Peptide Chemistry

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Structural Studies of Copper(I) Complexes of Amyloid-β Peptide Fragments: Formation of Two-Coordinate Bis(histidine) Complexes**

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Extensive evidence points to oxidative stress as a key event in the pathogenesis and exacerbation of Alzheimer's Disease (AD).[1] Transition metals, such as Zn, Fe, and Cu, are present in elevated concentrations in AD brain deposits, composed primarily of 40- or 42-mer amyloid beta (Aβ) peptides. The redox-active copper(II) ion binds to the unstructured, hydrophilic N terminus of Aβ;[1g,2] and the ability of copper to promote the formation of reactive oxygen species (ROS) and cause neuronal death by interaction with Aβ has been demonstrated in vitro.[1a,c,3,4] ROS formation is proposed to occur by interaction of reduced Cu^{I}–Aβ with O_2 or H_2O_2. However, few direct studies of Cu^{I} binding or reactivity with Aβ peptides or fragments have been reported.[5,6]

We have studied the interactions of the hydrophilic N-terminal region of the Aβ peptide with Cu^{I}. An understanding of the full redox competency of Cu–Aβ, leading to ROS formation and oxidative stress (that is, to cause events associated with the onset of AD), is incomplete without elucidation of the structure/function relationships of the reduced (active) copper(I)–peptide complexes. We report herein studies on the interaction of Cu^{I} ions with small portions of the Aβ peptide incorporating specific metal-binding (His6, His13, His14) or potentially redox-active (Tyr10) residues (Figure 1). Of considerable interest are the contiguous His13 and His14 residues. We have previously reported studies on Cu^{I} complexes of modified (by end-capping and/or regiospecific N^{ε}- or N^{δ}-alkylation) His–His dipeptides which, significantly, adopt a two-coordinate, near-linear N_{His}–Cu^{I}–N_{His} environment.[6] In this report, we demonstrate that Cu^{I} complexes of longer Aβ peptide fragments adopt the same apparent two-coordinate structure in the solid state and aqueous solution. Preliminary reactivity investigations, described here, indicate that the His13–Cu^{I}–His14 moiety is the active part of the structure, responsible for copper-Aβ reactivity.

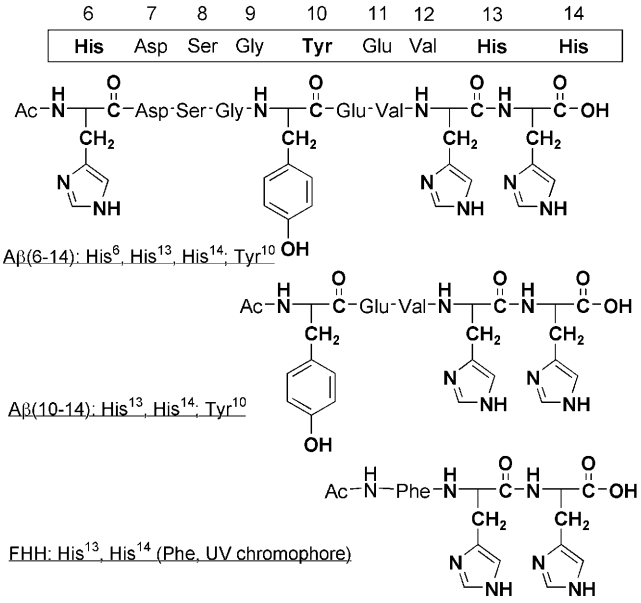

Figure 1. Aβ peptides used for studies with Cu^{I} ions.

A range of peptides (Figure 1) were synthesized and purified by reverse-phase (RP) HPLC to a single peak. Their identity and purity were confirmed by ESI mass spectrometry. The peptides were stored either as lyophilized powders or as stock solutions in doubly distilled deionized water, both at −80 °C.[7] Copper(I)–peptide complexes were prepared directly from Cu^{I} starting materials in the absence of reductants, and their formulation confirmed using ESI-MS. Structural information was obtained by spectroscopic techniques for both solid and solution states (see below). Solid samples of Cu^{I}–Aβ(6–14) and Cu^{I}–Aβ(10–14) were prepared by incubating stoichiometric amounts of the respective peptides with a $[Cu^{I}(CH_3CN)_4]^{+}$ salt in DMF and isolated by precipitation with diethyl ether, filtration, and drying under reduced pressure. Their formulation was confirmed using ESI-MS.[8] Mishandled samples turned deep blue, indicating oxidation to Cu^{II}, whereas the Cu^{I} complexes remained white-to-gray when air was excluded, indicating reduced metal–peptide complexes.

