Amyloid β Peptides

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Introduction of a Fluorescent Probe to Amyloid-β to Reveal Kinetic Insights into Its Interactions with Copper(II)**

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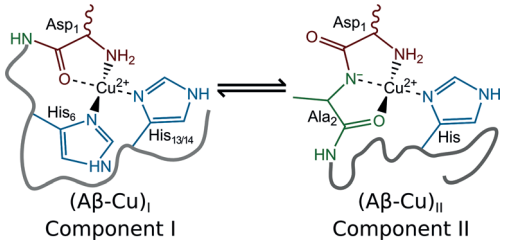
Abstract: *The kinetics of the interactions between amyloid-β (Aβ) and metal ions are crucial to understanding the physiological and pathological roles of Aβ in the normal brain and in Alzheimer's disease. Using the quenching of a fluorescent probe by Cu^{2+}, the mechanism of Aβ/Cu^{2+} interactions in physiologically relevant conditions has been elucidated. Cu^{2+} binds to Aβ at a near diffusion-limited rate, initially forming component I. The switching between component I and II occurs on the second timescale, with a significant energy barrier. Component I is much more reactive towards Cu^{2+} ligands and likely responsible for initial Aβ dimer formation. Clioquinol (CQ) is shown to sequester Cu^{2+} more effectively than other tested ligands. These findings have implications for the potential roles of Aβ in regulating neurotransmission, and for the screening of small molecules targeting Aβ–metal interactions.*

Amyloid plaques in the brain are one of the hallmarks of Alzheimer's disease (AD).[1] Amyloid-β (Aβ) encounters Zn^{2+} and Cu^{2+} during synaptic transmission, where metal ions promote Aβ aggregation,[2] and it has been proposed as a crucial step in the amyloid cascade.[3] Aβ oligomers are mostly responsible for the toxicity.[4] Preventing Aβ–metal interactions to inhibit oligomer formation has been proposed as a disease-modifying strategy for AD. Small molecules that serve this purpose are termed metal–protein attenuating compounds (MPACs). PBT2, a CQ derivative, is currently in phase II trials.[2b,5,6]

Aβ may play a role in synaptic regulation.[7] Owing to the transient nature of the release of neurotransmitters and neurometals, regulatory roles are likely controlled by its kinetics.[8] Glutamatergic neurotransmission operates on timescales of milliseconds to seconds, and the response of the receptor is in part modulated by Cu^{2+}/Zn^{2+}.[9,10] However, whether the kinetics of Aβ–metal interactions allow it to impact the modulation of receptor function is unknown.

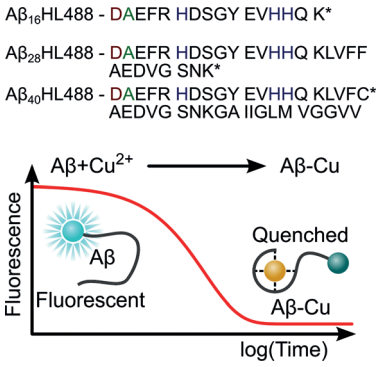
Aβ/Cu^{2+} binding in equilibrium has been studied extensively.[11] EPR has revealed two primary coordination modes with human Aβ (hAβ), termed component I and II (Scheme 1), which coexist under physiological pH and differ in protonation state.[12,13] NMR spectroscopy suggested that these states are highly dynamic and in fast equilibrium.[14]



Scheme 1. Cu^{2+} coordination to Aβ at physiological pH.

To uncover the role of Aβ–metal interactions in oligomer formation and reactive oxygen species generation,[15] a quantitative understanding of the kinetic processes underlying these interactions is essential. Chemical kinetics has provided insights into amyloid fibril assembly and inhibition.[16] However, kinetic studies of Aβ–metal interactions have been hindered by the low sensitivity of the methods.[17] The coexistence of two major Aβ–Cu components further complicates the identification of the kinetic mechanism which is crucial for rational drug design.

Herein we introduce a bright probe onto Aβ peptides, which quenches when Cu^{2+} binds (Scheme 2). This improves



Scheme 2. Labeled Aβ peptides, and the concept of measuring binding kinetics from fluorescence quenching. Residues involved in Cu^{2+} coordination are colored. HiLyte 488 attachment is denoted by a ⋆.

