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Heme Binding Induces Dimerization and Nitration of Truncated β-Amyloid Peptide Aβ16 Under Oxidative Stress**

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Several observations suggest a link between (ferric) heme and Alzheimer's disease (AD), for example, an increase in heme oxygenase 1,[1,2] a loss of complex IV,[3–6] and abnormal iron metabolism. These observations suggest that heme metabolism is altered in age-related disorders. As a possible explanation, hemin has been proposed to interact with β-amyloid peptides (Aβ),[7] and it is now well established that Aβ peptides bind ferric heme.[8] The adducts of hemin–Aβ peptides also exhibit increased peroxidase activity with respect to free hemin,[9] but these complexes have not been characterized. Herein we provide thermodynamic and spectroscopic data showing that hemin *b* can coordinate two Aβ16 molecules to form the low-spin, six-coordinated complex [hemin$(Aβ16)_2$], and this is in equilibrium with the high-spin [hemin(Aβ16)] species, the relative amounts of which strongly depend on peptide concentration and temperature. In the presence of H_2O_2, hemin-Aβ16 complexes produce dimerization of the peptide through dityrosine cross-linking, and in addition the peptide undergoes endogenous nitration at Tyr10 when nitrite is also present in solution. This result is important because both cross-linking and nitration at Tyr10 critically enhance Aβ aggregation and plaque formation,[10] thus showing that the binding of heme to Aβ can indeed be a factor contributing to neuroinflammation and Aβ aggregation. The endogenous peroxidative (H_2O_2) and nitrative (H_2O_2/NO_2^-) activities exhibited by the hemin–Aβ16 peptide add to, and are likely more deleterious than, the oxidation[9] and nitration[11] of external substrates which have been previously reported.

Upon adding Aβ16 to a 50 mM aqueous phosphate buffer solution of hemin *b* at pH 7.4 and 300 K, the broad Soret band of the complex near $\lambda = 385$ nm progressively changes to give a sharp, red-shifted band at $\lambda = 414$ nm, with additional visible bands at $\lambda = 535$ and 566 nm (Figure 1C, and see the Supporting Information). The conversion from the high-spin to the low-spin hemin species is complete after addition of

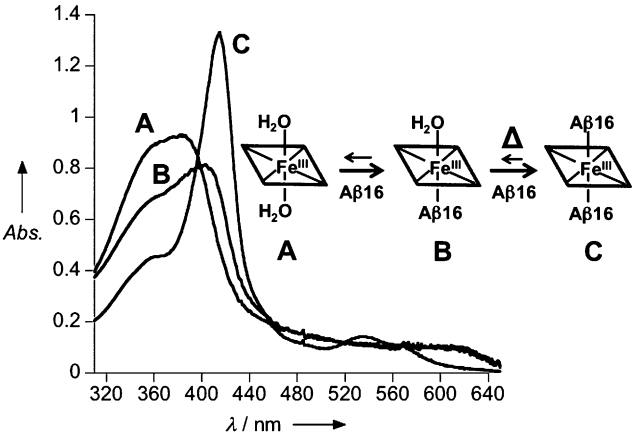

Figure 1. UV/vis spectra recorded in 50 mM phosphate buffer solution at pH 7.4. A) Hemin *b* (1.2×10^{-5} M). B) Hemin *b* in the presence of an excess (20 equiv) of Aβ16 peptide at 319 K. C) The same as (B) but at 300 K, the spectral changes are fully reversible. The Δ symbol indicates an increase in temperature.

20 equivalents of Aβ16, and spectrophotometric data analysis (see the Supporting Information) confirms a 2:1 Aβ16/hemin stoichiometry, thus indicating the formation of the [hemin-$(Aβ16)_2$] complex. The Mössbauer and ^{1}H NMR spectra are consistent with a low-spin ($S = 1/2$) iron(III) center coordinated by two axial imidazole ligands. However, below saturating amounts of Aβ16, the Mössbauer spectrum clearly indicates the presence of a minor high-spin species (Figure 2).