For these solid samples, X-ray absorption spectroscopy (XAS) was used as a powerful (yet unexploited, in the case of Cu–Aβ complexes) tool for the determination of oxidation state, coordination environment, and bond lengths in the derived metal complexes.[9,10] For both Aβ(6–14) and Aβ(10–14) complexes, the occurrence of the 1s→4p transition at 8983–84 eV (Figure 2) definitively indicated that copper was

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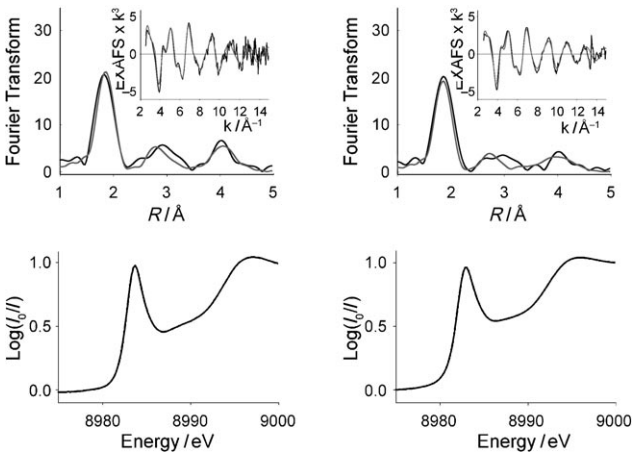

Figure 2. EXAFS (top, including insets) and XANES (bottom) spectroscopic data for Cu^{I}–Aβ(6–14) (left) and Cu^{I}–Aβ(10–14) (right). Fourier transforms: black, fits: gray.

in the +1 oxidation state. Extended X-ray absorption fine structure (EXAFS) spectroscopic data fits for the Cu^{I}–Aβ(10–14) complex, with only two histidine residues (Figure 1), indicated two nitrogen ligands from imidazole donors, as further supported by back-scattering from the ring carbons and nitrogen. The data were consistent with these donors being the *only* ligands bound to the Cu^{I} ion. The intensity of the pre-edge (1s→4p) feature (Figure 2) was further indicative of two-coordination, to the exclusion of other (i.e., three-coordinate) geometries.[9,10] In addition, the short Cu–N bond lengths—at 1.878 Å—are characteristic of linear, two-coordinate geometry in copper(I)–nitrogen ligand complexes, by comparison to crystallographically characterized synthetic copper(I) complexes.[11] The data also conform to the structures identified previously in our $Cu^{I}(His)_2$ dipeptide complexes, in which intramolecular binding of the imidazole moieties of the dipeptide affords tight, linear Cu–N two-coordinate geometry.[6]

Further results obtained for Cu^{I}–Aβ(6–14) firmly demonstrate the propensity for Cu^{I} to adopt near-linear two-coordinate geometry: EXAFS spectroscopic analysis of solid Cu^{I}–Aβ(6–14) indicated formation of the same structure, despite the presence of a third potential histidine ligand. For Cu^{I}–Aβ(6–14), the Fourier Transform with fit is shown in Figure 2. The data could only be fit to two N/O scatterers, thus indicating the presence of only two ligands at the Cu^{I} center; these were identified unambiguously as His nitrogen atoms by backscattering. The $Cu–N_{His}$ bond lengths of 1.876 Å and the X-ray absorption near-edge structure (XANES) absorption intensity clearly indicate two-coordination (and three-coordination).

Binding of CO to Cu^{I} was used as a probe of solution structure. Results indicated that the $2N_{imid}$ structure persists in solution, even for the three-His-containing complex Cu^{I}–Aβ(6–14). CO complexes were formed for each of the three peptides [Figure 1: Aβ(6–14) and Aβ(10–14), discussed above, and the tripeptide FHH, discussed in more detail below] in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered (pH 7.4) D_2O and characterized using FTIR spectroscopy. The stretching frequency of copper(I)-bound CO is diagnostic for the overall coordination number in cationic copper(I) species,[6,11e,12] and has been noted in cuprous enzymes.[13] All three complexes had stretching frequencies greater than 2110 cm^{-1}, varying by no more than 2 cm^{-1} (Table 1). The high frequency is clearly indicative

Table 1: Structural data for Cu^{I} complexes of His-containing peptides.

