Use of a new water-soluble Zn sensor to determine Zn affinity for the amyloid-β peptide and relevant mutants

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1-Materials and methods.

Chemicals.
Reagents were commercially available and were used as received. The Zn(II) ion source was Zn(SO$_4$)(H$_2$O)$_7$. A unique stock solution at 0.1 M was prepared.

Peptides.
Aβ16 peptide (sequence DAEFRHDSGYEVHHQK and referred to as Aβ in the following) and the modified counterparts (Ac-Aβ, Ac-DAEFRHDSGYEVHHQK; H6A-Aβ, DAEFRADSGYEVHHQK, H13A-Aβ, DAEFRHDGYSVVAHK; H14A-Aβ, DAEFRHDSGYVAHK; D1N-Aβ, NAEFRHDSGYHHQK; E3Q-Aβ, DAQFRHDSGYHHQK; D7N-Aβ, DAEFRHNSGYHHQK; E11Q-Aβ, DAEFRHDGYSVHHQK and Y10F-Aβ) were bought from GeneCust (Dudelange, Luxembourg) with purity grade > 98%.

Stock solutions of the peptides were prepared by dissolving the powder in milliQ water (resulting pH ~ 2). Peptide concentration was then determined by UV-visible absorption of Tyr10 considered as free tyrosine (at pH 2, $(\varepsilon_{276}-\varepsilon_{296}) = 1410$ M$^{-1}$cm$^{-1}$). For the Y10F-Aβ mutant, the absorption of the two Phe ($(\varepsilon_{258}-\varepsilon_{280}) = 390$ M$^{-1}$cm$^{-1}$) was used. pH of the peptide stock solutions were then adjusted at a pH value the closest possible to pH 7.1 to avoid any pH drift upon addition of the peptide during the competition experiments. Indeed the $K_a$ value of the L$_2$-Zn complex is highly dependent with pH, and as a direct consequence, the measurements must be (and have been) done under the very same pH conditions.

Ligand Na$_2$H$_2$L$_2$.
The sodium salt of the H$_2$L$_2$ ligand ($N,N'$-Bis[(5-sulfonato-2-hydroxy)benzyl]-N,N'$-dimethyl-ethane-1,2-diamine) was prepared as previously described.$^1$ 0.1 M stock solution was prepared according to the molecular mass determined by elemental analysis (resulting pH ~8).

Methods.
UV-Vis spectra were recorded on an Agilent 8453 spectrometer at 25°C in 1 cm path length quartz cuvette.

Protonation and Zn binding constants. Equilibrium constants for protonation and Zn(II) complexation reactions with the ligand H$_4$L$_2$ were determined by protometric titrations in a 0.1 M KNO$_3$ aqueous medium. Protometric titrations were fully automated using an automatic titrator composed of a microprocessor burette Metrohm Dosimat 665, a metrohm 6 0234100 glass electrode with an incorporated Ag/AgCl reference and a pH-meter metrohm 713 connected to a computer. All measurements were performed within a thermoregulated cell at 25 °C under an argon stream to avoid the dissolution of carbon dioxide. An HNO$_3$ solution at exactly 10$^{-2}$ M was used to calibrate the electrode. For a classical titration, a total of 120 to 150 points (volume of titrant, pH) was taken. All the used commercial reagents were of the highest purity (> 99%) and were used without further purification. The stock solution of ligand was prepared in distilled water at a concentration of 1 mM in presence of 5 mM HNO$_3$. A Zn(NO$_3$)$_2$ solution was prepared at a concentration of 10$^{-2}$ M in presence of 3.10$^{-2}$ M HNO$_3$. Zn(II) concentration was precisely determined by ICP-AES (Liberty Series II, Varian). The titrating solution of carbonate-free base KOH was prepared from a standardized 1M solution (Prolabo).
Acidity constants for the ligand H₄L₂ were determined by titrating 20 mL of 0.75 and 1 mM ligand with 0.1 M KOH. The stability constants of Zn(II) with H₄L₂ were determined by titrating a solution of 0.83 mM Zn(II) and 1.0 mM ligand (1:1.2 metal:ligand molar ratio) with 0.1 M KOH. The protonation and formation constants were calculated from potentiometric data by HYPERQUAD software and the computer program HYSS was used to plot the species distribution curves in the pH range 2-12. Five independent titrations were used in determining the final values. The value of used pKₖw was 13.77. The global acidity constants β₀lh of H₄L₂ and global stability constants βₘlh of Zn(II) complexes are defined by equations (1) and (2):

