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Key roles of Arg^5, Tyr^{10} and His residues in Aβ–heme peroxidase: Relevance to Alzheimer's disease

Naihao Lu [a,*], Jiayu Li [b], Rong Tian [b], Yi-Yuan Peng [a,b,*]

[a] Key Laboratory of Functional Small Organic Molecule, Ministry of Education and College of Life Science, Jiangxi Normal University, 99 Ziyang Road, Nanchang, Jiangxi 330022, China
[b] Key Laboratory of Green Chemistry, Jiangxi Province and College of Chemistry and Chemical Engineering, Jiangxi Normal University, 99 Ziyang Road, Nanchang, Jiangxi 330022, China

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ABSTRACT

Recent reports show that heme binds to amyloid β-peptide (Aβ) in the brain of Alzheimer's disease (AD) patients and forms Aβ–heme complexes, thus leading a pathological feature of AD. However, the important biological relevance to AD etiology, resulting from human Aβ–heme peroxidase formation, was not well characterized. In this study, we used wild-type and mutated human $Aβ_{1-16}$ peptides and investigated their Aβ–heme peroxidase activities. Our results indicated that both histidine residues (His^{13}, His^{14}) in $Aβ_{1-16}$ and free histidine enhanced the peroxidase activity of heme, hence His residues were essential in peroxidase activity of Aβ–heme complexes. Moreover, Arg^5 was found to be the key residue in making the $Aβ_{1-16}$–heme complex as a peroxidase. Under oxidative and nitrative stress conditions, the $Aβ_{1-16}$–heme complexes caused oxidation and nitration of the Aβ Tyr^{10} residue through promoting peroxidase-like reactions. Therefore, these residues (Arg^5, Tyr^{10} and His) were pivotal in human Aβ–heme peroxidase activity. However, three of these residues (Arg^5, Tyr^{10} and His^{13}) identified in this study are all absent in rodents, where rodent Aβ–heme complex lacks peroxidase activity and it does not show AD, implicating the novel significance of these residues as well as human Aβ–heme peroxidase in the pathology of AD.

1. Introduction

Alzheimer's disease (AD) is one of the most common progressive neurodegenerative disorders in the elderly. Many studies have demonstrated that excess amyloid β-peptides (Aβ) in the brain are believed to be the culprits in the neurodegeneration of AD [1–3]. The aggregation of Aβ peptides and generation of reactive oxygen species (ROS) are the two markers of AD and can be responsible for the early oxidative damage observed in AD [3,4]. The role of metal ions (e.g., Cu^{2+}, Fe^{3+}, and Zn^{2+}) in modulating the Aβ aggregation and in generating ROS is being actively investigated due to the fact that brains of AD patients contain abnormally higher levels of metal ions [3,5–7].

Heme, which is a ferroprotoporphyrin IX complex, is essential to the function of a number of proteins. Recent studies show that heme binds to Aβ to form an Aβ–heme complex, which can stabilize the structure of Aβ and inhibit Aβ aggregation [8–11]. However, depletion of biologically required heme by Aβ binding can result in symptoms such as increases in heme synthesis and iron uptake, abnormal iron homeostasis, dysfunction in mitochondrial complex IV, and oxidative stress, etc [8]. On the other hand, the Aβ–heme complex also exhibits increased peroxidase activity with respect to free heme, and can catalyze the oxidation of specific neurotransmitters (such as 3,4-dihydroxyphenylalanine, serotonin) by H_2O_2 [8,9]. This peroxidase activity could be a probable reason for the oxidative damage and abnormal neurotransmission observed in AD patients. The above symptoms are the characteristic pathological features of AD, and thus the formation of Aβ–heme complex opens up a new dimension in AD pathologic research.