Complex	Donors[a]	$Cu–N_{Imid}$ [Å]	υ_{CO}[b] [cm^{-1}]
$[Cu^{I}L_{\delta}]^{+}$[c]	2 His	1.876	2110[d]
$[Cu^{I}L_{H}]^{+}$[c]	2 His	1.869	2105[e]
$[Cu^{I}A\beta(6–14)]^{+}$	3 His	1.876	2110[f]
$[Cu^{I}A\beta(10–14)]^{+}$	2 His	1.878	2112[f]
$[Cu^{I}FHH]^{+}$	2 His	N/A	2110[f]
$[Cu^{I}L_{\delta}(MeImid)]^{+}$[c]	2 His	1.896	2075
	1 Imid	2.008[g]	

[a] N-Donor ligands available for coordination to Cu^{I}. [b] For corresponding peptide–Cu^{I}–CO complex. [c] Copper(I) complexes of His–His dipeptides; L_{δ} contains two trityl-protected imidazole ε nitrogen atoms, whereas L_H incorporates two unprotected imidazole moieties. See Ref. [6]. [d] Dichloromethane solution. [e] Methanol solution. [f] With HEPES buffer, pH 7.4, D_2O. [g] Two $Cu–N_{His}$ bonds 1.896 Å, $Cu–N_{Imid}$ bond 2.008 Å.

of the presence of only two N donors coordinating to the Cu^{I} ion. The results recalled our previous finding that His–His dipeptide moieties strongly favor near-linear two-coordination (Table 1).[6]

The similarity in structure deduced for these complexes, [Cu^{I}–Aβ(6–14) and Cu^{I}–Aβ(10–14), by EXAFS and IR spectroscopy (CO binding); and also Cu^{I}(FHH), by IR] strongly suggests that His13 and His14 constitute the two N-donor ligands to the Cu^{I} center. Whereas the unique redox properties of a Cu^{I} ion in a linear, two-coordinate environment have been noted in model complexes[11a,e,14] and the structure has been proposed to be important in some copper-enzyme active sites,[15] the possibility of a $Cu^{I}(His)_2$ site involved in Aβ chemistry has been overlooked.

With our structural results in mind, we have begun studying the redox reactivity of these systems. Preliminary experiments on the ability of Cu^{I}–Aβ fragment complexes to produce ROS have been carried out. The first step in Cu–Aβ ROS production has been proposed to be Cu^{II} reduction followed by reaction with O_2 to produce H_2O_2.[16] Hydrogen peroxide has been formed in vitro from Cu–Aβ complexes, but only in the presence of very large excesses of reducing agents, such as ascorbate,[16,17] or by electrochemical reduction of Cu^{II}.[4] Direct reactivity of Cu^{I}–Aβ with O_2, by way of the reactions shown in Scheme 1, has not been studied, until now.

Production of H_2O_2 from oxygenated Cu^{I}–peptide solutions was monitored using the horseradish peroxidase (HRP)/Amplex Red assay. Hydrogen peroxide is produced from solutions of Cu^{I}–Aβ over the course of one hour, in amounts significantly greater than Cu^{I}-only or peptide-only control reactions.[8,18] Most intriguingly, all three systems, whether incorporating the third His residue (His6) or not, or incorporating the potentially redox-active Tyr10 or not, produce assayable H_2O_2 in similar yields and rates of

$$2\ A\beta\text{–}Cu^{I} + O_2 + 2\ H^{+} \longrightarrow 2\ A\beta\text{–}Cu^{II} + H_2O_2 \quad (1)$$

$$A\beta\text{–}Cu^{I} + O_2 + 2\ H^{+} \longrightarrow A\beta^{\bullet}\text{–}Cu^{II} + H_2O_2 \quad (2)$$

Scheme 1. Potential reactions of Cu–Aβ with dioxygen to form H_2O_2.

formation. Mechanistically, reduction of O_2 to H_2O_2 requires two electrons (Scheme 1). Thus, in the absence of an exogenous reductant (as in these experiments), stoichiometry requires that a second electron must be provided either by a second copper ion in the Cu^{I}–Aβ moiety or by the peptide itself, potentially by tyrosine oxidation [Eq. (2)].

Based on our results (Figure 3), the similar efficiency of Cu^{I}(FHH) in H_2O_2 production, compared to that of the Tyr-containing species, suggests that electrons are supplied only

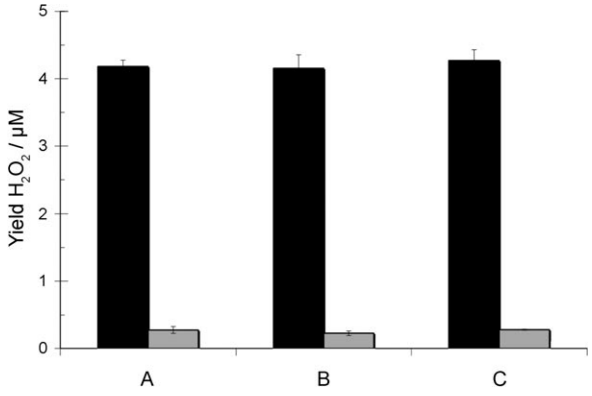

Figure 3. Yields of H_2O_2 from reactions of O_2 with 25 μM copper(I)–peptide solutions, as determined by HRP/Amplex Red assay. Cu^{I} complex: black, peptide-only: gray. A) Aβ(6–14); B) Aβ(10–14); C) FHH. Error bars represent standard errors from five trials.

