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Affinity of Cu^{+} for the Copper-Binding Domain of the Amyloid-β Peptide of Alzheimer's Disease

Heather A. Feaga, Richard C. Maduka, Monique N. Foster, and Veronika A. Szalai*

Department of Chemistry & Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250, United States

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The role of metal ions in Alzheimer's disease etiology is unresolved. For the redox-active metal ions iron and copper, the formation of reactive oxygen species by metal amyloid complexes has been proposed to contribute to Alzheimer's disease neurodegeneration. For copper, reactive oxygen species are generated by copper redox cycling between its 1+ and 2+ oxidation states. Thus, the AβCu^{I} complex is potentially a critical reactant associated with Alzheimer's disease etiology. Through competitive chelation, we have measured the affinity of the soluble copper-binding domain of the amyloid-β peptide for Cu^{I}. The dissociation constants are in the femtomolar range for both wild-type and histidine-to-alanine mutants. These results indicate that Cu^{I} binds more tightly to monomeric amyloid-β than Cu^{II} does, which leads us to propose that Cu^{I} is a relevant in vivo oxidation state.

Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that afflicts one in five people over the age of 85. The number of Alzheimer's victims is expected to double over the next 20 years.[1] Extracellular plaques containing the amyloid-β (Aβ) peptide are the hallmark of this disease upon post-mortem examination of brain tissue. Soluble oligomers of Aβ, rather than Aβ fibrils in senile plaques, correlate most strongly with the degree of dementia.[2,3] Aβ plaques contain redox-active metal cations including copper, which has been found at a concentration of 400 μM.[4]

The role of metal ions in AD etiology is unresolved, in part, because conflicting effects of metal ions have been reported. Increased[5−8] or decreased[9,10] toxicity of Aβ has been observed in the presence of transition-metal cations. For the redox-active metal ions iron and copper, the formation of reactive oxygen species (ROS) by metal amyloid complexes has been proposed to contribute to AD neurodegeneration.[6−8,11−15] Recent work indicates that Aβ quenches hydroxyl radicals produced through Fenton chemistry involving iron and copper ions, thereby serving to protect cells from ROS-mediated damage.[16,17] We have previously shown that the monomeric, soluble form of the Aβ peptide binds Cu^{+} in a linear bis-His geometry that is kinetically slow to

*To whom correspondence should be addressed. E-mail: veronika.szalai@nist.gov.

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react with oxygen to form ROS.[18] Such two-coordinate Cu^+ complexes are thermodynamically stable.[19] A remaining question is the affinity of Aβ for copper in both oxidation states. Affinities of monomeric Aβ for Cu^{2+} range widely in the literature, but a recent review indicates that the conditional association constant range is 0.01−1.0 nM, with a proclivity for the higher end of the range.[20] Through competitive chelation experiments, we have measured the affinity of the copper-binding domain of the Aβ peptide for Cu^+. Our interpretation of these data is that Cu^+ should be considered the relevant in vivo oxidation state.

Methods and Materials

Materials. The Aβ16 peptide was a kind gift from Dr. Jason Shearer, University of Nevada—Reno. The peptide was synthesized on a Protein Technologies PS3 peptide synthesizer using Fmoc solid-phase synthesis chemistry, purified by high-performance liquid chromatography, and stored at −80 °C. The amino acid sequence of Aβ16 is DAEFRHDSGYEVHHQK. The Aβ16 mutants H6A, H13A, and H14A were purchased from EZBiolab (Carmel, IN). Mass spectra of all of the peptides are provided in Figure S1 in the Supporting Information. Disodium bathocuproinedisulfonic acid (BC) and disodium bicinchoninic acid (BCA), Cu^+ chelators, were purchased from Acros Organics (Morris Plains, NJ). A Cu^{2+} solution was prepared by dissolving clean copper wire in nitric acid and water as described previously.[21] Sodium ascorbate (asc) was purchased from Spectrum Quality Products, Inc. (New Brunswick, NJ).