Atamna et al. compared heme-binding between human Aβ and rodent Aβ, and found that human Aβ, unlike rodent (i.e., mouse, rat, etc.) Aβ, tightly bound to heme and formed a peroxidase-like complex. Although both human Aβ and rodent Aβ could form aggregates equally, rodents lack AD-like neuropathology [12]. These findings suggest that formation of Aβ–heme peroxidase contributes to human Aβ's neurotoxicity and the increased human susceptibility to AD. The amino acid sequence of rodent Aβ is identical to that of human Aβ except for three amino acids (Arg^5Gly^5, $Tyr^{10}Phe^{10}$, $His^{13}Arg^{13}$) within the hydrophilic region (Fig. 1A), which

* Corresponding authors. Address: Key Laboratory of Functional Small Organic Molecule, Ministry of Education and College of Life Science, Jiangxi Normal University, 99 Ziyang Road, Nanchang, Jiangxi 330022, China (N. Lu). Fax: +86 791 8120391.
E-mail addresses: naihaolu@gmail.com (N. Lu), yiyuanpeng@yahoo.com (Y.-Y. Peng).

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implies possible important roles of the three residues of human Aβ in AD pathology [12]. Furthermore, these amino acids Arg, Tyr, and His are found to participate in heme-binding in heme-proteins and peroxidases [12–15], which drive us to propose that Aβ–heme peroxidase is a key molecular link between these residues present in human Aβ and the increased human susceptibility to AD. Recent results showed that Arg^5 residue was required for Aβ–heme peroxidase activity and His^{13} and His^{14} residues were the heme-binding ligands [16,17].

It is now well established that heme binds to Aβ peptides and the adducts of Aβ–heme complex show higher peroxidase activity than free heme, but the important biological relevance to AD etiology, resulting from human Aβ–heme peroxidase formation, have not been well characterized. In this study, we used wild-type and mutated human $Aβ_{1-16}$ peptides and investigated their Aβ–heme peroxidase activities, and found that Arg^5 and His residues (His^{13}, His^{14}) were critical for human Aβ–heme peroxidase activity. In the presence of oxidative stress (H_2O_2), $Aβ_{1-16}$–heme complex produced dimerization of the peptide through dityrosine cross-linking, and in addition Aβ underwent endogenous nitration at Tyr^{10} when nitrite ($NaNO_2$) was also present. This result was important for the potential pathogenic role of Tyr^{10} in AD, because both dityrosine cross-linking and Tyr^{10} nitration critically accelerated Aβ aggregation and plaque formation [5,14,15], thus implying that the formation of Aβ–heme peroxidase complex under oxidative stress conditions could be a promoting factor for the Aβ aggregation process. Hence, three of these residues (Arg^5, Tyr^{10} and His^{13}) absent in rodent Aβ and formation of human Aβ–heme peroxidase can play a vital role in AD pathology, while rodent Aβ–heme complex lacks peroxidase activity and it does not show AD.

2. Materials and methods

2.1. Materials

L-Histidine (His), L-arginine, ferriprotoporphyrin IX chloride (hemin, which is referred to as "heme" here), and 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and rabbit polyclonal antibody against 3-nitrotyrosine were purchased from Sigma. Soluble Aβ peptides ($Aβ_{1-16}$, $Aβ_{17-40}$, and $Aβ_{1-40}$) and the mutated peptides were synthesized by GL Biochem (Shanghai, China) with >95% purity. The $Aβ_{1-16}$ mutants used were Arg^5Asn (R5 N), His^{13}Ala (H13A), His^{14}Ala (H14A), and double mutant His^{13}Gly, His^{14}Gly (H13G-14G).

2.2. Binding of Aβ with heme

Aβ peptides were dissolved in 16% (v/v) CH_3CN/water, while heme solution was prepared in dimethyl sulfoxide [11]. The stock of heme and Aβ was diluted in 100 mM phosphate-buffered saline (PBS, pH 7.0). Aβ–heme complexes were prepared by incubating 1 equiv of both heme and Aβ solutions at room temperature (~25 °C) for 30 min. Then, the complexes were used for peroxidase activity determination.

