex may be the actual
species able to move copper around during neurotransmission.
The fact that the CuIIAβ4–x complex, although so much weaker,
was formed 2.5 times faster, prompts further research into possible
synaptic roles of CuII interactions with various Aβ species.

Furthermore, the observed hierarchical binding of CuII to
Aβ4–16 resembles the kinetics of the binding of many intrinsically
disordered proteins (IDPs).25 The methodology used in this study
may be applicable to the fundamental understanding of the
emerging “coupled binding and folding” paradigm.26

Conflicts of interest
There are no conflicts to declare.

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