Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2243–2248

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

BINDING OF ZN(II), CU(II), AND FE(II) IONS TO ALZHEIMER'S Aß PEPTIDE STUDIED BY FLUORESCENCE

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Received 10 February 1999; accepted 28 June 1999

Abstract: Binding of Zn(II), Cu(II) and Fe(II) ions to Aβ1-40, Aβ1-42 and a single tryptophan mutant of Aβ1-40 in solution at pH 7.4 was studied by fluorescent titration. Job plots and fitting of titration curves revealed formation of 1:1 and 1:2 peptide-metal complexes. For dimeric peptides Aβ1-40 and AβF4W the order of metal to peptide affinities is Fe < Cu > Zn, which is in agreement with the Irving–Williams series of complex stability. The affinity of Aβ1–42 for Fe increases dramatically upon aggregation: K_D changes from ca. 100 to ca. 0.2 μM.

Introduction

Amyloid β protein (Aβ), a 39-43 amino acid peptide, is a primary component of the amyloid fibrils, which are deposited in the brains of patients with Alzheimer's disease (AD). It has been proposed that amyloid deposition is a critical event in the formation of senile plaques and a major causative factor in AD.[1] Exposure of Aß to certain metals (Al, Fe, and Zn) was found to induce the aggregation of the peptide.[2] In the case of zinc, a high-affinity binding site (K_D = 107 nM) and a low-affinity binding site (K_D = 3.2–5.2 μM)[3–5] were identified by membrane radioisotope binding assay. The concentration of zinc required for Aβ aggregation is controversial. Other groups reported that a zinc concentration of at least 10^{-4} M is required to induce significant aggregation of Aβ.[6,7] Thus, it is difficult to assess the significance of zinc binding as a potential pathological inducer of amyloid aggregation without resolving what the actual affinity is. Atwood et al.[8] found that Cu^{2+} unlike other biometals, induced aggregation of physiological concentrations (low nM) of Aβ1-40 and Aβ1-42 as the solution pH was lowered from 7.4 to 6.8. The binding affinity of Cu^{2+} for Aβ1-40 and Aβ1-42, based upon shift in absorbance at 214 nm, was estimated as 4.0 and 0.3 μM. Ferritin, the main intracellular iron storage protein in the brain, is found in high concentration in AD brains compared with age matched controls.[9,10] This is thought to be attributable to the increase in ferritin-rich activated microglia in AD brains, which cluster around amyloid plaques.[11,12] It has also been proposed that iron accumulation in Alzheimer's disease serves as a source of damaging free radicals.[13] The knowledge of thermodynamic stability of amyloid peptide–metal complexes, especially in solution under physiological conditions, is of an obvious importance for the interpretation of metal effects on the formation and properties of amyloid deposits. Other groups have studied the metal binding of Aß amyloid by indirect measurements that rely on aggregation as an end point[2–7]. In order to define the role of metals in amyloid aggregation, here we report metal-induced changes in the tyrosine fluorescence of Aβ1-40 and Aβ1-42, as well as a tryptophan substitution of Aβ1-40, for monitoring the metal binding in solution.

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00357-1

Materials and Methods

All peptides and single tryptophan Aβ peptide analogs were synthesized by fluoren-9-ylmethoxy carbonyl chemistry using a continuous flow semiautomatic instrument as described previously.[14] We introduced a single tryptophan in Aβ1-40 peptide at position 4 (AβF4W). No differences were found in the aggregation properties of Aβ1-40 and AβF4W.[14]