Sample Preparation. A solution of the Aβ16 peptide (wild-type or mutant peptide) was prepared in water and stored at −20 °C. The peptide concentration of this solution was ascertained on the basis of the absorbance at 214 nm in comparison with a bovine serum albumin (BSA) calibration curve.

After complex formation with either Aβ or a competitive chelator, Cu^{2+} was reduced with ascorbate to Cu^+ on the basis of the colorimetric assay. Copper needs to be ligated to the peptide prior to reduction; attempts to directly metalate Aβ using $[Cu(CH_3CN)_4]PF_6$ have been unsuccessful. We believe this is because Cu^+ binding is slow, which means that Cu^+ disproportionates before the peptide can be fully loaded with copper for the titration experiments. All samples were deoxygenated with nitrogen gas and sealed in 1 cm quartz cuvettes (Starna). The absorption titrations were monitored with a Jasco V-560 UV/vis spectrophotometer. In all forward titrations, the concentration of $[Cu(BC)_2]^{3-}$ in each sample solution was quantified colorimetrically using the absorbance at 483 nm (ε = 13 300 M^{-1} cm^{-1}).[22] Stock solutions of BC (5.0 mM), BCA (5.4 mM), and asc (34.9 mM) were prepared by weighing the appropriate amount of desired solid on an analytical balance followed by dissolution in Milli-Q water (resistivity of >18 mΩ-cm; total organic content of <33 ppb).

Determination of the Cu^+Aβ16 Dissociation Constant. The determination of binding constants of Cu^+ to metalloproteins through competitive chelation has been outlined in the literature.[22] Cu^+ binds strongly to BC to form a stable, red-orange complex, $Cu(BC)_2^{3-}$, with a formation constant, β_2, of $10^{19.8}$.[22]

$$Cu^+ + 2BC^{2-} \leftrightarrow Cu(BC)_2^{3-}$$

The total concentration of $Cu(BC)_2^{3-}$ is determined by the absorbance at 483 nm. The concentrations of other species involved in this equilibrium are as follows:

$$[Cu]_{free} = \frac{[Cu(BC)_2^{3-}]}{([BC]_{total} - 2[Cu(BC)_2^{3-}])^2\beta_2}$$

Upon the addition of Aβ16, the following equilibrium is established:

$$Cu(BC)_2^{3-} + A\beta16 \leftrightarrow 2BC^{2-} + Cu^+A\beta16$$

Previously, we have shown a 1:1 binding ratio between Cu^+ and Aβ16.[18] The change in the absorbance at 483 nm provides the concentration of $Cu(BC)_2^{3-}$ because the Cu^+Aβ16 species does not absorb at this wavelength. By mass balance, it is clear that the following must be true:

$$[CuA\beta16] = [Cu]_{total} - [Cu(BC)_2^{3-}] - [Cu]_{free}$$

The fraction of Aβ16 bound to Cu^+, θ, is

$$\theta = \frac{[Cu^+A\beta16]}{[A\beta16]_{total}} \quad (1)$$

θ values of 0.5 are ideal for the accurate determination of binding constants. Small values of θ will give minimum dissociation constants.[23]

The dissociation constant for Cu^+Aβ16 is then

$$K_d = \left\{\frac{1-\theta}{\theta}\right\}[Cu]_{free} \quad (2)$$

In addition to analysis of titrations using an Excel spreadsheet and the equations above, titrations were analyzed with *SpecFit/32*, a program that calculates unknown binding constants given a model of the titration type and known binding constants. The basis spectrum of the colored $Cu(BC)_2^{3-}$ complex is also an input. Both 1:1 and 1:2 Cu/Aβ models were used to fit the data.

Multiple, reproducible trials of the competitive chelation between BC and Aβ16 for Cu^+ were performed. Prior to the addition of Aβ16, the sample cuvette contained [BC] = 60 μM, [Cu] = 25 μM, and [asc] = 1 mM in an aqueous solution. The reference cuvette contained [BC] = 60 μM and [asc] = 1 mM. Spectra were collected every 10 min until two consecutive spectra were identical, indicating equilibration of the system. To the sample were added 2.00 μL additions of 10.75 mM Aβ16. The sample was bubbled with nitrogen and allowed to equilibrate between additions until two consecutive spectra were identical. Equilibration generally required at least 30 min. The total Aβ16 concentration was brought to 95 μM by the end of each titration. Table S1 in the Supporting Information contains a representative data set; Figure S2 in the Supporting Information shows the binding isotherm.