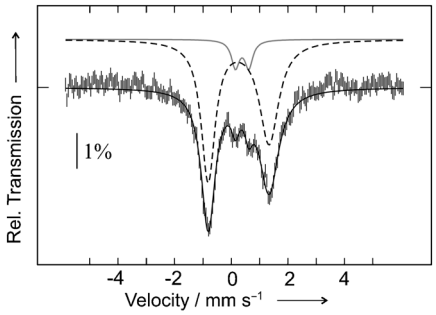

Figure 2. Mössbauer spectrum of hemin *b* (2 mM), in the presence of 6 equivalents of the Aβ16 peptide, recorded at 78 K. Quadrupole doublet simulation reveals the presence of a major low-spin ferric heme (90% of total iron, dashed line: $\delta = 0.26$ mm s^{-1}, $\Delta E_Q = 2.15$ mm s^{-1}) and a minor high-spin ferric heme (gray line: $\delta = 0.38$ mm s^{-1}, $\Delta E_Q = 0.47$ mm s^{-1}). The $S = {}^1/_2$ ground state of the main component was confirmed by low-temperature measurements (see the Supporting Information).

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Fitting of the titration data using standard methods (see the Supporting Information)[12] indicates two binding steps:

$$\text{Step 1}: \quad [\text{hemin}(H_2O)_2] + A\beta16 \rightleftharpoons [\text{hemin}(A\beta16)(H_2O)] + H_2O \tag{1}$$

$$\text{Step 2}: \quad [\text{hemin}(A\beta16)(H_2O)] + A\beta16 \rightleftharpoons [\text{hemin}(A\beta16)_2] + H_2O \tag{2}$$

The binding constant $\beta_2 = 6.6 \times 10^8\,\text{M}^{-2}$ thus obtained (at 300 K) is much larger than that found for hemin *b* with L-histidine ($9 \times 10^2\,\text{M}^{-2}$), or other His-containing peptides under the same conditions (which range from 8.1×10^3 to $4.7 \times 10^7\,\text{M}^{-2}$),[12] thus showing that the peptide remarkably contributes to the stability of the complex.

Performing ^{1}H NMR and UV/visible studies at various temperatures showed that the equilibrium between Aβ16 and hemin *b* is strongly affected by temperature. In fact, in the presence of moderate amounts of Aβ16, low temperature favors the low-spin [hemin(Aβ16)$_2$] species, whereas at higher (e.g. physiological) temperature the high-spin complex [hemin(Aβ16)] is favored (see Figure 1 C and the Supporting Information). The origin of this behavior is clearly explained by the thermodynamic parameters. Both binding steps are exothermic ($\Delta H_1^\circ = -72 \pm 5\,\text{kJ mol}^{-1}$, $\Delta H_2^\circ = -95 \pm 20\,\text{kJ mol}^{-1}$), but formation of [hemin(Aβ16)$_2$] ($\Delta S_2^\circ = -240 \pm 70\,\text{J K}^{-1}\text{mol}^{-1}$) has a higher entropic cost than that of [hemin(Aβ16)] ($\Delta S_1^\circ = -146 \pm 17\,\text{J K}^{-1}\text{mol}^{-1}$). This larger entropic component may be due to the fact that the association of the second peptide to the heme causes additional reduction of the conformational mobility of the bound peptides, probably because they can interact directly. An increase in temperature thus favors the high-spin complex by decreasing K_2 more than K_1 (Table 1), however, the equilibrium between high-spin and low-spin species can be reversed by relatively small changes in temperature (see Supporting Information).

Table 1: Equilibrium constants for hemin binding to Aβ16 in 50 mM phosphate buffer solution pH 7.5 at different temperatures.

T [K]	log K_1	log K_2[a]	log β_2
275	5.96 ± 0.04	5.55	11.51 ± 0.07
287	5.50 ± 0.01	4.23	9.73 ± 0.02
300	4.80 ± 0.02	4.03	8.82 ± 0.02
308	4.56 ± 0.02	3.44	8.00 ± 0.04

[a] Obtained as the difference between log β_2 and log K_1.

