Substrate mediated reduction of copper-amyloid-β complex in Alzheimer's disease†

Victor A. Streltsov* and Joseph N. Varghese

Received (in Cambridge, UK) 6th March 2008, Accepted 15th April 2008
First published as an Advance Article on the web 14th May 2008
DOI: 10.1039/b803911a

X-Ray absorption near-edge spectroscopy (XANES) has been used to probe the substrate mediated reduction of Cu^{2+} in Aβ-Cu^{2+} complexes by ascorbate and the neurotoxin 6-hydroxydopamine (6-OHDA), however dopamine and, in particular, cholesterol are incapable of reducing soluble monomeric Aβ-Cu^{2+} complexes.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by the presence of amyloid plaques. The major constituent of AD plaques is the amyloid β-peptide (Aβ, up to 42 amino acids: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) which is cleaved from the membrane-bound amyloid precursor protein (APP) *via* the β/γ-secretase pathway. There is evidence[1,2] that the major source of neurodegeneration observed in AD is related to the toxicity (oxidative stress) from reactive oxygen species (ROS) produced in the brain by the Aβ peptide bound to primarily copper ions. The Aβ-Cu^{2+} complexes can be involved in extensive redox chemical reactions that produce H_2O_2 and other ROS from molecular oxygen.[2,3] However, there are inconsistencies in the interpretation of these redox reactions, which are whether Cu^{2+} can be reduced by Aβ itself,[4] with Met35 residue proposed as an electron donor[3,4] or whether the Cu^{2+} reduction is mediated by biological reducing agents.[5] Catechols (*e.g.* dopamine), vitamin C (ascorbate) and cholesterol were considered to be important substrates for Aβ-Cu^{2+} redox activity.[2,5] However, to be capable of reducing Aβ-Cu^{2+} these biological agents should have a lower (more negative) reduction potential (E) than Aβ-Cu^{2+}. Cyclic voltametry[2,5] produced a highly positive formal reduction potential of Aβ(1–42)-Cu^{2+} ($\sim$0.50–0.55 V *vs.* Ag/AgCl or $\sim$0.74–0.79 V *vs.* NHE). On the contrary, two other independent studies[6,7] reported significantly lower E values (0.28–0.34 V *vs.* NHE) for three Aβ(1–16, 1–28 and 1–42)-Cu^{2+} complexes. These values are in the range of redox potentials for Cu-binding peptides[6] and appeared to be lower than the E values (0.37–0.38 V) for some important neurotransmitters, such as dopamine, epinephrine and norepinephrine, suggesting that these substrates are incapable of reducing Aβ-Cu^{2+}.[7] On the other hand, all measured Aβ-Cu^{2+} E values[5–7] are higher (more positive) than that for ascorbic acid (0.051–0.058 V *vs.* NHE),[7] and therefore consistent with the strong reducing properties of ascorbate in Aβ-Cu^{2+} complexes.[2,5,7,8]

In the reduced state, the Cu^{1+} is in a d^{10} closed shell electronic configuration, and thus its geometric and electronic configuration cannot be probed by EPR or optical methods. On the other hand, X-ray absorption spectroscopy (XAS) is direct method of probing the electronic and structural nature of the metal sites.[9] In this study, X-ray absorption near-edge spectroscopy (XANES) was employed to investigate reducing properties of several substrates (ascorbate, cholesterol, neurotransmitter dopamine and neurotoxin 6-hydroxydopamine (6-OHDA)) in solution with Aβ(1–16)-Cu^{2+} complexes. As shown in many studies,[6,7,10] the truncated Aβ(1–16) peptide is relatively hydrophilic and highly soluble in the range of conditions compared to full-length wild type Aβ(1–42) peptide. Most importantly, both the full-length and truncated monomeric peptides exhibit similar Cu^{2+} binding and ROS production properties. A remarkable similarity among the voltammetric properties of three Aβ(1–16, 1–28 and 1–42)-Cu complexes excludes the possibility that monomeric Aβ peptides and, in particular, the Met35 residue can donate electrons for copper reduction.[6,7] Also, no reduction of Cu^{2+} by Aβ binding was observed in our previous[11] and this current XAS studies. Therefore, biological reducing agents have to be recruited to mediate the ROS generation by monomeric Aβ-Cu^{2+} complexes.[5]