The titration also was performed in the reverse direction, starting with Cu^+ bound to the peptide. A sample cuvette containing 98 μM Aβ16, 25 μM Cu, and 1.5 mM asc under nitrogen was allowed to equilibrate for 3 h. BC was then titrated into the sample and reference cuvettes. Another experiment was conducted to compete Aβ16 with BCA for Cu^+ (Figure S3 in the Supporting Information). To confirm that conducting the competitive chelation experiments in water[22−24] does not affect the magnitude of log(β), we also conducted a matched pair of reverse competitive chelation titrations in water and in a 20 mM

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Table 1. K_d Values from Forward $[Cu(BC)_2]^{3-} + A\beta16$] and Reverse ($Cu^+A\beta16 +$ BC) Titrations

titration type	$K_d \pm$ std dev (M)
forward[a]	$1.48 \pm 2.13 \times 10^{-15}$
forward[b]	$1.56 \pm 2.16 \times 10^{-15}$
forward[c]	$1.82 \pm 0.46 \times 10^{-15}$
reverse[c]	$2.74 \pm 2.10 \times 10^{-15}$

[a] K_d calculated using equations in the text applied to all points in the titration. [b] K_d calculated using equations in the text applied to cases where $\theta > 0.2$. [c] K_d calculated from log(β) generated by *SpecFit/32* analysis of the titration data.

Table 2. K_d Values for $Cu^+A\beta16$ Histidine Mutants

mutant	avg $K_d \pm$ std dev (M)
H6A	$8.91 \pm 3.65 \times 10^{-15}$
H13A	$11.1 \pm 7.18 \times 10^{-15}$
H14A	$10.4 \pm 7.73 \times 10^{-15}$

N-ethylmorpholine buffer solution at pH 7.0. The log(β) value obtained in a buffer solution (13.6 ± 0.4; K_d range = 10−63 fM) is a similar order of magnitude is that obtained for a matched titration conducted in water (14.3 ± 0.2; K_d range = 3−8 fM).

For forward titration data analyzed using equations outlined in the text, the error is reported as the standard deviation of the K_d values calculated at each point in the titration. For data sets analyzed using *SpecFit/32*, we report absolute K_d errors calculated from the relative error for each titration. The relative error was determined by propagation of the log(β) error provided by *SpecFit/32*. Note that this propagates the error from the titration data points all the way through to the K_d values. Averaging individual K_d values, obtained directly by converting log(β) values, and then reporting the error as the standard deviation from the average K_d give smaller errors (Table S2 and Figure S4 in the Supporting Information), but this method is not as analytically rigorous as that we used to calculate the errors listed in Tables 1 and 2.

Results

Competition with Bathocuproinedisulfonic Acid. A method of competing ligands was used to determine the binding affinity between Cu^+ and Aβ16. Successive additions of Aβ16 decreased the absorbance of the $Cu(BC)_2^{3-}$ complex, indicative of a transfer of Cu^+ from BC to Aβ16 (Figure 1A). Conversely, successive additions of BC to the $Cu^+A\beta16$ complex displayed increasing absorbance because of formation of the $Cu(BC)_2^{3-}$ complex (Figure 1B).

From the forward titration data in Figure 1A, the average K_d calculated on the basis of eq 2 and all points in the titration is $1.48 \pm 2.13 \times 10^{-15}$ M (Tables 1 and S1 in the Supporting Information). When only additions with the highest fraction bound ($\theta > 0.2$) are averaged, K_d is $1.56 \pm 2.16 \times 10^{-15}$ M. K_d from *SpecFit/32* for these same data is $1.82 \pm 0.46 \times 10^{-15}$ M.