2.3. Peroxidase activity measurement

ABTS was used as the substrate to measure the peroxidase activity of heme [11,15]. The assay mixture (in PBS, pH 7.0) contained 5 μM heme, 1 mM ABTS, 0.5 mM H_2O_2, and in the presence or absence of Aβ (or free histidine). The peroxidase activity was measured by monitoring the increase in absorbance at 734 nm. The values were the absorptions subtracted by that at 0 min. The kinetic constants (k_{obs}) were obtained from the initial rates of the reactions and calculated from absorbance vs. time [15].

2.4. Dimerization and nitration of Aβ

In the presence or absence of sodium nitrite ($NaNO_2$, 1 mM), the reaction was carried out by adding H_2O_2 (1 mM) to a solution containing heme (20 μM) and $Aβ_{1-16}$ (100 μM) in PBS (pH 7.0). The mixture was incubated for 60 min at 37 °C in the dark and then analyzed for dimerization and nitration of $Aβ_{1-16}$. Significant protein modifications were observed in short time incubation when high concentrations of heme-H_2O_2-NO_2^- were used in many in vitro experiments [10,11,15]. These high concentrations were, therefore, chosen in our studies to conveniently compare the different effects of Aβ–heme complexes.

Dityrosine (3,3′-dityrosine), an oxidation product of tyrosine produced by reaction between tyrosyl radicals, is a highly stable marker of tyrosyl radical activity and an intensely fluorescent compound. The formation of dityrosine (Aβ–Aβ dimeric peptide) was analyzed by measuring fluorescence spectra [5]. Dityrosine fluorescence was excited at 320 nm and monitored at 350–500 nm. Fluorescence intensity was measured at 410 nm. The spectra were recorded with a fluorescence spectrophotometer PerkinElmer-LS55.

Dot blotting was usually performed in detecting protein tyrosine nitration [14]. A rabbit polyclonal antibody against 3-nitrotyrosine was used for detection of the nitrated Aβ peptide in this study.

2.5. Statistical analysis

All of the experiments were performed at least three times. The results were reported as the means ± SD of at least triplicate determinations. One-way ANOVA was used for statistical analyses, and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. His residues were essential in peroxidase activity of Aβ–heme complexes

It is well-known that heme binds to Aβ peptides in the hydrophilic portion (residues 1–16) and His residues are found to be the possible binding ligands [16–18]. Thus, $Aβ_{1-40}$, $Aβ_{1-16}$ and $Aβ_{17-40}$ fragments were used to investigate the possible relationship between histidine-bound complex and peroxidase-like active site. By following the catalytic oxidation of the substrate ABTS by H_2O_2, the peroxidase activities of free heme and Aβ–heme complexes were investigated. Compared with the free heme, both $Aβ_{1-40}$-heme and $Aβ_{1-16}$-heme complexes showed obviously higher and similar peroxidase activities, while $Aβ_{17-40}$-heme complex showed no enhanced peroxidase activity (Fig. 1B). The result further indicated that the hydrophilic N-terminal of Aβ was involved in heme binding [16–18], and played an important role in promoting the peroxidase activity of heme. This peroxidase activity of Aβ–heme complexes (with k_{obs} of 4.2×10^{-3} min^{-1}) was ~3 times faster compared to that of free heme (with k_{obs} of 1.5×10^{-3} min^{-1}), similar to a previous study [16].

Recent studies have shown that His^{13} and His^{14} residues in the N-terminal hydrophilic region of Aβ are the heme-binding ligands, and His^{13} binds to the iron center of heme preferentially when both residues are present [16–18]. Due to the similar active site environments of the heme-bound complexes in $Aβ_{1-40}$ and $Aβ_{1-16}$, $Aβ_{1-16}$ and site-directed mutants of $Aβ_{1-16}$ including single mutants (His^{13}Ala, His^{14}Ala), and double mutant (His^{13}Gly, His^{14}Gly) were thus used to observe the effect of His residues on peroxidase activity of Aβ–heme complexes. As shown in Fig. 1C, the double mutant (His^{13}Gly, His^{14}Gly) showed no enhanced