Curve fitting. Data were fitted to their respective theoretical equations by using the nonlinear least-squares fitting procedure implemented with the Origin 3.5 program. In all figures the curves are theoretical best-fitting plots constructed with the parameters given in the text. In cases where a large excess of the metal (M) over the peptide is employed, the equations for binding isotherms are:[15]

for 1:1 complexation:

$$I = (I_0 + I_C K[M])/(1+K[M]) \qquad (1)$$

where I_0 and I_C are the fluorescence intensities of free and complexed peptide, [M] is the total metal concentration and K is the association constant:

$$K=1/K_d. \qquad (2)$$

for consecutive 1:1 and 1:2 complexation with association constants K_1 and K_2:

$$I = (I_0 + I_{C1}K_1[M] + I_{C2}K_1K_2[M]^2)/(1+K_1[M] + K_1K_2[M]^2) \qquad (3)$$

where I_{C1} and I_{C2} are the fluorescence intensities of 1:1 and 1:2 stoichiometric complexes, respectively.

For 1:1 complexation with similar peptide and metal concentrations:[15]

$$I = I_0 + 0.5(\Delta I/[P])\times([P] + [M] + K_d - (([P] + [M] + K_d)^2 - 4[P][M])^{1/2}) \qquad (4)$$

where [P] and [M] are the total concentrations of peptide and metal, ΔI is the difference in fluorescence intensities of free and complexed peptide.

Method of continuous variations. The continuous variations method, or Job-plot analysis, was originally developed to determine the composition of metal–ligand complexes in solution.[15,16] This method was recently used to study the Thioflavin T binding to Aß1-40 amyloid fibrils by stopped-flow kinetics.[17] The binding stoichiometry was determined by this method, in which the sum of the concentration of Aβ1-40 or AβF4W and the metal was kept at 50 μM, (Zn^{2+}), 10 μM (Cu^{2+}), and 20 μM (Fe^{2+}); for Aß1-42: 30 μM (Zn^{2+}), 10 μM (Cu^{2+}), and 20 μM (Fe^{2+}), respectively.

Results and Discussion

Peptide complexation with Zn and Cu. Job plots of the fluorescence intensity corrected for that of free peptide vs. peptide molar fraction for all peptides with Zn showed maxima corresponding to peptide-to-metal stoichiometry between 1:1 and 1:2. Therefore, the fitting of binding isotherms (Figure 1A) was performed both with equations 1 and 3. For Aβ1-42 and AβF4W, fitting for the 1:2 model was divergent, meaning that under the conditions of the titration experiment, contribution of the second complex is negligible and equation 3 becomes overparametrized. For Aβ1-40 convergent fits were obtained with both equations, but the

improvement of the fit with equation 3 was statistically insignificant and the respective binding constants were determined with very low precision. Therefore, in further consideration only the K_D value for 1:1 complex is taken into account. All dissociation constants are summarized in Table 1.

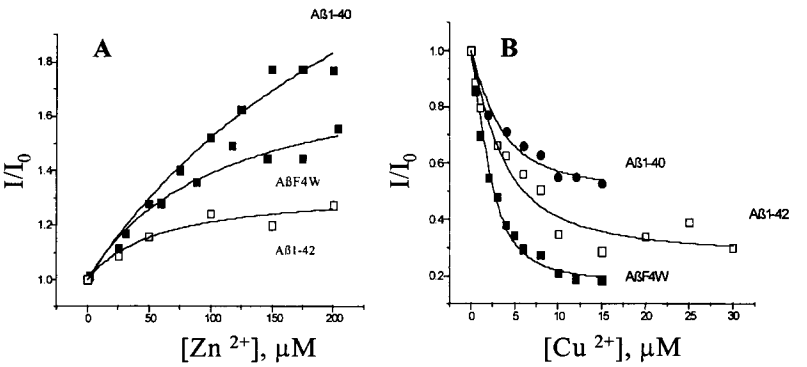

Figure 1. Fitting of the results for Aß (3 μM) with Zn^{2+} (A) and Cu^{2+} (B) in 10 mM Tris HCl/ 0.1 M NaCl (pH 7.4); λex = 280 nm for Aß1-40 and Aß1-42 and λex = 295 nm for AßF4W, λem = 310 nm for for Aß1-40 and Aß1-42 and λem = 350 nm for AßF4W.