Reverse titrations using wild-type peptide yielded $2.74 \pm 2.10 \times 10^{-15}$ M for K_d when analyzed with *SpecFit/32*. Inclusion of a 1:1 $[Cu(BC)]^-$ species was required to obtain satisfactory fits. Although the equilibrium constant for $[Cu(BC)]^-$ is not known, changing its value in *SpecFit* did not change K_d of $Cu^+A\beta16$. This same observation was made for competitive chelation titration data for Cu^+ binding to other cuproproteins analyzed using *SpecFit*.[25]

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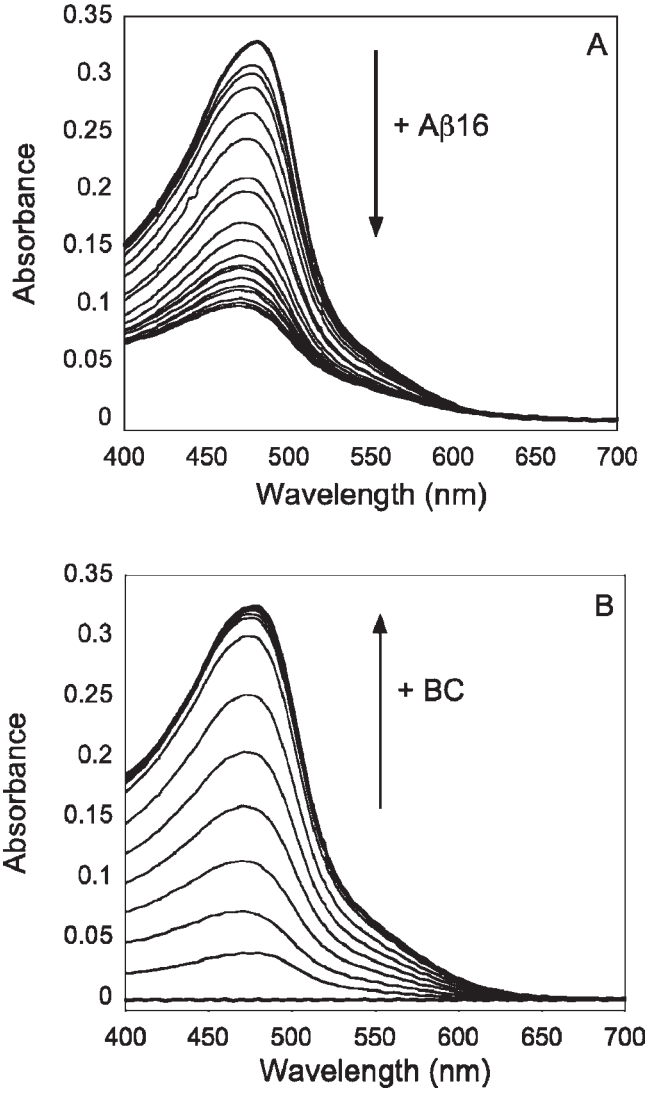

Figure 1. Competitive chelation of BC and Aβ16 for Cu^+. (A) Forward titration: addition of Aβ16 to the $[Cu(BC)_2]^{3-}$ complex. (B) Reverse titration: addition of BC to the $Cu^+A\beta16$ complex.

For the mutant peptides Aβ16H6A, Aβ16H13A, and Aβ16H14A, the K_d values range from 8.9 to 11.1×10^{-15} M as determined by *SpecFit/32* fitting of reverse titrations (Table 2 and Figure 2).

Applying a 1:2 Cu/Aβ model in *SpecFit* to the wild-type data sets produced an average log(β) of 19.0 ± 0.2, which is higher than that for the 1:1 model. The errors on the fits do not improve, however, which suggests that application of a 1:2 model, in our view, is not justified.