\[ \text{mM} + \text{lL} + \text{hH} \rightarrow \text{M}_{\text{m}}\text{L}_{\text{l}}\text{H}_{\text{h}} \]  
(Eq. 1)

\[ \beta_{\text{mn}} = \frac{[\text{M}_{\text{m}}\text{L}_{\text{l}}\text{H}_{\text{h}}]}{[\text{M}]^{\text{m}}[\text{L}]^{\text{l}}[\text{H}]^{\text{h}}} \]  
(Eq. 2)

in which m, l and h are values in the general ligand (m=0) and complex formula for [MₘLₙHₜ]. M, L and H correspond to the metal ion, the ligand L, and the protons, respectively (in sake of clarity, the charges are omitted). The calculated uncertainties for log β and pKa were determined on the basis of the standard deviation.

**Competition experiments between L₂ and Aβ peptides** have been monitored by UV-Vis in a hepes buffer 50 mM. In a typical experiment, the ligand L₂ (60 µM, in theory), Zn(II) (50 µM, in theory) was mixed and 1 to 10 equivalents of peptide (compared to the theoretical Zn(II) concentration) added and the UV-Vis spectrum of each of the 12 samples recorded. To be in reproducible pH condition, the same stock buffer solution (adjusted to pH 7.1) was used for a given set of experiments. Two different sets of data, in which all the experiments were repeated 2 or 3 times for each peptide, were obtained with two different starting buffer stock solutions, and the results were coherent. All the competition experiments were performed at 25°C.

**Analysis of the data were performed with a 2-step procedure for each experiment.**

**Step 1.** Real concentration in L₂ was determined using absorbance at 252 nm, with a ε_{252} = 6130 M⁻¹ cm⁻¹ at pH 7.1. Then, real concentration in Zn added was determined using absorbance of the L₂-Zn complex at 252 nm and considering the formation of the L₂-Zn complex with a ε_{252} = 30000 M⁻¹ cm⁻¹. The latter value was determined using Zn titration of L₂ (See Figure S1).

**Step 2.** Change in the absorbance at 252 nm was reproduced according to an in-house procedure using the above-determined L₂ and L₂-Zn concentrations as starting parameters.

Absorbance was calculated according to:

\[ \text{Abs} = ([\text{peptide}] - [\alpha]) \cdot \varepsilon_{252nm}^{\text{peptide}} + [\alpha] \cdot \varepsilon_{252nm}^{\text{peptide-Zn}} + ([\text{L}_2] - [\text{Zn}] + [\alpha]) \cdot \varepsilon_{252nm}^{L_2 - Zn} + ([\text{Zn}] - [\alpha]) \cdot \varepsilon_{252nm}^{Zn} \]

where α stands for the progression of the following reaction: peptide + Zn → peptide-Zn.

\[ \alpha = \frac{-b + \sqrt{\Delta}}{2a} \]

\[ b = \frac{K_{\text{peptide-Zn}}}{L_2 - Zn_d} \cdot ([L_2] - [Zn]) + ([\text{peptide}] + [Zn]) \]

with

\[ \Delta = b^2 - 4 \cdot a \cdot c ; \]

\[ a = \frac{K_{\text{peptide-Zn}}}{L_2 - Zn_d} - 1 ; \]

\[ c = -[\text{peptide}] \cdot [Zn] \]
was adjusted to obtain the best reproduction of the experimental data.

2. Zn titration of the L₂ ligand

![Graph showing UV-Vis spectrum and absorbance value](image)

**Figure S1.** Titration curve of Zn in L₂. Evolution of the L₂ UV-Vis spectrum (left panel) and absorbance value at 252 nm (right panel) upon addition of increasing amount of Zn. [L₂] = 50 µM in hepes buffer 50 mM pH 7.1, ℓ= 1cm, T = 25°C.