The Job plots for Cu^{2+} ion showed the stoichiometry closer to 1:1 with some possible contribution from a 2:1 peptide–metal complex (probably this reflects formation of a single binding site per dimer). The fitting of binding isotherms (Figure 1B) to the 1:1 model with equation 4 was always satisfactory, and dissociation constants are given in Table 1. Our results with Zn are in agreement with Esler et al.[6] We did not find any high-affinity binding of Zn as reported by Bush et al.[3,5] If there is a high-affinity zinc binding site, it is not observable by fluorescence spectroscopy.

Results for Zn^{2+} (Aß1-40) in the presence of Cu^{2+} (2 μM) show there is no competition between metals. The fluorescence intensity increased, but it "saturated" at a level lower than that for free peptide and much lower than would be observed for a Zn^{2+} complex with peptide. Furthermore, the K_d was even smaller than that for free peptide (Table 1) while in the case of competition it must be larger. It seems that even a certain cooperativity exists in the binding of these metals. The K_d of Zn^{2+} for Aβ1-40 is approximately an order of magnitude smaller in the presence of Cu^{2+}, than for a free peptide.

In the case of Cu^{2+}, we found a similar affinity for Aß1-42 (K_d = 2.0 μM) and Aß1-40 (K_d = 1.6 μM), while Atwood et al.[8] reported a ten–fold higher affinity for Aß1-42 (K_d = 0.3 μM) than for Aß1-40 (K_d = 4 μM).

Peptide complexation with Fe. Results for Fe^{2+} were more complicated and Job plots were rather scattered in this case. Binding isotherms are shown in Figure 2.

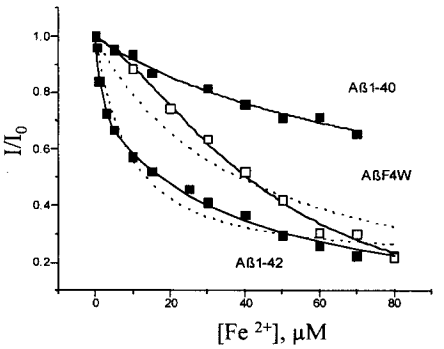

Figure. 2. Fitting of the results for Aß with Fe. For Aβ1-40 fitting was adjusted to the 1:1 model. For Aβ1-42 and AßF4W dotted lines show the fitting to 1:1 model, solid lines - 1:2 model.

In the case of Aβ1-40, fitting to the 1:1 model was satisfactory. However, for Aβ1-42 and AßF4W, the binding isotherms clearly showed a much better fit to a 1:2 complexation model. The first dissociation constant with Aβ1-42 is too small for an accurate calculation; only an upper estimate could be made in this case. Binding of Fe^{2+} to AβF4W demonstrated a positive cooperativity that was noted above for Zn^{2+} binding in the presence of Cu^{2+}. The second dissociation constant is ca. six-fold smaller than the first one. For Aβ1-42 the second constant was much larger than the first.

Addition of Zn causes fluorescence enhancement, while both Cu and Fe quench the fluorescence partially or completely. A quenching effect is typical for transition metal ions.[18] Observation of the quenching effect with these metals suggests the possibility of direct contact between bound metal ion and peptide fluorophore. The enhancement effect of Zn probably reflects a conformational change in the peptide that brings the fluorophore into a more hydrophobic environment.[18] This is in agreement with a smaller enhancement effect for the less soluble Aβ1–42 peptide, which presumably exists in solutions in an aggregated form at the concentration used in the titration experiments (3 μM). Another possibility is the coordination of Zn with peptide histidine or lysine residues, which are effective quenchers of both tyrosine and tryptophan fluorescence.[19] In the cases of Aβ1–40 and AβF4W, the order of metal to peptide affinities was Fe < Cu > Zn, which is in agreement with the Irving–Williams series of complex stability.[20] However, for Aβ1–42 the affinity of Fe for the peptide at one of its binding sites increases dramatically. To test the possible contribution of oxidized Fe^{3+} form, we performed titration experiments in the presence of ascorbic acid, Figures 3A and 3B. Evidently, the quenching effect is even stronger with ascorbate. The dissociation constants calculated by fitting the plots in figures 3A and 3B were 13 and 0.33 μM for Aβ1–40 and Aβ1–42, respectively. Therefore, the unusually high affinity of Aβ1–42 peptide for Fe^{2+} is not associated with its oxidation to Fe^{3+}.