Competition with Bicinchoninic Acid. Given that our measured dissociation constant for $Cu^+A\beta16$ is in the femtomolar range, BCA should be a better competitive chelator than BC because K_d of the $Cu(BCA)_2^{3-}$ complex is 2.16×10^{-15} M^{-2} (measured in a HEPES buffer).[25] Such a similarity in the dissociation constants makes for ideal competition. The $Cu(BCA)_2^{3-}$ complex has a peak absorbance at 562 nm ($\varepsilon = 7700$ M^{-1} cm^{-1}).[24]

The addition of Aβ16 to a cuvette containing $Cu(BCA)_2^{3-}$ did not decrease the $Cu(BCA)_2^{3-}$ absorption spectrum significantly. An additional experiment indicated that, as reported by another group, ascorbate is not a strong enough reductant to reduce all Cu^{2+} to Cu^+ in

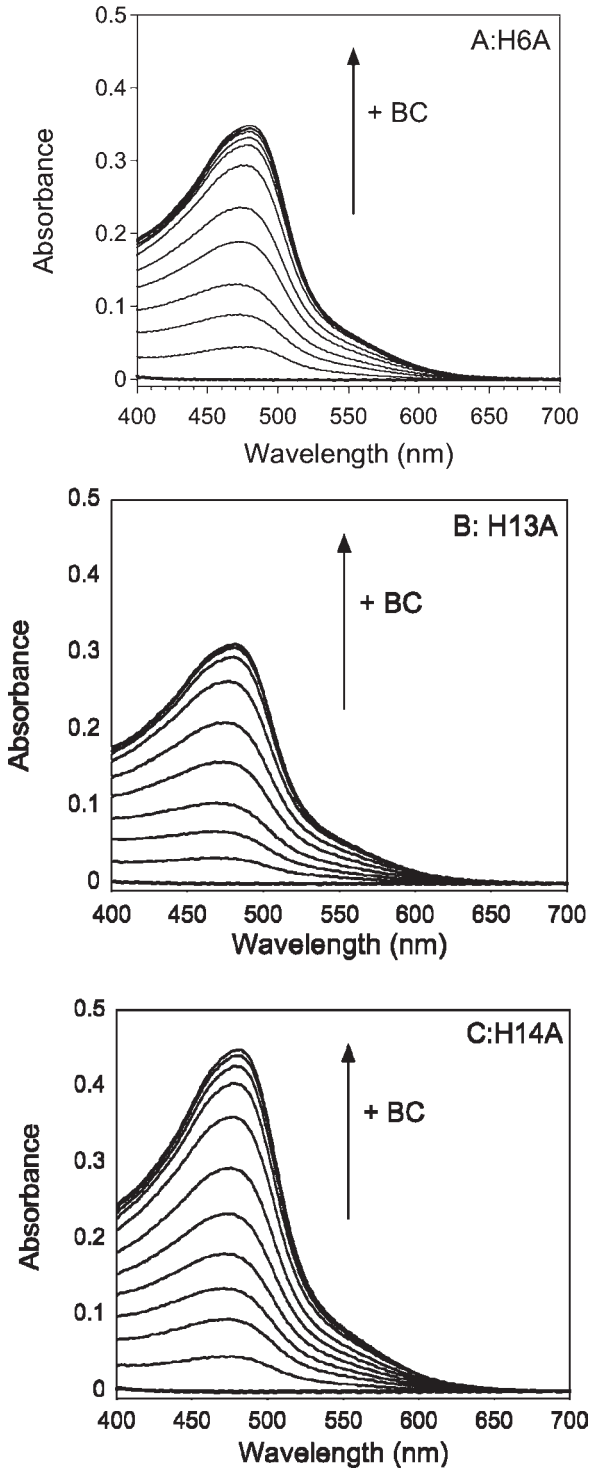

Figure 2. Competitive chelation of BC and Aβ16 mutants for Cu^+. Reverse titrations in which BC was added to Cu^+Aβ16 mutants (A) H6A, (B) H13A, and (C) H14A.

the presence of BCA (Figure S3 in the Supporting Information).[26]

Discussion

The competitive chelation experiment we chose is similar to that performed for Cu^+ metallochaperones.[22−27] These experiments bypass the instability of Cu^+ in aqueous solution by ensuring that it is bound to either a chelator or a protein during the entire course of the titration. For these experiments to work properly, several criteria need to be met. The first is that both chelators must be present in excess over the copper so that effective competition can be established. Second, both chelators ideally should have an affinity for copper in the same range. Finally, a signal (optical, typically) must be available for quantification of at least one of the chelated species.