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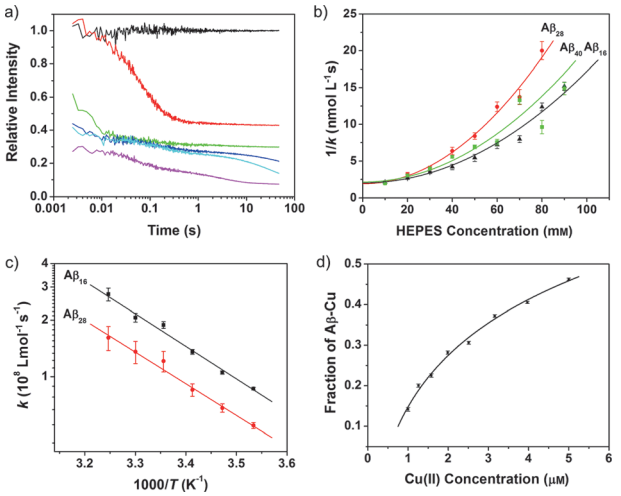


Figure 1. Kinetics of Cu^{2+} binding to Aβ peptides. a) Reaction time traces of 10 nM $Aβ_{16}$ with Cu^{2+} (in μM) from top to bottom lines: 0, 0.25, 1, 5, 20, 80. b) HEPES dependence of the k_{on}. The HEPES independent k_{on} values were $5.0(2)\times10^8$, $5.3(7)\times10^8$, and $5.4(6)\times10^8$ $L mol^{-1} s^{-1}$ for $Aβ_{16}$, $Aβ_{28}$, and $Aβ_{40}$. c) Arrhenius plots for the binding of Cu^{2+} with $Aβ_{16}$ and $Aβ_{28}$. The activation energies determined were 33(1) kJ mol^{-1} and 32(3) kJ mol^{-1}, with 50 mM HEPES. d) Competition for Cu^{2+} between 100 nM $Aβ_{16}$ and 5 μM HSA. Maximal fractions of Cu^{2+} bound $Aβ_{16}$ as a function of Cu^{2+} concentration are shown.

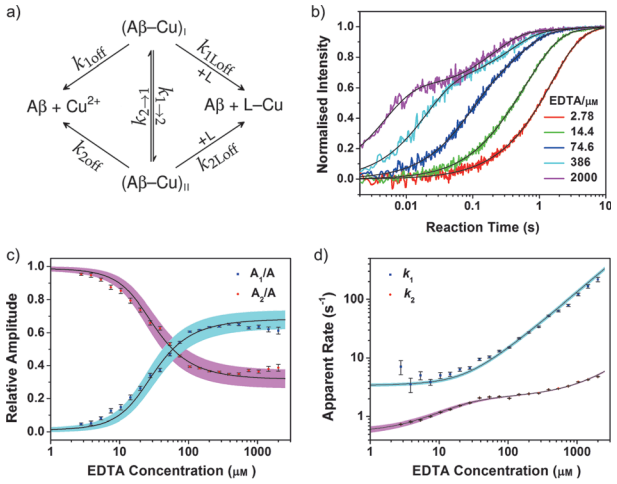


Figure 2. Kinetics of interconversion between the two components of $hAβ_{16}$–Cu as probed by their reactions with EDTA. a) Kinetic scheme proposed for the reaction. b) Traces showing the existence of two ligand dependent phases, with multi-exponential fits. c) and d) The relative amplitudes and the apparent rates of the two phases obtained from the raw data. The solid lines are the fits of the reaction scheme. Error boundaries are shaded. The complexes were prepared at 1:1 stoichiometry (100 nM) and mixed in equal volume with EDTA (4 μM to 4 mM).

the detection sensitivity by about 10^3 over its tyrosine fluorescence,[17] enabling measurements to be carried out at physiologically relevant nM Aβ.

Figure 1 a shows stopped-flow kinetic traces of Aβ binding to Cu^{2+}. The fastest phase, corresponding to the first Cu^{2+} binding, can only be measured when Cu^{2+} is less than 1 μM. Figure 1 b shows the association rate constants (k_{on}) obtained at different HEPES concentrations. The HEPES-independent k_{on} value is about 5×10^8 $L mol^{-1} s^{-1}$ for all three hAβ peptides, showing that binding is governed by the N-terminus. Both murine $Aβ_{16}$ (mAβ) and $hAβ_{40}$ on GM1 micelles have similar rates (Supporting Information, Figure S1). These k_{on} values are close to the limit for diffusion-controlled reactions (10^9 $L mol^{-1} s^{-1}$). The activation energy of the process was determined (Figure 1 c). They are low at about 30 kJ mol^{-1} for both $Aβ_{16}$ and $Aβ_{28}$. These results predict that Cu^{2+} binding in the synapse would finish within about 1 ms and that Aβ could kinetically out-compete Cu^{2+} ligands during neurotransmission. To confirm this, we measured the effect of HSA on the kinetics. We found that Aβ can compete with HSA for Cu^{2+} binding, but HSA can sequester Cu^{2+} from Aβ at longer timescales (more than 100 ms) (Figure 1 d; Supporting Information and Figure S2 therein).

