Amyloid β Peptides

DOI: 10.1002/anie.201006335

Rapid Formation of a Preoligomeric Peptide–Metal–Peptide Complex Following Copper(II) Binding to Amyloid β Peptides**

*Jeppe T. Pedersen, Kaare Teilum, Niels H. H. Heegaard, Jesper Østergaard, Hans-Werner Adolph, and Lars Hemmingsen**

Copper(II)-induced extracellular aggregation of amyloid β peptides (Aβ) is implicated in the pathogenesis of Alzheimer's disease (AD).[1] The exact role of the Cu^{II}–Aβ interaction is not fully understood but it has been demonstrated that Cu–Aβ oligomeric complexes may catalyze the formation of neurotoxic reactive oxygen species.[2] Cu^{II} also induces the rapid formation of insoluble Aβ oligomers that dissolve if Cu^{II} is removed.[3] Low-order oligomers both with and without Cu^{II} are key to the neurodegeneration associated with AD.[4] In the brain, Aβ are primarily found as 40-residue ($Aβ_{1–40}$) and 42-residue ($Aβ_{1–42}$) peptides. It is known that Cu^{II} binds to the N-terminal part of Aβ and the 16-residue fragment $Aβ_{1–16}$ is well established as a nonfibrillating model for the Cu–Aβ complex. $Aβ_{1–16}$ coordinates Cu^{II} with the side chains of the amino acid residues D1, H6, H13, and H14 and presumably the N-terminal amino group;[5] two different pH-dependent coordination modes exist.[6] The importance of the elucidation of the mechanism of Cu^{II} binding to Aβ and its specific role in the aggregation process is emphasized by the fact that the Cu^{II} concentration is elevated in the amyloid plaques in brains from AD patients,[1a] and that Cu^{II} concentration transiently can reach micromolar concentrations in the extracellular space.[7] However, detailed knowledge of the dynamics of the initial events in Cu^{II} binding to Aβ and its relation to the formation of higher-order oligomers and aggregates is lacking. This information could be essential for the development of therapeutic strategies that could convert a toxic oligomerization pathway into a nontoxic pathway. Herein we address the kinetic mechanism of Cu^{II} binding to both $Aβ_{1–16}$ and $Aβ_{1–40}$, and copper(II)-induced $Aβ_{1–40}$ oligomerization by a combination of stopped-flow spectroscopy (fluorescence spectroscopy and light scattering), NMR relaxation, and dynamic simulations. Global fitting and dynamic simulations resulted in a unifying model for the initial steps of Cu^{II} binding to Aβ, which includes a transient peptide–metal–peptide (Aβ–Cu–Aβ) complex that does not participate in the subsequent aggregation.

Binding of Cu^{II} to $Aβ_{1–16}$ induces a large change in the environment of Y10 in $Aβ_{1–16}$ that allows characterization of the binding kinetics by stopped-flow fluorescence spectroscopy. $Aβ_{1–16}$ (40 μM) was mixed with solutions of Cu^{II} with varying concentrations in HEPES buffer (0–40 μM), thus resulting in biphasic time traces (Figure 1a). The fast phase (< 30 ms) that exhibits a large decrease in signal intensity shows observed rates from approximately 35 to $1.7 \times 10^2\ s^{-1}$