Scheme 1. Sequence of Aβ16 Highlighting Histidines Potentially Involved in Creating a bis-His Cu^+ Coordination Site

DAEFRH_6DSGYEV$H_{13}H_{14}$QK

Our K_d for the Cu^+Aβ16 complex is in the femtomolar range (Table 1). When the same forward titration data set was analyzed using either *SpecFit* or equations in the text, the K_d values were within error. Moreover, *SpecFit* analysis of both forward and reverse titration data gives dissociation constants for the Cu^+Aβ16 complex that are all within error of one another.

To probe the importance of each of the histidine residues in creating the Cu^+ coordination site, alanine was substituted for each histidine (Scheme 1). Of the mutant peptides, the average K_d of the Aβ16H6A mutant is closest to that of the wild-type peptide. The average K_d values of the Cu^+Aβ16-H13A and Cu^+Aβ16H14A complexes are slightly higher than those of the H6A mutant, but not dramatically so; all of the dissociation constants are within error of each other. We have hypothesized that H13 and H14 create the bis-His coordination site for Cu^+ in soluble forms of the Aβ peptide.[18] The lack of significant variation in the Cu^+ affinities of the mutant peptides was thus a bit surprising. The K_d invariance is, however, consistent with a model in which any two of the three histidines in the Aβ sequence can bind Cu^+. The Cu^{2+}Aβ complex, which is the starting state for our experiments, is pleomorphic with regard to the identity of histidine residues coordinated to Cu^{2+} (two out of three in the dominant species present at physiological pH).[20,28,29] This Cu^{2+}Aβ coordination environment heterogeneity could be the source of heterogeneity in the Cu^+ coordination environment. Another possible explanation is that the Cu^+ coordination environment in the mutant peptides differs entirely from that in the wild-type peptide. Preliminary X-ray absorption data for Cu^+ coordinated to these mutants support this second suggestion (Shearer, J. Personal communication).

In previous work, we found that the monomeric Cu^+Aβ complex is a stable, unreactive form of the copper amyloid complex.[18] Recent work on the mechanism of ascorbic acid reaction with the Cu^{2+}Aβ complex also indicates that reoxidation of Cu^+Aβ is slow.[30] Using Aβ16, which represents the soluble copper-binding fragment of the Aβ peptide, we show that the Cu^+Aβ complex dissociates less readily than Cu^{2+}Aβ. On the basis of a thermodynamic square scheme (Scheme 2), the dissociation constant of the Cu^+Aβ16 complex can be calculated. This scheme assumes a midpoint

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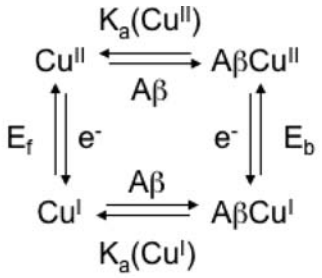
Scheme 2. Thermodynamic Square Scheme



$$E_b - E_f = 0.059 \times \log\left(\frac{K_a(Cu^I)}{K_a(Cu^{II})}\right)$$

potential for the $Cu^{2+/+}A\beta$ complex of 0.280 V vs NHE,[31] a midpoint potential for free $Cu^{2+/+}$ of 0.158 V vs NHE, and K_d of the $Cu^{2+}A\beta$ complex in the 0.1 to 1 nM range.[20,32] A more recently reported midpoint potential for the $Cu^{2+/+}A\beta$ complex is −0.06 V vs SCE,[29] which is lower than the previously published value. Also, the most recently reported K_d of the $Cu^{2+}A\beta$ complex is 57 ± 5 nM.[33] Depending on the combination of parameters used [the midpoint potential for the free $Cu^{2+/+}$ couple (0.158 or 0.360 V), K_d of the $Cu^{2+}A\beta$ complex (ranging from 0.1 to 57 nM), and the midpoint potential of the $Cu^{2+/+}A\beta$ couple (ranging from 0.180 to 0.280 V vs NHE)], the K_d of the $Cu^{+}A\beta$ complex is calculated to range from 0.9 fM to 64 μM. Using a measured $Cu^{+}A\beta$ K_d of 2 fM from our work and inserting the range of values reported/used by others for the free $Cu^{2+/+}$ couple and the $Cu^{2+}A\beta$ K_d, the midpoint potential of the $Cu^{2+/+}A\beta$ couple ranges from 400 to 700 mV vs NHE. Recently, Hureau et al. showed that the $CuA\beta$ midpoint potential is concentration-dependent.[29] On the basis of that result, they conclude that a square scheme is insufficient to describe the redox chemistry of the $CuA\beta$ complex. As the spread in the above calculations indicates, our results support that idea.