The progress of the reaction at the stoichiometry point (SP) is given by $\frac{Abs(SP) - Abs_0}{Abs_{max} - Abs_0}$ and leads to an affinity value of the $L₂ + Zn \rightarrow L₂-Zn$ reaction equals to $K_a = 1.4 \times 10^6$ M⁻¹.
3. Speciation diagram of L₂-Zn

![Speciation diagram of L₂-Zn](image)

**Figure S2:** Solution speciation diagram for the L₂-Zn system with [L₂] = 1 mM and [L₂][Zn²⁺] = 1.2:1. Charges are omitted for the sake of clarity.

**Table S1.** Logarithmic values of the overall acidity constants of the ligand (β₀₁₇) and of the overall stability constants of Zn(II) complexes (βₘ₁₇). (Standard deviation is indicated in parenthesis for the last digit.)

<table>
<thead>
<tr>
<th>log βₘ₁₇</th>
<th>L₂</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>log β₀₄₄</td>
<td>34.24 (7)</td>
<td>5.05</td>
</tr>
<tr>
<td>log β₀₃₃</td>
<td>29.19 (6)</td>
<td>7.79</td>
</tr>
<tr>
<td>log β₀₂₂</td>
<td>21.40 (4)</td>
<td>9.72</td>
</tr>
<tr>
<td>log β₀₁₁</td>
<td>11.68 (4)</td>
<td>11.68</td>
</tr>
<tr>
<td>log β₁₁₁</td>
<td>19.70 (4)</td>
<td>-</td>
</tr>
<tr>
<td>log β₁₁₀</td>
<td>14.03 (1)</td>
<td>-</td>
</tr>
<tr>
<td>log β₁₁⁻¹</td>
<td>2.81 (2)</td>
<td>-</td>
</tr>
</tbody>
</table>

[a] \( \beta_{ₘ₁₇} = \frac{[M_mL_1H_h]}{[M]^m[L]^l[H]^h} \) where mM + 1L + hH \( \xrightarrow{\text{MmL1Hh}} \)

The dissociation constant at pH 7.1 was determined according to:

\[
K_d = \frac{[M] \sum_h [LH_h]}{\sum_h [MLH_h]} = \frac{\sum_h \beta_{₀₁₇}[H]^h}{\sum_h \beta_{₁₁₇}[H]^h}
\]

A \( K_d \) (1/Kₐ) value of 1.2 \( 10^6 \) M⁻¹ was found and used for further competition experiments with peptides.
4. UV-Vis spectra of the Y10F-Aβ16 competition with L₂

![UV-Vis spectra](image)

**Figure S3.** UV-Vis spectra of a solution of L₂ (dotted line), in presence of Zn (solid bold line) and after addition of increasing amount of Y10F-Aβ peptide (Absence of the absorption at 275 nm, compared to Figure 1 in the full text). The arrows indicate the variation of the UV-Vis spectra upon Aβ16 addition. [L₂] = 60 µM, [Zn] = 50 µM, [Y10F-Aβ16] = 50-500 µM, Hepes buffer 50mM, pH 7.1, 25°C.

5. Competition data

![Competition data](image)

**Figure S4.** Experimental normalized Absorbance (dotted black points) and their reproduction (red lines) of L₂-Zn system upon addition of increasing equivalents of peptides. [L₂] = 60 µM, [Zn] = 50µM and [peptides] = 50 - 500 µM, Hepes buffer 50mM, pH 7.1, 25°C.
Figure S5. Experimental normalized Absorbance (dotted black points) and its reproductions (dotted red lines) of L₂-Zn system upon addition of increasing equivalents of Aβ16, with a) $K_a = 1.35 \times 10^5$ M$^{-1}$; b) $K_a = 1.08 \times 10^5$ M$^{-1}$; c) $K_a = 0.91 \times 10^5$ M$^{-1}$. $[L_2] = 60 \, \mu$M, $[Zn] = 50\mu$M and [peptides] = 50 - 500 µM, Hepes buffer 50mM, pH 7.1, 25°C.

Figure S5 shows the sensibility of the fitting procedure. The best reproduction of the experimental data is curve b) obtained for $K_a = 1.08 \times 10^5$ M$^{-1}$. Curves a) and c) are calculated with ± 20% of difference in the $K_a$ value and lead to unsatisfying reproduction of the experimental data. This thus indicates that the error bar on the $K_a$ value are below 20%.
**7-Zn coordination model**

![Scheme S1](image)

**Scheme S1.** Zn coordination model proposed here (A), from ref. 4 and 5 (B), ref. 6 (C) and ref. 7 (D).

**References**