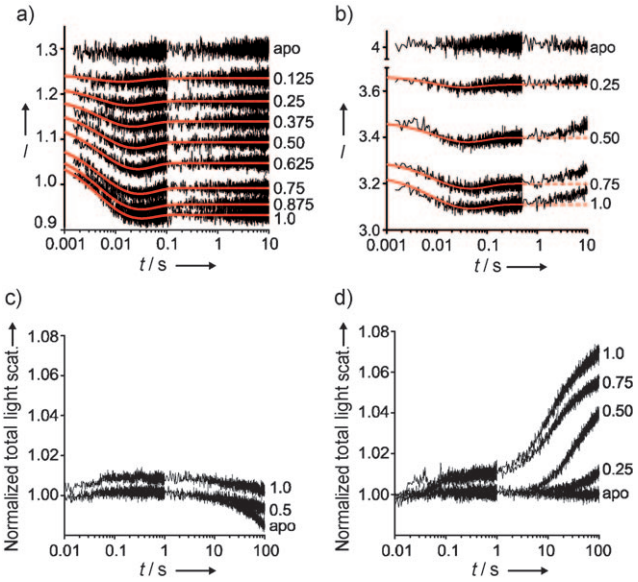

Figure 1. Tyrosine fluorescence of a) $Aβ_{1–16}$, b) $Aβ_{1–40}$, and normalized light scattering of c) $Aβ_{1–16}$ and d) $Aβ_{1–40}$ after rapid mixing with Cu^{II} in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) + 100 mM NaCl buffer solution, pH 7.4 at 37 °C. The peptide concentration was kept constant at 40 μM and the Cu^{II} concentration was increased stepwise to a molar Cu^{II}/Aβ ratio of 1 as indicated. Fluorescence data were globally fit (red traces) to a two-step model using KinTek Explorer. Broken red traces (b) show extrapolated fit for $Aβ_{1–40}$ beyond 500 ms to visualize the second phase of the fluorescence increase. Four equivalents of glycine were present in the Cu^{II} solution (the light-scattering intensity does not directly reflect the aggregation number, see the Supporting Information).

[*] Prof. H.-W. Adolph, Prof. L. Hemmingsen
Department of Basic Sciences and Environment
Faculty of Life Sciences, University of Copenhagen
Thorvaldsensvej 40, 1871 Frederiksberg (Denmark)
Fax: (+45) 3533-2398
E-mail: lhe@life.ku.dk
J. T. Pedersen, Prof. J. Østergaard
Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen(Denmark)
Prof. K. Teilum
Structural Biology and NMR Laboratory, Department of Biology
University of Copenhagen(Denmark)
MD DSc N. H. H. Heegaard
Department of Clinical Biochemistry and Immunology
Statens Serum Institut, Copenhagen (Denmark)

[**] This work was supported by Statens Serum Institut and the Drug Research Academy at the Faculty of Pharmaceutical Sciences, The Villum Kann Rasmussen Foundation, and The Danish Research Council for Independent Research | Natural Sciences.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201006335.

WILEY ONLINE LIBRARY
© 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

depending on the Cu^{II} concentration. The slow phase (ca. 30–100 ms) exhibits a small increase in signal intensity and has an observed rate of approximately 30 s^{-1}, which is independent of Cu^{II}. As two well-separated reaction phases characterize the binding kinetics of Cu^{II} to $A\beta_{1-16}$, a mechanism must include at least three states. This prerequisite is consistent with the result that at least two conformations of the Cu^{II}–Aβ are in a dynamic equilibrium.[6f] We used the numerical program KinTek Explorer[8] for stopped-flow data analysis to globally fit all reaction traces to a two-step mechanism [Scheme 1, Equation (1)]. The first step in this model is the

$$A\beta + Cu^{II} \underset{0.101\ s^{-1}}{\overset{4.9\ \mu M^{-1}s^{-1}}{\rightleftharpoons}} A\beta\text{–}Cu \underset{20.0\ s^{-1}}{\overset{10.2\ s^{-1}}{\rightleftharpoons}} [A\beta\text{–}Cu]^* \quad (1)$$

$$[A\beta\text{–}Cu]^* + A\beta \underset{2.8\times10^2\ s^{-1}}{\overset{0.66\ \mu M^{-1}s^{-1}}{\rightleftharpoons}} A\beta\text{–}Cu\text{–}A\beta \quad (2)$$

Scheme 1. Proposed two-step (1) and three-step (1 + 2) mechanism for initial binding of Cu^{II} to $A\beta_{1-16}$. Rate constants are determined from stopped-flow data, NMR relaxation decay, and simulation. [Cu–Aβ]* indicates an alternative conformation of the complex. See also the Supporting Information.

bimolecular binding of Cu^{II} to free $A\beta_{1-16}$ in consistency with the observed increase of the rate with an increase in Cu^{II} concentration; the second step is an intramolecular rearrangement of the initial Cu–Aβ complex to an alternative conformation [Cu–Aβ]* that accounts for approximately 33% of the Cu–Aβ complexes at equilibrium (see the Supporting Information). The fluorescence of [Cu–Aβ]* is less quenched than that of Cu–Aβ, and may correspond to the coordination mode favored by high pH values, which has been found to account for approximately 20–25% of the Cu–Aβ complexes at pH 7.4.[6e]

The model satisfactorily describes the stopped-flow kinetic data up to one equivalent of Cu^{II} (Figure 1a and Table S1 in the Supporting Information). Supporting the model, we find that the affinity ($K_d = (14 \pm 1)$ nM) of $A\beta_{1-16}$ for Cu^{II} derived from the kinetic parameters agrees with values reported by others[9] (see the Supporting Information). At superstoichiometric concentrations of Cu^{II}, $A\beta_{1-16}$ is known to bind a second Cu^{II} ion albeit 100 times weaker than the first Cu^{II} ion;[10] this event is not detected in our experiments.