The literature data on heme binding to full length Aβ40/Aβ42 peptides are quite variable, with binding constants ranging between 10^4 and 10^5[11] to $7 \times 10^6\,\text{M}^{-1}$.[8b] Our K_1 value of $6.5 \times 10^4\,\text{M}^{-1}$ for [hemin(Aβ16)] at room temperature is thus comparable to the lower end of the range, although it may be expected that the nonpolar C-terminal end of the peptide could further contribute to the stability of the complex. In general, the moderate affinity of Aβ for ferric heme limits its biological relevance under normal reaction conditions, where proteins like hemopexin[13a] and heme oxygenase,[13b] with affinities in the nanomolar range,[14] will rapidly detoxify it. However, during pathological states and brain injury, free heme levels increase,[15] even up to the micromolar range,[15a] and under these conditions the physiological mechanisms of its clearance from the circulation become insufficient, thus allowing heme uptake by other proteins/peptides like Aβ, which particularly accumulates in the brain of AD patients. As a matter of fact, brain iron accumulation is a common feature of AD, Parkinson's disease, and Huntington's disease.[15c,d]

Given the temperature dependence of the equilibrium between [hemin(Aβ16)] and [hemin(Aβ16)$_2$] we then investigated to what extent this affects the peroxidase activity of the complexes. These experiments were carried out with hemin *b* in the presence of variable amounts of the Aβ16 peptide (0, 5, and 20 equiv) using ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] as a representative substrate (see the Supporting information). Even though the peroxidase activity of hemin–Aβ complexes was studied before,[9,11,16] no numerical data showing the real biological significance of this activity have been reported so far. As expected, the catalytic activity of hemin–Aβ16 complexes (as a mixture of 1:1 and 1:2 adducts) increases with temperature (Table 2, left column), but it also increases slightly with an increase in the ratio of Aβ16 to hemin (see the Supporting Information). This data suggests that the coordinated histidine residues in [hemin(Aβ16)$_2$] do not inhibit the interaction between H_2O_2 and the iron(III) center, possibly because of the lability of the iron axial ligands.

Table 2: Kinetic constants for the catalytic oxidation of ABTS by hydrogen peroxide in 50 mM phosphate buffer solution pH 7.4.[a]

Complex	*T* [K]	k_{cat} [s^{-1}]	Complex[b]	k_{cat} [s^{-1}]
[hemin(Aβ16)]	278	0.016	hemin	0.01
[hemin(Aβ16)]	298	0.042	hemin-GH	0.20
[hemin(Aβ16)]	310	0.060	HRP[c]	45.5

[a] [ABTS] = 3 mM, [H_2O_2] = 2.5 mM, [hemin] = 15 μM, [Aβ16] = 30 μM. The activity of free Aβ is indeed negligible, with $k_{cat} < 10^{-3}\ s^{-1}$. [b] *T* = 298 K. [c] At pH 7.0, data from: H. B. Dunford in *Peroxidases in Chemistry and Biology, Vol. 2* (Eds.: J. Everse, K. E. Everse, M. B. Grisham), CRC, Boca Raton, FL, **1991**, pp. 1–24.

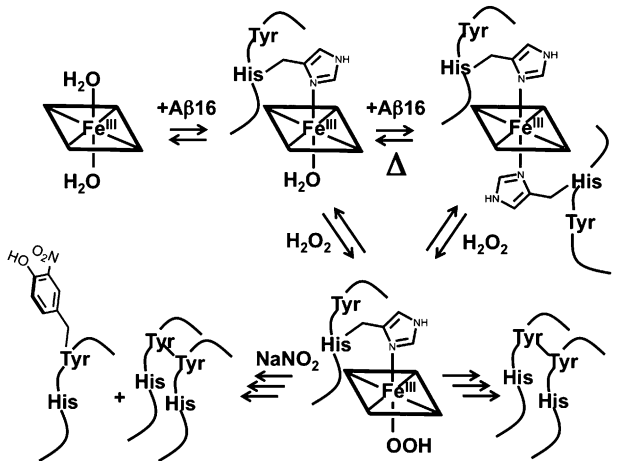
However, as shown by the comparative data reported in Table 2 (right column), the most important outcome of these experiments is that the catalytic activity of hemin–Aβ16 complexes is extremely low. It is only slightly larger than that of free hemin, and much smaller than that of chelated hemin-glycyl-L-histidine (hemin-GH),[17] where a GlyHis-OMe residue is covalently linked to one propionate arm of the porphyrin. Here, the position of the imidazole group is optimized to give a strain-free axial bond with the iron center,[18] and this effect strengthens the interaction between the heme iron and the sixth ligand in the trans position. Therefore, either the imidazole–Fe^{III} interaction in hemin–Aβ16 is not optimal, or the steric arrangement of the peptide around the heme prevents a suitable interaction with the substrate for efficient electron transfer to the heme high-valent intermediate. In any case, it is clear that hemin–Aβ16 is a very modest peroxidase catalyst and the small k_{cat} value