We have prepared Aβ(1–16)-Cu^{2+} complexes in phosphate buffer saline (PBS) as described[11] and mixed them with 10-fold molar ratio of substrates dissolved in water, except for cholesterol which was dissolved in chloroform and added at a 5-fold molar ratio. Aβ(1–16) was obtained from Auspep Pty Ltd. Immediately after preparation, the samples were injected into solution cells and rapidly frozen. The Cu^{2+} concentration in solution cells was up to 2.2 mM. Two additional Aβ(1–16)-Cu samples with added dopamine and ascorbate were incubated at room temperature for 1 and 10 h, respectively and then measured again. There were no marked changes in XAS spectra of these samples after the incubation. A series of Cu K-edge (8980.4 eV) XAS scans were obtained from samples in a fluorescence mode at 15–20 K using a helium displex cryostat. The experiments were conducted at the PNC-CAT 20BM bending magnet beamline at the Advanced Photon Source (APS), USA. The experimental set up and data processing were identical to those reported previously.[11] Energy calibration was accomplished using the first inflection point of a Cu foil spectrum measured simultaneously with each scan. Up to 15 40 min scans were measured for each sample. No X-radiation reduction of Cu^{2+} to Cu^{1+} was detected by comparing edge spectra for consecutive scans. Each scan was

CSIRO Molecular and Health Technologies and P-Health Flagship, Parkville, VIC 3052, Australia. E-mail: victor.streltsov@csiro.au; Fax: +613 9662 7101; Tel: +613 9662 7311
† Electronic supplementary information (ESI) available: Samples preparation, data collection and enlarged regions of XANES spectra. See DOI: 10.1039/b803911a

collected from a freshly exposed region of the sample by moving the sample stepwise within the cell window area. The XANES regions were extracted from the experimentally measured absorption coefficient using background subtraction and normalization methods implemented in the program ATHENA,[12] an interface to IFEFFIT.[13]

Fig. 1 compares absorption edges for an oxidized Aβ(1–16)-Cu^{2+} complex recently measured[11] with substrate treated peptide complexes. As expected for the Cu^{2+} site, the oxidized Aβ(1–16)-Cu^{2+} complex exhibits a featureless edge with a half-maximal position around 8990 eV.[14,15] Interestingly, a similar edge was produced by the Aβ-Cu complex with cholesterol, strongly suggesting that cholesterol did not reduce Cu^{2+} bound to soluble monomeric Aβ(1–16). On the other hand, both ascorbate and 6-OHDA treated Aβ-Cu complexes exhibited a well-resolved peak at about 8984 eV. XANES of Cu^{1+} is normally characterized by a pre-edge feature, the strong 1s $\rightarrow$ 4p transition peak, in the 8980–8985 eV region and it serves as an immediate and definitive indication of the +1 oxidation state of Cu.[14] Further convincing evidence of the conversion of Cu^{2+} to Cu^{1+} comes from the significant shift (>5 eV) in the position of the edge near 8987 eV after adding reducing agents, such as ascorbate or 6-OHDA. This difference in edge position is typically observed between Cu^{2+} and Cu^{1+} species.[14,16] These edge features strongly support the suggestion that ascorbate and 6-OHDA can reduce Aβ-Cu^{2+}. The high-intensity of the pre-edge peaks suggests that the majority of Cu is in the Cu^{1+} state under these conditions.

The edge spectrum of the Aβ-Cu complex treated with dopamine shows a small bulge (normalized absorption amplitude of 0.21) at about 8984 eV and a slight reduction of the white line intensity ($\sim$9000 eV) compare to oxidized Aβ-Cu^{2+} complex. This can be interpreted as a minor reduction of Cu^{2+} to Cu^{1+} in the Aβ-Cu complex which occurred when dopamine was added. No X-ray reduction was detected and no marked changes were observed in the spectrum after the retention of the sample at room temperature for about 1 h. No oxidation of dopamine prior to addition was detected by

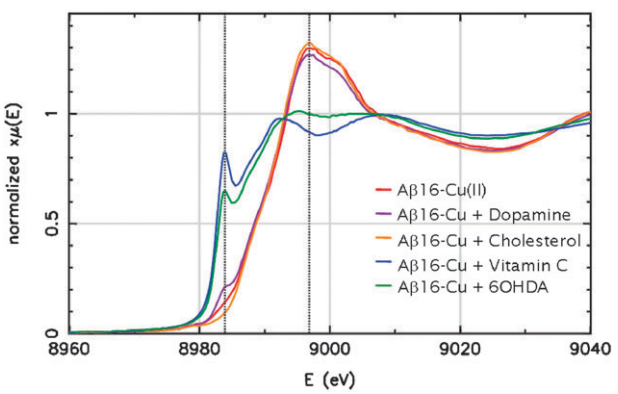

Fig. 1 The XANES regions of normalized absorption amplitude *vs.* energy *E* (vertical dotted lines indicate 8984 and 8997 eV) for oxidized (Aβ(1–16)-Cu^{2+}—red, with dopamine—purple and with cholesterol—orange) and reduced (Aβ(1–16)-Cu^{1+} with vitamin C—blue and with 6-hydroxydopamine (6-OHDA)—green) complexes. The oxidized Cu^{2+} complexes show less featureless edges than reduced Cu^{1+} complexes which exhibit a well-resolved peak at about 8984 eV.

mass-spectroscopy. Therefore, the dopamine can be considered as a very weak reducing agent or more likely that some limited reduction of Cu^{2+} occurred due to formation of Cu-dopamine complexes with associated oxidation of dopamine.[17]

A systematic study[14] of 19 Cu^{1+} and 40 Cu^{2+} complexes showed that the intensity and position of near-edge features are strongly correlated with oxidation state and coordination mode. The pre-edge peak positions of 8983.7 and 8983.6 eV and normalized absorption amplitudes of 0.82 and 0.64 for Aβ-Cu^{1+} complexes with ascorbate and 6-OHDA, respectively, are in the range of values for the Cu^{1+} centers with the coordination number of either 2 or 3, as well as close to mixed 2+3 or 2+4 coordinate shells in binuclear Cu^{1+} complexes.[14] The difference in the edge profiles of the reduced Aβ-Cu^{1+} complexes in Fig. 1 also suggests the formation of different coordination structures. It should be noted that in some samples with coordination 2 of the Cu^{1+} centres[18,19] and with strong interactions between molecular groups, such as in crystalline solids, the Cu^{1+} pre K-edge peak cannot provide reliable stereochemical information due to anomalous reduction of the peak by intense exciton formation.[19] However, this is unlikely to be the case for diluted solutions studied here.