To complement the stopped-flow kinetics, the Cu^{II} binding kinetics were measured by ^1H NMR spectroscopy (Figure S3) taking advantage of the paramagnetic relaxation enhancement (PRE) induced by Cu^{II}. The PRE of the longitudinal relaxation of ^1H spins in $A\beta_{1-16}$, $R_{1,PRE}$, was quantified by measuring R_1 on samples of $A\beta_{1-16}$ in the absence and presence of Cu^{II} ions at $A\beta_{1-16}$ concentrations of 150 and 300 μM (Figure 2a). The observed $R_{1,PRE}$ for $H^{\delta2}$ and $H^{\varepsilon1}$ in H6, H13, and H14 is simply the apparent rate constant, k_{app}, by which metal-free $A\beta_{1-16}$ coordinates the Cu^{II} ion (see the Supporting Information). Owing to the strong PRE, Cu-loaded Aβ is invisible in the spectra. Thus the time courses shown in Figure 2a reflect the rate by which Aβ molecules that are Cu-free at time zero experience the transient binding

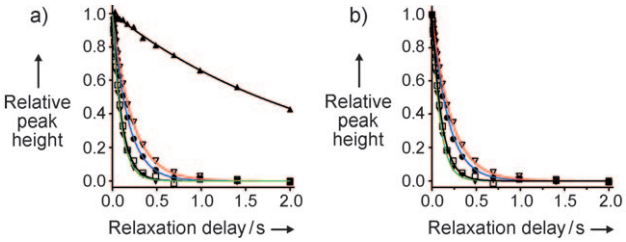

Figure 2. Longitudinal relaxation decays of H6 $H^{\delta2}$ in $A\beta_{1-16}$ at varying concentrations of peptide and Cu^{II} a) measured by ^1H NMR and b) simulated. (▲) 300 μM $A\beta_{1-16}$, (▽) 300 μM $A\beta_{1-16}$ + 15 μM Cu^{II}, (●) 150 μM $A\beta_{1-16}$ + 15 μM Cu^{II}, (□) 300 μM peptide + 30 μM Cu^{II}, and (▼) 150 μM $A\beta_{1-16}$ + 30 μM Cu^{II}. All samples were measured in 10% D_2O, pH 7.4 at 37°C. Solid lines are a) fits of monoexponential decays to the experimental data points or b) simulated traces with $k_3 = 0.66\ \mu M^{-1}s^{-1}$ and $k_{-3} = 276\ s^{-1}$, see Scheme 1, Equation (2).

of Cu^{II}. In all the NMR experiments the Cu^{II}/Aβ molar ratio is less than or equal to 0.1. Nonetheless, the decay of the NMR signal reflects that all Aβ molecules in the sample have experienced Cu^{II} coordination to H6. Thus, either the Cu^{II} is rapidly bound and dissociated or the Cu–Aβ and the [Cu–Aβ]* complexes bind to another peptide in a metal-ion-bridged species.