indicates that this activity cannot be of any significance in vivo. In addition, it can be noted that the catalytic activity exhibited by heme model complexes which carry a positively charged distal residue mimicking the role of arginine in peroxidases,[19] as well as that of enzymes like horseradish peroxidase (HRP; Table 2) is larger by several orders of magnitude. This data implies that the proposed role exerted by Arg5 as an acid catalyst promoting cleavage of the peroxide O–O bond[16a] is negligible; no increase in peroxidase activity occurs upon adding free arginine to hemin–Aβ16 (see the Supporting Information).

Given that the peroxidase activity of hemin–Aβ16 complexes toward external substrates is modest, we investigated whether this reactivity could be of importance when addressed on the endogenous peptide. Upon reaction of hemin–Aβ16 with H_2O_2 at physiological temperature, analysis by HPLC/ESI-MS of the peptide fragments showed formation of an Aβ16–Aβ16 dimeric peptide. In the absence of hemin the peptide dimer was not observed. To establish the nature of the covalent bond between the two chains, the peptide product mixture was hydrolyzed (6 M HCl) and analyzed by HPLC/ESI-MS. This enabled us to identify dityrosine in the amino acid mixture, and is consistent with the formation of a 3,3′-dityrosine cross-link between the two Aβ16 chains (see the Supporting Information). A similar type of cross-link was previously observed by the copper(II)-mediated oxidation of amyloid peptide in the presence of H_2O_2[20] and by oxidation of the tau protein using peroxynitrite.[21] Aβ-Aβ cross-linking increases the structural strength of the peptide, thus making it highly resistant to proteolysis and promoting its oligomerization.[20]

The reactivity of hemin–Aβ16 towards H_2O_2 was further studied in the presence of nitrite (1 mM), which typically simulates the conditions of oxidative and nitrative stress.[22] HPLC/ESI-MS analysis of the peptide products showed a reduction in the amount of Aβ16-Aβ16 dimer and the formation of a new product corresponding to a mass increment of 45 amu with respect to native Aβ16. Moreover, the color of the peptide solution visually changed to yellow, thus indicating that the peptide underwent nitration. This was confirmed by the ^{1}H NMR spectrum of the peptide isolated by HPLC, and showed the characteristic signature of nitrated tyrosine (see the Supporting Information). In the absence of hemin, Tyr10 nitration was not observed. Exogenous nitration of two proteins, enolase and glyceraldehyde-3-phosphate dehydrogenase, by heme-Aβ peptides in the presence of nitrite and H_2O_2, was recently reported but the effect on Aβ16 itself was apparently not investigated.[11,23]

This work demonstrates that Aβ16 interacts with hemin *b* forming complexes with one or two Aβ16 molecules, the ratio of which depends on the Aβ16/hemin ratio and on temperature. In spite of the presence of two axial ligands, the six-coordinated [hemin(Aβ16)$_2$] complex binds and activates hydrogen peroxide, probably because the two axial ligands are quite labile, thus allowing a fast exchange between the three histidines at positions 6, 13, and 14 of the peptide. However, the peroxidase activity of hemin–Aβ16 towards external substrates is weak and biologically nonrelevant. In contrast, the endogenous reactivity exerted on the Tyr10 residue leads to the easy formation of a peptide dimer containing dityrosine cross-linking. In addition, Tyr10 is competitively nitrated when nitrite is also present (Scheme 1). Both modifications strongly enhance the aggregation of Aβ peptides,[10,20] thus the association of Aβ with hemin *b* under oxidative stress conditions could be a promoting factor for the Aβ aggregation process.



Scheme 1. Endogenous modification of Aβ16 induced by hemin *b* in the presence of H_2O_2 and H_2O_2/nitrite. Reproducing the effect of oxidative and nitrative stress.

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