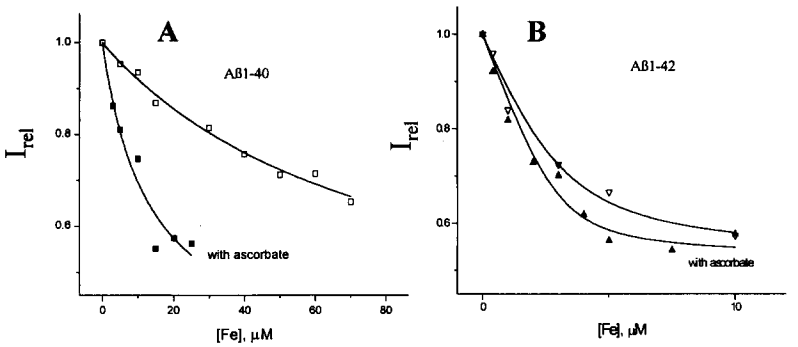

Figure 3. Titration of Aß 1–40 (A) and Aß1-42 (B) peptide in the absence and in the presence of ascorbate.

The strongly increased affinity of Fe^{2+} for the aggregated (Aβ1–42), as compared to its dimeric form, indicates that physiological concentrations of iron stabilize the aggregate. This high-affinity binding of iron to aggregated Aß1-42 may explain the observation that the removal of iron from senile plaques by deferoxamine is very slow.[13] The observation of tight binding of Fe^{2+} to the aggregated peptide not only suggests its possible role in stabilizing the aggregated state, but also explains why this cation, which is less powerful complexing cation than Cu^{2+} and even than Zn^{2+}, has elevated levels in senile plaques.[21] In the case of Zn, the K_d for aggregated Aβ1–42 was also smaller than that of non-aggregated Aβ1–40, but it is still one order of magnitude larger than that reported from membrane assays (see Introduction). This is in a qualitative agreement with the necessity of rather high Zn concentrations to induce the precipitation. The dissociation constants of the complexes of various peptides forms with Cu ions found in the present study are in good agreement with those reported previously; and no difference in affinity between dimeric and aggregated forms of the peptide is observed for this metal. Our results are of interest in light of the recent observation that Cu, Fe and Zn concentrations are elevated in senile plaques in AD.[21]

Table 1. (errors are standard deviations)

Metal	peptide	K_d, µM	K_{d1}, µM	K_{d2}, µM	I_C/I_0
Zn	AßF4W	90 ± 30			1.77
	Aβ1–40	300 ± 100			2.16
	Aβ1–42	57 ± 28			1.34
	Aß1-40(+Cu)	26 ± 14			
Cu	AßF4W	0.61 ± 0.17			0.185
	Aβ1–40	1.6 ± 0.9			0.53
	Aβ1–42	2.0 ± 0.8			0.26
Fe	AßF4W		130 ± 28	19 ± 6	0
	Aβ1–40	76 ± 20			0.28
	Aβ1–42		0.2	36 ± 10	0

Acknowledgments. This work was supported by Grant NS31230 from National Institute of Health and through access to the Laser Microbeam and Medical Program (LAMMP) and the Clinical Cancer Center Optical biology Shared Resource at the University of California at Irvine (facilities are supported by National Institute of Health Grants RR-01192 and CA-62203). W.G.R acknowledges support from the S.N.I. of Mexico.

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