Monoexponential R_1 decays were observed for the peak from H6 $H^{\delta2}$ (Figure 2a) and the two peaks from H13 + H14 $H^{\delta2}$ and H13 + H14 $H^{\varepsilon1}$. The $R_{1,PRE}$ determined from the three peaks gave average apparent rate constants, k_{app}, of $(10.7 \pm 0.5)\ s^{-1}$, $(9.6 \pm 0.5)\ s^{-1}$, $(5.9 \pm 0.8)\ s^{-1}$, and $(4.7 \pm 0.7)\ s^{-1}$ for total concentrations $c_{A\beta1-16}/c_{Cu}$ of 150 μM/30 μM, 300 μM/30 μM, 150 μM/15 μM, and 300 μM/15 μM, respectively. Thus, the observed rate depends on the total concentration of both $A\beta_{1-16}$ and Cu^{II}. This behavior is inconsistent with the two-step mechanism [Equation (1) in Scheme 1] that was initially found to be sufficient to describe the stopped-flow data. In that model, the rates observed in the NMR experiments would depend only on the concentration of free Cu^{II}, thus the expected K_d in the low or subnanomolar range[9] is determined by the ratio $c_{Cu}/(c_{A\beta1-16} - c_{Cu}II)$. Hence the overall rate-limiting step in Equation (1) would be the dissociation of Cu^{II} from Cu–Aβ (k_{-1}). Direct exchange of Cu^{II} between the Cu–Aβ complex and the pool of free Cu^{II} would also infer that the off-rate for the complex is in the order of 50–90 s^{-1}. However, k_{-1} derived from the stopped-flow data is two to three orders of magnitude too slow to explain the NMR experiments (see the Supporting Information). Instead we propose that the exchange of Cu^{II} proceeds by a metal-ion-bridged ternary complex (Aβ–Cu–Aβ) as seen for other proteins[11] and also suggested for Aβ.[12] This requires an extension of the binding model with Equation (2) in Scheme 1. In this model, we assumed for simplicity that [Cu–Aβ]* is the precursor for the ternary complex. However, from our current data we cannot determine if [Cu–Aβ]*, Cu–Aβ, or both are able to interact with free Aβ molecules. Rate constants for the proposed minimum three-step model were obtained by combining numerical fitting of the stopped-flow data with NMR relaxation data and simulations (see the Supporting Information). The Aβ–Cu–Aβ complex could not be detected in the

 © 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

stopped-flow experiments where the peptide concentration is 3.8–7.5 times lower than in the NMR experiments (see Supporting Information). The combined analysis of stopped-flow and NMR data thus demonstrates that the first higher-order species of $A\beta_{1-16}$ to form in the presence of Cu^{II} is a transient dimer that coordinates one equivalent of Cu^{II}.

To determine if our model also accounts for the initial events in binding of Cu^{II} to $A\beta_{1-40}$, a series of stopped-flow experiments were performed with $A\beta_{1-40}$. Two subsecond phases similar to those observed in $A\beta_{1-16}$ were detected upon Cu^{II} binding (Figure 1b). The kinetics of the two initial phases could be described by the same model used for the binding to $A\beta_{1-16}$ and with almost identical rate constants and affinities (see the Supporting Information). Thus the initial binding mechanism of Cu^{II} to $A\beta_{1-40}$ is most likely very similar to that in $A\beta_{1-16}$. Our model also explains previous findings of line broadening induced by substoichiometric amounts of Cu^{II} in NMR spectra of $A\beta_{1-40}$.[13] Such line broadening infers that Cu^{II} exchanges faster between different $A\beta_{1-40}$ molecules than explained by a kinetic model including only Equation (1) in Scheme 1.

On timescales longer than 1 s, $A\beta_{1-16}$ and $A\beta_{1-40}$ behave differently. For $A\beta_{1-40}$, a third phase in the fluorescence signal is observed, which is absent for $A\beta_{1-16}$ (Figure 1a and b). This late phase parallels what is observed by stopped-flow Rayleigh scattering of the Aβ–Cu^{II} binding process. In this experiment, where the formation of higher-order species is probed,[14] the light scattering of $A\beta_{1-16}$ did not change significantly upon mixing with Cu^{II} (Figure 1c), thus supporting that the dominant species is the monomer under the experimental conditions employed. In contrast, a pronounced increase in light scattering with a rate that is dependent on the Cu^{II} concentration is observed for timescales longer than 1 s for $A\beta_{1-40}$ (Figure 1d). The observation that the rate of oligomerization increases with the concentration of Cu^{II} and thus the concentration of Cu–Aβ and [Cu–Aβ]* suggests that one or both of these Aβ species are direct precursors of the observed oligomers as previously proposed.[15] The light-scattering signal for apo-$A\beta_{1-40}$ remains constant throughout the experiment (Figure 1d), hence indicating that spontaneous oligomerization does not take place on the seconds-to-minutes time scale. This behavior is consistent with previous observations.[16] However, we cannot conclude any further on the initial steps of the oligomerization pathway in the absence of Cu^{II}.