by the oxidation of copper. Furthermore, the similar rates and yields among all three species suggest His6 is not significantly involved in Cu^{I}–Aβ–O_2 reactivity. In other words, the uniformity in results from these preliminary experiments with the three copper–peptide species suggests that they react with O_2 to produce H_2O_2 by the same mechanism—Equation (1), wherein two separate Aβ–Cu^{I} moieties are involved and each Cu^{I}–Aβ species is a $Cu^{I}(His)_2$ complex.[19] Together, these results suggest that the $Cu^{I}(His)_2$ unit may not only be the predominant binding mode of Cu^{I} ions to Aβ peptides, but also the structure directly responsible for the behavior (including ROS production) of reduced Cu^{I}–Aβ species.

In summary, our EXAFS spectroscopy and CO-binding studies have clearly demonstrated the preference of Cu^{I} ions for two-coordinate geometry in binding to fragments of the Aβ N-terminal region through a contiguous His13–His14 motif. That this structure is retained, even in the presence of three histidine residues (His6, His13, His14) and additional potential donors (Tyr10, Asp7, Glu11, Ser8, backbone carbonyl O, amide N), is striking. The two-coordinate geometry of Cu^{I}–Aβ may prove critical to understanding the redox chemistry of Cu–Aβ, and thus to understanding oxidative stress in AD. Preliminary ROS results indicate that the two-coordinate $Cu^{I}(His)_2$ structure is significant for explaining the behavior (H_2O_2 production) of Cu^{I}Aβ, or that a third His in the sequence (His6) may not be crucial. All the current literature suggests His6 as a ligand for the oxidized Cu^{II} ion. These studies, absent of any Cu^{I}–Aβ structural information, conclude that a three-histidine binding environment is probably important for the Cu^{II}/Cu^{I}-promoted production of ROS. We have shown herein that this may not be the case. AD oxidative stress chemistry is dependent upon: 1) the Cu^{II}/Cu^{I} redox cycle, and 2) ROS production from Cu^{I}/H_2O_2 and/or Cu^{I}/O_2 chemistry. The energetics and kinetics of both may be highly tuned by the preferred stable copper(I)–bis(histidine) structure, which we have previously demonstrated has unique redox properties.[6] As such, the study of Cu^{I}–Aβ may hold additional information, key to the understanding of Cu–Aβ oxidative stress.

Experimental Section

Experimental procedures, including procedures for peptide synthesis and purification, preparation of solid and solution Cu^{I}–peptide samples, procedures for CO-binding and H_2O_2-producing experiments, and methods for EXAFS spectroscopic data collection and analysis, are available in the Supporting Information.

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[8] See Supporting Information.

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[18] a) When carried out by the same method as the copper(I)–peptide experiments[8] copper(I)-only control reactions give a yield of H_2O_2 that never exceeds (and is often less than) 40% of that detected for copper(I)–peptide (that is, < 1.5 μM, compared to > 4 μM for copper(I)–peptide). It should be noted that there is some unexplained dependence on experimental conditions, especially the order of addition of reagents; for example, if Cu^I solutions are added to aerobic, buffered HRP/Amplex Red assay, strong signalling of H_2O_2 is indicated; b) if Cu^I ions are in excess (2:1) relative to the peptide, the results are unchanged, qualitatively indicating that Cu^I ion binds the peptide strongly. Quantitative determinations are in progress; c) assayed yields of H_2O_2 are, at most, approximately 30%, considering background and small amounts of Amplex oxidation by copper(II)–peptide and copper(I)–peptide/O_2 (data not shown). It is unknown whether relative inefficiency of HRP/Amplex Red trapping is responsible for the sub-stoichiometric yield, or if the copper(I)/(II)–peptide itself consumes some peroxide. Catalase attenuates the signal. We are currently carrying out experiments to "trace" all copper(I) electron equivalents.
[19] A recent publication implicates a soluble "dimer" (with two Aβ moities and, thus, possibly two Cu ions) as the minimal unit responsible for AD toxicity. See G. M. Shankar, S. Li, T. H. Mehta, A. García-Muñoz, N. E Shepardson, I. Smith, F. M. Brett, M. A. Farrell, M. J. Rowan, C. A. Lemere, C. M. Regan, D. M. Walsh, B. L. Sabatini, D. J. Selkoe, *Nature Medicine* **2008**, *14*, 837 – 842.