To determine the interconversion kinetics between the two Aβ–Cu coordination modes, we used the difference in reactivity with EDTA to separate the two species (Figure 2 and Supporting Information). The observed traces were fitted by exponentials (Figure 2 b) as predicted by the reaction scheme (Figure 2 a). The amplitudes and rates of the two phases (Figure 2 c,d) were then fitted to the model to give the parameters shown in the Supporting Information, Table S1. The model predicts that at high EDTA concentrations, when the EDTA reactions are much faster than the interconversion, the relative amplitudes of the two phases represent the proportion of two species at equilibrium. Indeed, the ratios between component I/II are 0.63:0.37 and 0.71:0.29 for $Aβ_{40}$–Cu and $Aβ_{16}$–Cu, in good agreement with EPR measurements.[19] The dissociation rate of component I is about 1 s^{-1}. In contrast, component II is more stable and the rate is too slow to be determined. The reaction rate constant of component I with EDTA is 100 times faster than that of component II. The difference in reactivity and the slow dissociation of component II implies that component II prolongs the lifetime of the Aβ–Cu^{2+} complex, and that Aβ dimerization proceeds by component I. $hAβ_{40}$–Cu as well as GM1 micelle-associated $hAβ_{40}$–Cu are similar (Supporting Information and Figure S3 therein).

The apparent K_d was derived to be 1.1(1) nmol L^{-1} and 1.3(2) nmol L^{-1} for $hAβ_{16}$ and $hAβ_{40}$, but 0.38(3) nmol L^{-1} for $mAβ_{16}$, in broad agreement with previous reports.[20,21] The association timescales (ca. 0.2 ms) in the presence of the physiological 10 μM Cu^{2+} and the dissociation (ca. 1 s) of the Aβ–Cu complex fall within the time response of glutamate receptors in neurotransmission (ca. 0.1 ms to ca. 10 s).[9]

To assess which species forms initially and confirm the interconversion rate, we used double-mixing stopped flow. The temporal evolution of the populations shows that component I forms first, converting into component II (Figure 3 a–c). The sum of the interconversion rate constants ($k_{1\rightarrow2}+k_{2\rightarrow1}$) at 298 K is 3.5(1) s^{-1} for $hAβ_{16}$, which is consistent with the values in the Supporting Information, Table S1. For mAβ, the interconversion is faster, namely 7.3(2) s^{-1}. Although component II is dominant at equilibrium for mAβ, binding is still by component I. To ensure that populations of the two species are pH-dependent, the same

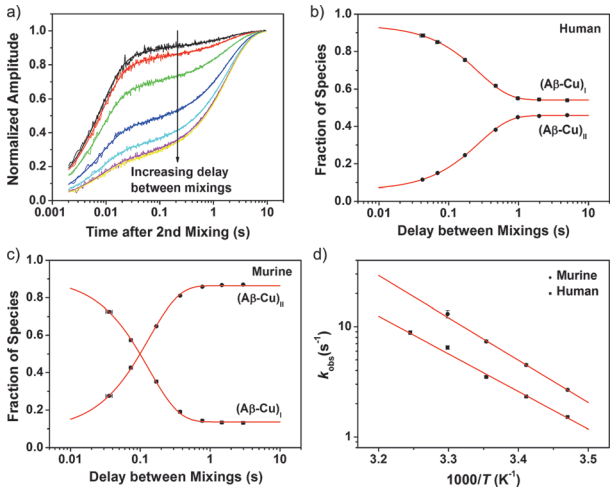


Figure 3. Kinetics of Aβ/Cu interactions probed by double mixing stopped flow. 100 nM $A\beta_{16}$ was first mixed with 2 μM Cu^{2+} (1:1 volume) and then after a variable delay, with 400 μM EDTA (1:1 volume). a) Kinetic traces of mAβ at 298 K, pH 7.5. b) and c) Time-dependent evolutions of the fractions of the two components of hAβ–Cu and mAβ–Cu after formation. The relaxation rate constants ($k_{1\rightarrow 2}+k_{2\rightarrow 1}$) determined are 3.5(1) s^{-1} for hAβ–Cu and 7.3(2) s^{-1} for mAβ–Cu. d) Arrhenius plot to determine the switching activation energies: 65(3) kJ mol^{-1} for hAβ–Cu, and 73(2) kJ mol^{-1} for mAβ–Cu.