Our current minimal model of the initial steps in Cu^{II} binding to $A\beta_{1-40}$ is summarized in Figure 3. It should be emphasized that although the identified Aβ–Cu–Aβ complex is the multimeric Aβ species that kinetically is most directly accessible from the monomeric state, it is unlikely to be a direct precursor for higher-order oligomers or aggregates. As increasing Cu^{II} concentrations above 0.5 equivalents inevitably lead to lowered concentrations of the Aβ–Cu–Aβ complex, a reduced rate of oligomerization of Aβ would be expected if this complex was a precursor—in contrast to what is observed. The model provides an explanation for the observation that low concentrations of Cu^{II} destabilize $A\beta_{1-40}$ oligomers and inhibit fibrillation.[17] As the Aβ–Cu–Aβ complex apparently does not oligomerize or aggregate, this may suggest selective stabilization of this complex as a strategy to avoid Aβ oligomerization and aggregation.

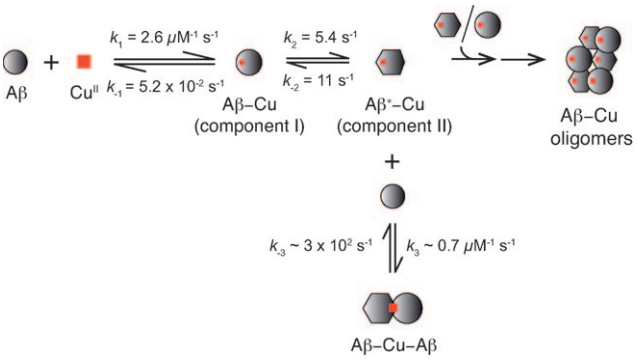

Figure 3. Schematic minimal mechanism for initial binding of Cu^{II} to Aβ followed by the copper(II)-induced Aβ oligomerization based on this study. k_1, k_{-1}, k_2, and k_{-2} are determined from fitting the $A\beta_{1-40}$ stopped-flow data while k_3 and k_{-3} are derived from $A\beta_{1-16}$ NMR relaxation and simulation. Components I and II refer to the complexes formed at low and high pH values, respectively.[6a,b]

Received: October 8, 2010
Revised: December 28, 2010
Published online: February 17, 2011

Keywords: Alzheimer's disease · amyloid β peptides · copper · kinetics · peptides

[1] a) M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, W. R. Markesbery, *J. Neurol. Sci.* **1998**, *158*, 47–52; b) L. M. Miller, Q. Wang, T. P. Telivala, R. J. Smith, A. Lanzirotti, J. Miklossy, *J. Struct. Biol.* **2006**, *155*, 30–37.

[2] a) G. F. da Silva, L. J. Ming, *Angew. Chem.* **2007**, *119*, 3401–3405; *Angew. Chem. Int. Ed.* **2007**, *46*, 3337–3341; b) X. Huang, M. P. Cuajungco, C. S. Atwood, M. A. Hartshorn, J. D. Tyndall, G. R. Hanson, K. C. Stokes, M. Leopold, G. Multhaup, L. E. Goldstein, R. C. Scarpa, A. J. Saunders, J. Lim, R. D. Moir, C. Glabe, E. F. Bowden, C. L. Masters, D. P. Fairlie, R. E. Tanzi, A. I. Bush, *J. Biol. Chem.* **1999**, *274*, 37111–37116; c) X. D. 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, A. I. Bush, *Biochemistry* **1999**, *38*, 7609–7616.

[3] A. Olofsson, M. Lindhagen-Persson, M. Vestling, A. E. Sauer-Eriksson, A. Ohman, *FEBS J.* **2009**, *276*, 4051–4060.

[4] a) D. P. Smith, G. D. Ciccotosto, D. J. Tew, M. T. Fodero-Tavoletti, T. Johanssen, C. L. Masters, K. J. Barnham, R. Cappai, *Biochemistry* **2007**, *46*, 2881–2891; b) D. P. Smith, D. G. Smith, C. C. Curtain, J. F. Boas, J. R. Pilbrow, G. D. Ciccotosto, T. L. Lau, D. J. Tew, K. Perez, J. D. Wade, A. I. Bush, S. C. Drew, F. Separovic, C. L. Masters, R. Cappai, K. J. Barnham, *J. Biol. Chem.* **2006**, *281*, 15145–15154; c) Y. Yoshiike, K. Tanemura, O. Murayama, T. Akagi, M. Murayama, S. Sato, X. Y. Sun, N. Tanaka, A. Takashima, *J. Biol. Chem.* **2001**, *276*, 32293–32299; d) G. M. Shankar, S. Li, T. H. Mehta, A. Garcia-Munoz, 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, *Nat. Med.* **2008**, *14*, 837–842.