The absorption edges of the oxidized Aβ-Cu^{2+} with cholesterol and dopamine resemble, in energy and position, those of Aβ-Cu^{2+} (Fig. 1). Aβ-Cu^{2+} complexes have very weak peaks at 8979–8980 eV which have been assigned as originating from 1s $\rightarrow$ 3d quadrupolar allowed transitions in Cu^{2+} species.[14,15] The increase in the intensity of the XANES peak at about 8997 eV (assigned as a 1s $\rightarrow$ 4p or a 1s $\rightarrow$ continuum resonance[15]) observed for the Cu^{2+} complexes relative to that for the Cu^{1+} complexes (see Fig. 1 and ESI†), is consistent with formation of the new coordination structure. There are three other important features in these XANES region of Cu^{2+} spectra, which are represented by shoulders at 9002, 9010 and 9045 eV. These features were assigned to be due to multiple scattering from equatorial ligands of tetragonally-distorted octahedral coordination of Cu^{2+} species[16,20] and depend, for example, on the orientation of the imidazole rings with respect to the CuN_4 plane in Cu^{2+} imidazole complexes.[15] Recent studies (reviewed in ref. 10) indicated the involvement of the three histidines His6, His13 and His14 in coordination of Cu^{2+} in Aβ-Cu^{2+} complexes. The fourth ligand is most likely an oxygen atom donor. There is a strong evidence that tyrosine (Tyr10) is not the oxygen atom donor, while the glutamic acid (Glu11) and the N-terminal aspartic acid (Asp1) are involved when Zn^{2+} is bound to human Aβ(1–16)[21] and Aβ(1–28)[22] and when Cu^{2+} is bound to rat Aβ(1–28).[23] Furthermore, in an analysis[24] of metal binding sites in metalloproteins, aspartate and glutamate were often found in the coordination sphere of Cu or Zn, while tyrosine was rare. It is reasonable to suggest that the same His6,13,14 and Glu11 or Asp1 residues can be also involved in Cu^{1+} binding in reduced Aβ-Cu complex, however with different coordination geometry. Further XAS analysis,[11] including EXAFS regions, for Aβ-Cu^{2+} and Aβ-Cu^{1+} is required to verify the involvement of those residues in copper coordination.

In this study, we have used XANES to probe directly the substrate mediated reduction of Cu^{2+} in Aβ(1–16)-Cu^{2+} complexes by ascorbate and the neurotoxin 6-OHDA.

However dopamine and, in particular, cholesterol are incapable of reducing soluble monomeric Aβ(1–16)-Cu^{2+} complexes at least under the current experimental conditions. This conclusion is valid for the full-length monomeric Aβ(1–42)-Cu^{2+} complex with high affinity Cu^{2+} binding site, since the redox behaviour of truncated and full length peptides are similar.[6,7] The results are in agreement with assignment of the redox potentials for Aβ-Cu^{2+}, ascorbic acid and dopamine.[6,7]

Finally, we note that inconsistencies in the interpretation of the redox reactions of the Aβ-Cu complexes and in particular whether the Cu^{2+} reduction can be mediated by dopamine or cholesterol, can be related to the indirect detection methods. These methods are usually based on measurement of H_2O_2 production using either TCEP (tris(2-carboxyethyl)phosphine) or DCFH (2′-7′-dichlorofluorescein) and its diacetate (DCFHDA) based assays. But TCEP may serve as the source of electrons to form H_2O_2 from O_2 because it is also a reducing agent.[5] Other findings[25] demonstrated that the DCFH/DCFHDA assay could be subject to a serious artifact. The H_2O_2 production is inherent in the oxidation of DCFH, which, therefore, cannot be used to prove the formation of ROS.[25]

We would like to acknowledge that the use of the APS was supported by the US Department of Energy, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357, and the APS access was supported by the Australian Synchrotron Research Program, which is funded by the Commonwealth of Australia under the Major National Research Facilities Program.