The likelihood of in vivo formation of the $Cu^{2+}A\beta$ species is a contentious issue that can only be partly resolved by establishing the K_d value of the $Cu^{2+}A\beta$ species. The magnitude of that K_d has important consequences. If the dissociation constant for the $Cu^{2+}A\beta$ complex is too high, formation of $Cu^{2+}A\beta$ is precluded in the presence of human serum albumin (HSA) found in cerebral spinal fluid (typically present in the micromolar range); $A\beta$ is present in nanomolar concentrations.[34] HSA binds Cu^{2+} with a pM K_d in an ATCUN motif.[35] This strong Cu^{2+} coordination mode (amino terminus, two deprotonated backbone amides, and the imidazole side chain from a histidine residue) does not support sustained redox cycling of Cu^{2+}.[36] Although cerebrospinal/extracellular fluid contains significant concentrations of ascorbate,[37,38] which might lead one to argue that redox-cycling of the $Cu^{2+}A\beta$ complex is possible, sequestration of Cu^{2+} by HSA would undermine this idea.[33] The corollary that redox cycling of $Cu^{2+}A\beta$ creates ROS contributing to oxidative stress in AD, therefore, also would be untenable. One important caveat is that the $Cu^{2+}A\beta$ K_d being compared to the Cu^{2+}HSA K_d is for soluble, monomeric $Cu^{2+}A\beta$. K_d of Cu^{2+} bound to fibrils is in the picomolar range,[13] which means fibrils potentially are viable competitive chelators for Cu^{2+} even in the presence of HSA, especially given the high local concentration of Cu^{2+} binding sites in fibrils (a multivalent effect). To our knowledge, the affinity of Cu^{2+} for well-characterized, stable oligomers, which are the most neurotoxic $A\beta$ species, has not been reported. The Cu^{+} coordination environment and oxidative susceptibility in well-defined oligomers also are unknown, although our preliminary investigations of such species indicates a dramatic change in the Cu^{+} binding site and oxygen reactivity.[39] Given these uncertainties, the issue of whether a neurotoxic $CuA\beta$ complex can exist in vivo remains difficult to resolve without direct measurement of the redox potential and Cu^{+}/Cu^{2+} speciation in extracellular fluid/cerebrospinal fluid.

We propose that monomeric $A\beta$ preferentially binds Cu^{+} rather than Cu^{2+} in vivo. Our previous work demonstrated that the $Cu^{+}A\beta$ complex is sluggish to react with O_2, which decreases turnover to produce ROS. Here, our data indicate that the affinity of monomeric $A\beta$ for Cu^{+} is higher than that for Cu^{2+}. Unlike in plasma where extracellular copper is presumed to be Cu^{2+}, the concentration of ascorbate present in extracellular fluid is sufficient to generate Cu^{+}.[37,38] If Cu^{+} is present in extracellular fluid, competition between HSA and $A\beta$ for copper is less important because the strong square-planar geometry of the ATCUN motif in HSA does not support the lower coordination number/tetrahedral coordination geometry favored by Cu^{+}. Naturally, further testing, particularly in vivo, of this potentially controversial hypothesis remains to be performed.

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Supporting Information Available: Binding isotherm and full table of data collected for $[Cu(BC)_2]^{3-}$ + $A\beta16$ forward titration, mass spectra of $A\beta$ peptides, K_d error analysis, and ascorbate reduction experiment for $Cu(BCA)_2^{2-}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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