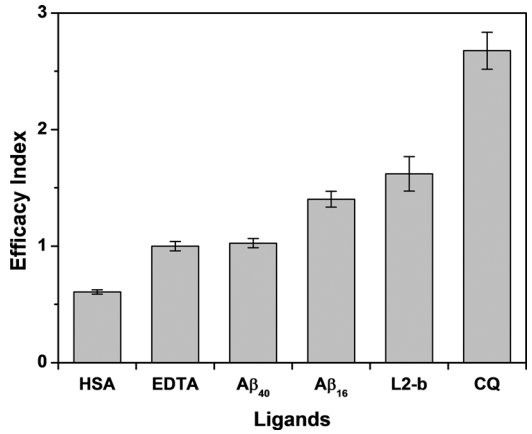
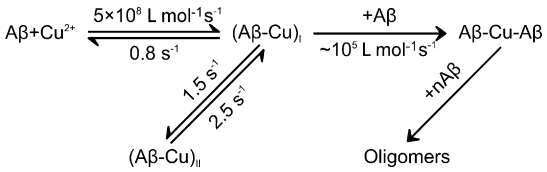


Figure 4. Efficacy index of the ligands: the rates of Cu^{2+} sequestration relative to that of EDTA at 10 μM ligand concentration.



Scheme 3. Mechanism and kinetics of Aβ/Cu^{2+} interactions.

experiment was repeated for $hA\beta_{16}$ at pH 8.0, where component II population increased (Supporting Information, Figure S4). Our observations agree with the pK_a values of 7.7 and 6.2 reported for the hAβ and mAβ.[21]

The activation energies of the coordination switching were determined to be 65(3) kJ mol^{-1} for $hA\beta_{16}$–Cu, and 73(2) kJ mol^{-1} for $mA\beta_{16}$–Cu (Figure 3d). These sizeable values may be due to the difficulty in deprotonating the amide bond (Scheme 1 and Supporting Information).

Finally we investigated the efficiency of Cu^{2+} extraction from Aβ–Cu by various ligands. L2-b is a bifunctional ligand designed to target both Aβ and metal ions.[22] To quantify the relative efficiency, we introduce an efficacy index, defined as the reaction rate at 10 μM ligand concentration relative to that of EDTA. The efficacy index for CQ shows it more rapidly removes Cu^{2+} from Aβ (Figure 4; Supporting Information and Table S2 therein). The efficacy index is not correlated with their K_d values. We hope that this index will provide a kinetics-based tool for the screening and refinement of MPACs.

The rate constant of the reaction of Aβ–Cu with unlabeled Aβ (ca. 10^5 L mol^{-1} s^{-1}) provides an estimate for Cu^{2+}-induced Aβ dimer formation. Metal-free Aβ dimerization rate constant is more than two orders of magnitude slower circa 10^2–10^3 L mol^{-1} s^{-1}.[23] Thus dimers can form considerably faster with neurometals. The reaction of mAβ–Cu with Aβ is much slower, namely about 10^4 L mol^{-1} s^{-1} (Supporting Information, Figure S5), despite mAβ binding to Cu^{2+} more strongly. This may explain kinetically why mice do not naturally develop AD.

Scheme 3 summarizes the kinetic mechanism of Aβ interactions with Cu^{2+}. Given the fast binding of Aβ to Cu^{2+}, the affinity of its C-terminal sequence to membranes,[18] and the reaction rates of ligands with Aβ–Cu, we propose that its function may be to capture Cu^{2+} and transport it on the membrane to receptors, such as prion protein or NMDA receptors (see further discussion in Supporting Information).

In conclusion, we have determined the kinetic mechanism of Aβ with Cu^{2+}, and found that kinetics is crucial for Aβ to interact with neurometals on the timescales of neurotransmission. We have also discovered that component II forms an inert reservoir of monomeric Aβ, which must revert to component I before forming oligomeric species. Our study suggests that on the short timescales relevant to neurotransmission, Cu^{2+} transport via Aβ may be governed kinetically. This kinetic approach can be applied to the screening of MPACs targeting Aβ–metal interactions.[24] This study demonstrates the potential of kinetic studies to find the molecular interactions of Aβ–metal and oligomerization, a critical step to the full understanding of Aβ toxicity. The methodology may also be applied to other Cu^{2+} binding proteins, such as PrP^C and α-synuclein.

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