Notes and references

1. A. I. Bush, *Trends Neurosci*., 2003, **26**, 207–214, and references therein.
2. K. J. Barnham, C. L. Masters and A. I. Bush, *Nat. Rev. Drug Discovery*, 2004, **3**, 205–214, and references therein.
3. D. G. Smith, R. Cappai and K. J. Barnham, *Biochim. Biophys. Acta*, 2007, **1768**, 1976–1990.
4. X. Huang, C. S. Atwood, M. A. Hartshorn, G. Multhaup, L. E. Goldstein, R. C. Scarpa, M. P. Cuajungco, D. N. Gray, J. Lim, R. D. Moir, R. E. Tanzi and A. I. Bush, *Biochemistry*, 1999, **38**, 7609–7616.
5. C. Opazo, X. Huang, R. A. Cherny, R. D. Moir, A. E. Roher, A. R. White, R. Cappai, C. L. Masters, R. E. Tanzi, N. C. Inestrosa and A. I. Bush, *J. Biol. Chem*., 2002, **277**, 40302–40308.
6. L. Guilloreau, S. Combalbert, A. Sournia-Saquet, H. Mazarguil and P. Faller, *ChemBioChem*, 2007, **8**, 1317–1325.
7. D. Jiang, L. Men, J. Wang, Y. Zhang, S. Chickenyen, Y. Wang and F. Zhou, *Biochemistry*, 2007, **46**, 9270–9282.
8. S. I. Dikalov, M. P. Vitek and R. P. Mason, *Free Radical Biol. Med*., 2004, **36**, 340–347.
9. J. J. Rehr and A. L. Ankudinov, *Coord. Chem. Rev*., 2005, **249**, 131–140.
10. V. A. Streltsov, *Eur. Biophys. J*., 2008, **37**, 257–263, and references therein.
11. V. A. Streltsov, S. J. Titmuss, V. C. Epa, K. J. Barnham, C. L. Masters and J. N. Varghese, *Biophys. J*., submitted; see also Supplementary Information.
12. B. Ravel and M. Newville, *J. Synchrotron Radiat*., 2005, **12**, 537–541.
13. M. Newville, *J. Synchrotron Radiat*., 2001, **8**, 96–100.
14. L. S. Kau, D. J. Spira-Solomon, J. E. Penner-Hahn, K. O. Hodgson and E. I. Solomon, *J. Am. Chem. Soc*., 1987, **109**, 6433–6442.
15. R. W. Strange, L. Alagna, P. Durham and S. S. Hasnain, *J. Am. Chem. Soc*., 1990, **112**, 4265–4268.
16. J. L. Fulton, M. M. Hoffmann, J. G. Darab, B. J. Palmer and E. A. Stern, *J. Phys. Chem. A*, 2000, **104**, 11651–11663.
17. I. Paris, A. Dagnino-Subiabre, K. Marcelain, L. B. Bennett, P. Caviedes, R. Caviedes, C. O. Azar and J. Segura-Aguilar, *J. Neurochem*., 2001, **77**, 519–529.
18. G. Lamble, A. Moen and D. G. Nicholson, *J. Chem. Soc., Faraday Trans*., 1994, **90**, 2211–2213.
19. A. Moen, D. G. Nicholson and M. Rønning, *J. Chem. Soc., Faraday Trans*., 1995, **91**, 3189–3194.
20. A. Filipponi, P. D'Angelo, N. V. Pavel and A. D. Cicco, *Chem. Phys. Lett*., 1994, **225**, 150–155.
21. S. Zirah, S. A. Kozin, A. K. Mazur, A. Blond, M. Cheminant, I. Segalas-Milazzo, P. Debey and S. Rebuffat, *J. Biol. Chem*., 2006, **281**, 2151–2161.
22. E. Gaggelli, A. Janicka-Klos, E. Jankowska, H. Kozlowski, C. Migliorini, E. Molteni, D. Valensin, G. Valensin and E. Wieczerzak, *J. Phys. Chem. B*, 2008, **112**, 100–109.
23. E. Gaggelli, Z. Grzonka, H. Kozlowski, C. Migliorini, E. Molteni, D. Valensin and G. Valensin, *Chem. Commun*., 2008, 341–343.
24. I. Dokmanic, M. Sikic and S. Tomic, *Acta Crystallogr., Sect. D: Biol. Crystallogr*., 2008, **D64**, 257–263.
25. M. G. Bonini, C. Rota, A. Tomasi and R. P. Mason, *Free Radical Biol. Med*., 2006, **40**, 968–975.