[5] a) C. D. Syme, R. C. Nadal, S. E. J. Rigby, J. H. Viles, *J. Biol. Chem.* **2004**, *279*, 18169–18177; b) C. J. Sarell, C. D. Syme, S. E. Rigby, J. H. Viles, *Biochemistry* **2009**, *48*, 4388–4402.

 © 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[6] a) P. Dorlet, S. Gambarelli, P. Faller, C. Hureau, *Angew. Chem.* **2009**, *121*, 9437 – 9440; *Angew. Chem. Int. Ed.* **2009**, *48*, 9273 – 9276; b) S. C. Drew, C. J. Noble, C. L. Masters, G. R. Hanson, K. J. Barnham, *J. Am. Chem. Soc.* **2009**, *131*, 1195 – 1207; c) S. C. Drew, C. L. Masters, K. J. Barnham, *J. Am. Chem. Soc.* **2009**, *131*, 8760 – 8761; d) C. Hureau, Y. Coppel, P. Dorlet, P. L. Solari, S. Sayen, E. Guillon, L. Sabater, P. Faller, *Angew. Chem.* **2009**, *121*, 9686 – 9689; *Angew. Chem. Int. Ed.* **2009**, *48*, 9522 – 9525; e) J. W. Karr, V. A. Szalai, *J. Am. Chem. Soc.* **2007**, *129*, 3796 – 3797; f) T. Kowalik-Jankowska, M. Ruta, K. Wisniewska, L. Lankiewicz, *J. Inorg. Biochem.* **2003**, *95*, 270 – 282.

[7] a) J. Kardos, I. Kovács, F. Hajós, M. Kálmán, M. Simonyi, *Neurosci. Lett.* **1989**, *103*, 139 – 144; b) M. L. Schlief, A. M. Craig, J. D. Gitlin, *J. Neurosci.* **2005**, *25*, 239 – 246.

[8] K. A. Johnson, Z. B. Simpson, T. Blom, *Anal. Biochem.* **2009**, *387*, 20 – 29.

[9] P. Faller, C. Hureau, *Dalton Trans.* **2009**, 1080 – 1094.

[10] L. Guilloreau, L. Damian, Y. Coppel, H. Mazarguil, M. Winterhalter, P. Faller, *J. Biol. Inorg. Chem.* **2006**, *11*, 1024 – 1038.

[11] U. Heinz, L. Hemmingsen, M. Kiefer, H. W. Adolph, *Chem. Eur. J.* **2009**, *15*, 7350 – 7358.

[12] a) C. S. Atwood, R. C. Scarpa, X. Huang, R. D. Moir, W. D. Jones, D. P. Fairlie, R. E. Tanzi, A. I. Bush, *J. Neurochem.* **2008**, *75*, 1219 – 1233; b) M. Rózga, A. M. Protas, A. Jablonowska, M. Dadlez, W. Bal, *Chem. Commun.* **2009**, 1374 – 1376.

[13] C. C. Curtain, F. Ali, I. Volitakis, R. A. Cherny, R. S. Norton, K. Beyreuther, C. J. Barrow, C. L. Masters, A. I. Bush, K. J. Barnham, *J. Biol. Chem.* **2001**, *276*, 20466 – 20473.

[14] D. Noy, I. Solomonov, O. Sinkevich, T. Arad, K. Kjaer, I. Sagi, *J. Am. Chem. Soc.* **2008**, *130*, 1376 – 1383.

[15] C. Talmard, L. Guilloreau, Y. Coppel, H. Mazarguil, P. Faller, *ChemBioChem* **2007**, *8*, 163 – 165.

[16] C. S. Atwood, R. D. Moir, X. D. Huang, R. C. Scarpa, N. M. E. Bacarra, D. M. Romano, M. K. Hartshorn, R. E. Tanzi, A. I. Bush, *J. Biol. Chem.* **1998**, *273*, 12817 – 12826.

[17] K. Garai, P. Sengupta, B. Sahoo, S. Maiti, *Biochem. Biophys. Res. Commun.* **2006**, *345*, 210 – 215.

 © 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim