Published in final edited form as:

Biochemistry. 2009 August 25; 48(33): 7939–7947. doi:10.1021/bi900907a.

Ternary Complexes of Iron, Amyloid- β and Nitrilotriacetic Acid:

Binding Affinities, Redox Properties, and Relevance to Iron-Induced Oxidative Stress in Alzheimer's Disease+

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Abstract

The interaction of amyloid- β (A β) and redox-active metals, two important biomarkers present in the senile plaques of AD brain, has been suggested to either enhance the A β aggregation or facilitate the generation of reactive oxygen species (ROS). The present study investigates the nature of the interaction between the metal-binding domain of A β , viz, A β (1-16), and the Fe(III) or Fe(II) complex with nitrilotriacetic acid (NTA). Using electrospray ionization mass spectrometry (ESI-MS), the formation of a ternary complex of A β (1-16), Fe(III), and NTA with a stoichiometry of 1:1:1 was identified. MS also revealed that the NTA moiety can be detached via collision-induced dissociation. The cumulative dissociation constants of both A β -Fe(III)-NTA and A β -Fe(II)-NTA were deduced to be $6.3 \times 10^{-21} \text{ M}^2$ and $5.0 \times 10^{-12} \text{ M}^2$, respectively, via measuring the fluorescence quenching of the sole tyrosine residue on A β upon the complex formation. The redox properties of these two complexes were investigated by cyclic voltammetry. The redox potential of the A β -Fe(III)-NTA complex was found to be 0.03 V vs. Ag/AgCl, which is negatively shifted by 0.54 V when compared to the redox potential of free Fe(III)/Fe(II). Despite such a large potential modulation, the redox potential of the A β -Fe(III)-NTA complex is still sufficiently high for occurrence of a range of redox reactions with cellular species. A β -Fe(II)-NTA electrogenerated from A β -Fe(III)-NTA was also found to catalyze the reduction of oxygen to produce H₂O₂. These findings provide significant insight into the role of iron and A β in the development of AD. The binding of iron by A β modulates the redox potential to a level where its redox cycling occurs. In the presence of a biological reductant (antioxidant), redox cycling of iron could disrupt the redox balance within the cellular milieu. As a consequence, not only ROS is continuously produced, but also oxygen and biological reductants can be depleted. A cascade of biological processes can therefore be affected. In addition, the strong binding affinity of A β toward Fe(III) and Fe(II) indicates A β could compete for iron against other iron-containing protein. Particularly, its strong affinity to Fe(II), which is eight orders of magnitude stronger than transferrin, would greatly interfere with the iron homeostasis.

A major hallmark of Alzheimer's disease (AD) is the deposition of aggregates of amyloid- β (A β) peptides in the senile plaques.(1) The *in vivo* aggregation/deposition of A β peptides is suggested to either enhance neurotoxicity or be a result of aberrant cellular processes.(2) The

[‡]This work is supported by research grants (Grant GM 08101 to FZ and R01 CA101864 to YW), and partially supported by the NIH-RIMI Program at California State University-Los Angeles (CSULA, P20 MD001824-01 to FZ) and NSF-LSAMP Program at CSULA (HRD 0331537 to RW). XL thanks the Chinese Academy of Sciences for financial support.

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amyloid cascade hypothesis has also been closely interweaved with its counterpart of oxidative stress.(2,3) A major form of oxidative stress is caused by reactive oxygen species (ROS) whose generation might be facilitated by redox-active metal ions such as Cu(II), Fe(III), and Fe(II). The fact that in the senile plaques there exist high levels of these metal ions (e.g., Cu(II) at ~0.4 mM and Fe(III) at ~0.9 mM)(4) supports this contention. Deleterious effects associated with ROS have been linked to extensive oxidative damage to proteins(5) and DNA,(6-8) declined levels of polyunsaturated fatty acids,(9) increased lipid peroxidation,(3,10) and mitochondrial dysfunction.(11,12)

On the basis that both A β peptides and redox-active metals are concentrated in senile plaques, intensive research efforts have focused on the formation, identification, and characterization of such A β metal complexes.(13-16) It is shown that the hydrophilic domain (residues 1-16) of the full-length A β can ligate Cu(II) through its histidine and possibly glutamate, aspartate and/or tyrosine residues.(4,14,17,18) A Raman analysis of postmortem brain samples has also confirmed the existence of the A β -Cu complex in senile plaques.(19) Although it is known that A β peptides are segments truncated from the amyloid protein precursor or APP (e.g., A β (1-16) is cleaved by α - and β -secretases *in vivo*(20)), the exact biological functions of A β peptides and APP are still not clear. Given that structurally the copper-binding domain of APP is similar to Cu chaperones and that Cu can modulate A β and APP cellular levels,(21) APP has been presumed to partake in intracellular copper homeostasis. Overexpression of APP results in Cu deficiency and consequently reduces activity of superoxide dismutase (SOD-1), (22) a key enzyme in scavenging reactive oxygen radicals. *In vitro* studies have shown that incubation of an A β /Cu(II) mixture with electron donors (e.g., ascorbic acid) under aerobic condition produces H₂O₂.(23,24) Recently, we successfully measured the redox potential of the A β -Cu(II) complex using voltammetric techniques. We and others also confirmed that H₂O₂ production can indeed occur via the A β -Cu(I)-catalyzed reduction of oxygen.(24,25) The amount of H₂O₂ is the highest in the presence of the electroreduced A β (1-16)-Cu(I) complex (i.e., A β (1-16)-Cu(I)).(25)

Similar to copper, iron is essential to a variety of brain functions. Iron in brain is highly regulated by proteins such as ferritin and transferrin.(26) The iron load increases with age and becomes acutely high in AD patients.(27,28) It was found that postmortem tissues from AD brains accelerate the ROS production *in vitro* when a reductant is present and the iron chelator deferoxamine appears to decelerate the ROS production.(29) Smith et al., through speciation of Fe(II) and Fe(III) in hippocampal tissues from several AD patients, have shown that iron accumulated in senile plaques can lead to generation of free radicals.(30) Thus, it is conceivable why little free and reactive forms of iron are present in normal brain and translocation of iron must be chaperoned.(31) Small inorganic and organic ligands are also important in transfixing and modulating the transfer of iron in and between various metalloenzymes.(32) For example, the bicarbonate anion in transferrin renders Fe(III) in a tight coordination sphere and is partially responsible for its high affinity to Fe(III) (ca. 10⁻¹⁹-10⁻²⁰ M).(33) Phosphate, carboxylates, and peptides serve as ligands for iron in the so-called "labile iron pool".(21,34) However, to the best of our knowledge, no *in vivo* studies have unequivocally demonstrated that iron is complexed by A β in the senile plaque. Moreover, owing to the rapid hydrolysis of Fe(III), *in vitro* formation of the putative A β -Fe(III) complex via simple mixing of Fe(III) and A β has been largely unsuccessful and is qualitative at the best. Without quantitative formation of Fe-containing A β complexes *in vitro* and knowledge about their redox potentials, the hypothesis that ROS are concertedly produced by A β and iron remains speculative. Considering that free Fe(III) is an even stronger oxidant than free Cu(II), the paucity of information about any Fe-containing A β complexes heightens the need to understand the relative contribution to oxidative stress in AD between iron and copper. Moreover, measuring the binding affinity of A β towards a Fe(III)-containing species with respect to that towards the Fe(II) variant should

also provide insight into the relative stability of the complexes when the iron centers are reduced.

We report, for the first time, the formation of a ternary complex containing A β (1-16), Fe(III), and nitrilotriacetic acid (NTA). The use of a preformed Fe(III)-NTA as the Fe(III) source for producing this ternary complex prevents the hydrolysis of free Fe(III) at physiological pH. The Fe(III)-NTA complex has long been used as a supply of Fe(III) for various biological studies. (35) Furthermore, the moderate binding affinity of NTA towards iron (1.3×10^{-16} M) (36) makes it an excellent mimetic of small ligands involved in metal-chaperone proteins. Electrospray ionization mass spectrometry (ESI-MS) was used to determine the binding stoichiometry among A β (1-16), Fe(III), and NTA and MSⁿ conducted in an ion trap allowed us to assess the relative binding strength between A β and NTA towards the iron center and the potential binding sites of A β . MS analysis of the isotopic peaks of the complex helped pinpoint the oxidation state of the iron center upon complex formation. The cumulative dissociation constants of both the A β -Fe(III)-NTA and A β -Fe(II)-NTA complexes were deduced through monitoring the quenching of the fluorescence of the sole tyrosine residue of A β during complex formation. Similar to our previous approach in interrogating the H₂O₂ generation facilitated by the electrogenerated A β complex of Cu(I), A β -Fe(II)-NTA was found to catalyze the reduction of oxygen to H₂O₂. The roles of Fe(III) and Fe(II) in participating in ROS generation and redox reactions with representative cellular species are discussed in the context of oxidative stress in AD.

Materials and Methods

Materials

Lyophilized A β (1-16) (DAEFRHDSGYEVHHQK, denoted as A β in the study) samples were either purchased from rPeptide (Bogart, GA) or synthesized and purified in house. Other chemicals were of analytical grade (Sigma-Aldrich). All the aqueous solutions were prepared using deionized water (Millipore 18 M Ω cm). Throughout the work, 10 mM FeCl₃ dissolved in 20 mM nitrilotriacetic acid (NTA) was used as the Fe(III) stock solution. A β (1-16) solutions were prepared freshly by dissolving lyophilized A β samples in deionized water. For experiment with Fe(II), FeSO₄·7H₂O (10 mM) was dissolved in a 20 mM NTA solution. To avoid the Fe(II) oxidation by oxygen, samples were prepared in a glove box constantly flushed with N₂ with aqueous solutions thoroughly purged with N₂.

Mass spectrometry

The mass spectra were collected on a LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electrospray ionization (ESI) source. A β was first dissolved in a water/methanol solution (V/V = 50/50) to yield a 50 μ M A β stock solution. The A β solution and an aliquot spiked with 50 μ M Fe(III)-NTA were separately analyzed.

Electrochemical measurements

Electrochemical experiments were performed on a CHI 832 electrochemical workstation (CH Instruments, Austin, Texas) using a home-made plastic electrochemical cell. A glassy carbon disk electrode and a platinum wire were used as the working and counter electrodes, respectively. All the potential values are reported with respect to the Ag/AgCl reference electrode. Prior to each experiment, the glassy carbon electrode was polished with diamond pastes of 15 and 3 μ m and alumina pastes of 1 and 0.3 μ m in diameter (Buehler, Lake Bluff, IL). The electrolyte solution was a 20 mM acetate/20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH, 7.0) containing 0.1 M NaNO₃. Aliquots of A β from stock solutions were diluted with the acetate buffer to desired concentrations. For

voltammetric studies, these A β solutions were spiked with Fe(III)-NTA or Fe(II)-NTA stock solution to predefined A β /iron molar ratios.

Spectrofluorometric measurements

Steady-state fluorescence measurements of the tyrosine residue at position 10 in A β were carried out at room temperature using a Cary Eclipse spectrofluorometer (Varian Inc., Palo Alto, CA). The procedure follows our earlier work on the measurement of the binding constants of the A β -Cu(II) complexes.(37) Based on the aforementioned MS studies, it can be assumed that change in fluorescence upon adding Fe(III)-NTA into the A β (1-16) solution reflects the amount of ternary complex produced. The dissociation constant (K_{d1}) was determined from the plot of fluorescence intensity against the total Fe(III)-NTA concentration, [L], using the following equation:

$$\Delta F = F_0 - F_L = \frac{F_0 - F_\alpha}{2[M_0]} \left[([L] + [M_0] + K_{d1}) - \left(([L] + [M_0] + K_{d1})^2 - 4[M_0][L] \right)^{1/2} \right] \quad (1)$$

where F_0 and F_L are the measured fluorescence intensities of A β (1-16) at 303 nm in the absence and presence of Fe(III)-NTA, respectively. F_α is the intensity of the solution in which the A β fluorescence is completely quenched by Fe(III)-NTA. $[M_0]$ is the A β concentration which was varied in the range of 10-100 μ M for the fitting procedure. Eq 1 holds when the concentration of metal binding sites exceeds the dissociation constant of the complex. For A β binding with Fe(II)-NTA, the experiment was again carried out in the glove box continuously circulated with N₂. The fluorescence measurement was performed with the cuvette containing the complex solution sealed inside the glove box.

Detection of hydrogen peroxide

The electrode potential was held at -0.05 V to reduce the A β -Fe(III)-NTA complex. H₂O₂ generated was monitored using the Fluoro H₂O₂ detection kit (Cell Technology Inc., Mountain View, CA), which was also utilized in our previous studies on the A β -Cu(I)-catalyzed H₂O₂ generation.(25) In the presence of H₂O₂ and horseradish peroxidase (HRP), 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) is rapidly oxidized to a fluorescent product, resorufin. The procedure involved adding 50 μ L of the sample solution into 50 μ L aliquot of the reaction cocktail, which contained 100 μ L of 10 mM ADHP, 200 μ L of 10 U/mL HRP, and 4.7 mL of reaction buffer. The mixture was then incubated at room temperature in dark for 10 min. Subsequently the fluorescence intensity of resorufin was measured at an excitation wavelength of 550 nm. By comparing the fluorescence intensity of resorufin in the sample solution to that of the control, the amount of H₂O₂ can be measured.

Results

Fe(III) retains its oxidation state in its complex with A β

We have demonstrated previously that high-resolution MS is a viable technique for determining both the oxidation state and the binding stoichiometry of the A β -Cu(II) complex.(25) The identification of the oxidation state of the metal center in the metal complex of A β is of particular interest, since the relevant biological redox reactions are largely dictated by the metal center (i.e., Cu(II) vs. Cu(I) or Fe(III) vs. Fe(II)).(38) Moreover, tyrosine (Tyr-10) and methionine (Met-35) are oxidizable and many studies have focused on the possible redox reactions between them and the metal center(s).(39-41) The oxidation potential of Met-35 is much higher than that of Tyr-10.(42) We and others have recently shown that the Tyr-10 residue remains intact when Cu(II) is complexed by A β and concluded that Cu complexation does not chemically modify any A β residues.(25)

Figure 1 shows a mass spectrum collected from a mixture of A β (1-16) and Fe(III)-NTA. The predominate peaks are of the 2+, 3+, and 4+ charge states. The peaks clustered around m/z 489.82 and 550.82 correspond to the quadruply charged A β (1-16) and A β (1-16)-iron-NTA complex, respectively. The intense peak at 489.82 is consistent with our previous positive-ion ESI-MS of A β (1-16) alone.(25) Increasing the molar ratio between Fe(III)-NTA and A β in the mixture only changed the relative abundances of ions corresponding to free A β and the complex, and did not generate peaks indicative of other binding stoichiometries. Therefore, our ESI-MS indicates that a ternary complex of A β -Fe-NTA has formed. The absence of an A β -Fe(III) binary complex peak in Fig.1 suggests that, even if present, it would not be a predominant species in solution. When ferric ion was added directly into an A β solution, no mass peaks corresponding to a Fe-containing A β complex were detected. This is expected since hydrolysis of Fe(III) at neutral pH is rapid and predominant.(43) The use of NTA inhibits the hydrolysis of Fe(III), allowing A β to bind to Fe(III)-NTA.

Previously, by comparing the experimentally measured isotopic peak profiles of various A β -Cu complexes to theoretical values corresponding to the two different Cu oxidation states (+2 vs. +1), we unambiguously concluded that Cu(II) retains its oxidation state in the complexed form.(25) The oxidation state of the iron center in the A β -Fe(III)-NTA ternary complex can be deduced similarly. Table S1 lists the experimentally determined isotopic peaks of the triply and quadruply charged species to the theoretical m/z values of the A β -Fe(III)-NTA and A β -Fe(II)-NTA complexes. If Fe(II) were produced during the complex formation, the complex would be doubly protonated to yield a 4+ ion; on the other hand, if the iron center had an oxidation state of 3+, the complex would only need to be singly protonated to reach a charge state of 4+. The deviations for the triply charged ions between the measured and the calculated m/z values of A β -Fe(III)-NTA are markedly smaller than those of the hypothetical A β -Fe(II)-NTA complex. This indicates that the iron center has the same oxidation state as that in the Fe(III)-NTA complex. The analysis of the isotopic peaks for the 4+ ions led to the same conclusion (Table S1). A mass spectrum of the complex collected in the high-resolution ultrazoom scan mode is shown in the inset of Figure 1.

Since the oxidation state of Fe(III) remains unchanged, the A β -Fe(III)-NTA complex must be incapable of oxidizing Tyr-10 to the semiquinone product. This conclusion is also confirmed by our voltammetric experiment (*vide infra*). Therefore, Met-35 in the full-length A β should not be oxidized, because, as aforementioned, methionine has a much higher oxidation potential than tyrosine.(42) The oxidation potential for the free Fe(III)/Fe(II) couple is 0.57 V vs. Ag/AgCl,(44) which is close to that of tyrosine (irreversible oxidation peak between 0.60 and 0.75 V) and substantially more positive than both the free Cu(II)/Cu(I) (-0.041 V vs. Ag/AgCl) and Cu(II)/Cu(0) (0.148 V vs. Ag/AgCl) couples.(45) Apparently the formation of a ternary metal complex has attenuated the relatively high oxidation potential of free Fe(III) to a low value at which Tyr-10 cannot be oxidized.

We also carried out MS-MS studies of the ternary complex and found that the NTA moiety can be readily eliminated from the complex upon collision-induced collision in the gas phase (Figure 2). In fact, triply charged ions corresponding to A β -Fe(III) (m/z 670.00) or its dehydrated form (m/z 664.00) dominate the product ion spectrum, suggesting that A β is a multidentate ligand that may bind Fe(III) more strongly than NTA. Therefore, although A β may not completely replace NTA in the coordination sphere, rearrangement of the binding sites and partial replacement of NTA sites by A β could occur in both solution and gas phases. Further tandem MS analysis of the fragments arising from cleavage of the daughter ion of m/z 670 revealed the facile formation of [b₁₃+Fe], [b₁₄+Fe], [b₁₅+Fe], and [y₁₅+Fe] ions as well as their dehydrated counterparts, though y₂, y₈, y₉, and b₇ ions are also produced in lower abundance (Fig. S1a). In contrast, the product-ion spectrum of the [M+3H]³⁺ ion of the uncomplexed A β revealed extensive cleavage along the entire peptide backbone (Fig. S1b).

The absence of abundant fragment ions emanating from the cleavage of the middle portion of the peptide might be attributed to the coordination of Fe(III) with multiple residues in this peptide segment. In this regard, the facile cleavages leading to the formation of abundant $[b_{13}+Fe]$, $[b_{14}+Fe]$, $[b_{15}+Fe]$, and $[y_{15}+Fe]$ ions indicate that the N-terminal aspartic acid residue and the three residues located close to the C-terminus of the peptide might not be substantially involved in Fe(III) binding. The determination of exact binding sites requires more investigation. However, histidines and carboxylic groups of aspartic and glutamic residues are possible binding sites. Particularly, histidines have been demonstrated to be involved in binding irons in postmodern senile plaque tissues of AD brain. (30)

Determination of Binding Affinity

We(37) and others(14,46) have shown that the Tyr-10 fluorescence is sensitive to structural changes brought about by Cu(II) complexation to the hydrophilic domain of A β . The fluorescence peak at 303 nm for A β in the presence of Fe(III)-NTA was therefore used to estimate the dissociation constant, K_{d1} , of A β -Fe(III)-NTA. The variation of A β fluorescence intensity as a function of the Fe(III)-NTA concentration (Figure 3) was analyzed by means of a nonlinear-least-square regression using eq 1. The observed dissociation constant is 3.2×10^{-5} M, which is about one order of magnitude smaller than that between the C-lobe of transferrin and Fe-NTA.(47) Therefore, we conclude that the binding between A β and Fe-NTA is quite strong. This is consistent with the above-mentioned MS that displays intense complex peaks.

When NTA is in excess, two possible complexes between Fe(III) and NTA, viz., Fe(III)-NTA and Fe(III)-NTA₂, could exist. Thus, depending on the relative abundance of the two Fe-NTA species, one of the following two equilibria will be predominant:

For simplicity, the charges of the ions involved in the A β -iron equilibria are omitted. In eq 3, the binding of A β to Fe(III)-NTA₂ competes out an NTA ligand. If such a competition were at work, then the measured binding constant would be dependent on the concentration of NTA. To pinpoint the equilibrium responsible for the observed A β -Fe(III)-NTA ternary complex, we measured the K values at various initial NTA concentrations. We also conducted the same measurements using the Fe(II)-NTA complex. The results are compiled in Table 1.

The fact that the K_{d1} values are not significantly dependent on the NTA concentration indicates that eq 3 is not important. This may be explained by the weaker ligation involving the second NTA group.(36)

The dissociation constant for eq 2 can be expressed by:

$$K_{d1} = \frac{[A\beta][Fe(III) - NTA]}{[A\beta - Fe(III) - NTA]} \quad (4)$$

The complexation equilibrium between Fe(III) and NTA can be written as follows:

and the corresponding dissociation constant is:

$$K_{d2} = \frac{[Fe(III)][NTA]}{[Fe(III) - NTA]} \quad (6)$$

Therefore the cumulative dissociation constant can be derived as:

$$K_d = \frac{[A\beta][Fe(III)][NTA]}{[A\beta - Fe(III) - NTA]} = K_{d1} \times K_{d2} \quad (7)$$

The commonly accepted value for K_{d2} is 1.3×10^{-16} M (36) and the average value measured here for K_{d1} is 5.0×10^{-5} M. Thus the cumulative dissociation constant (K_d) equals 6.3×10^{-21} M².

For the Fe(II)-NTA complexation with A β , the NTA concentration was also found to have little influence on K_{d1} . Equilibria equations analogous to eqs. 4 and 6 can be written for the A β -Fe(II)-NTA complex formation. The dissociation constant of Fe(II)-NTA has been reported to be 1.7×10^{-8} M. (36) Therefore, the cumulative dissociation K_d of A β -Fe(II)-NTA is estimated to be 5.0×10^{-12} M².

Redox potentials of the A β -Fe(III)-NTA and A β -Fe(II)-NTA complexes

Redox cycling of iron accompanied by production of ROS has long been considered to be responsible for the iron toxicity in biological system.(48) As mentioned earlier, little free Fe(III) or Fe(II) exist in an organism because most of these irons are either stored in the redox-inactive oxide forms or bound by siderophores.(21) Sequestration of Fe(III) or Fe(II) by protein (peptide) shifts the redox potential of Fe(III)/Fe(II) to much more negative values (Fe(III) center is more difficult to reduce or its oxidizing power is decreased), making the metal centers inaccessible to redox-active biomolecules. Thus, depending on the iron coordination spheres and the relative binding affinities of proteins/peptides to Fe(III) or Fe(II), the redox potentials of the resultant iron complexes will vary. As a result, the extent of ROS production is highly influenced by the Fe(II) and Fe(III) coordination chemistry. As mentioned in the Introduction, without knowledge about the redox potentials of the A β -Fe(III) or A β -Fe(II) complexes, it has been difficult to interpret biological redox reactions that might be induced or modulated by the iron-containing A β complexes.

Figure 4A is an overlay of a series of cyclic voltammograms (CVs) recorded under different experimental conditions. CVs of Fe(III)-NTA alone and A β (1-16) in the presence of Fe(III)-NTA are shown as the blue and black curves, respectively. To examine the effect of oxygen on the redox behavior of the A β -Fe(III)-NTA complex, we also bubbled O₂ into and collected CVs from a mixture of A β (1-16) and Fe(III)-NTA (red curve) and a Fe(III)-NTA solution (green curve). In the mixture of A β (1-16) and Fe(III)-NTA, a pair of quasi-reversible waves was observed with an oxidation peak at 0.094 V and a reduction peak at ca. -0.034 V. The redox peaks can be assigned to the reduction and reoxidation of the A β -Fe(III)-NTA complex and $E^0_{1/2}$ was therefore approximated to be 0.03 V. The redox behavior of the A β -Fe(III)-NTA complex is in contrast to the irreversible CV of Fe(III)-NTA. We also obtained a CV of Fe(II) and found the $E^0_{1/2}$ value (0.50 V; see also inset of Figure 4A) to be close to the theoretical value (0.57 V(44)). The small deviation between our measured and the theoretical values can be ascribed to the dependence of heterogeneous electron transfer rates on the pretreatment of the glassy carbon electrode surface.(49) Comparison of the theoretical $E^0_{1/2}$ value of Fe(III)/Fe(II) to that of A β -Fe(III)-NTA/A β -Fe(II)-NTA measured in this work shows a 0.54 V shift in the cathodic (negative) direction upon A β complexation. Such a shift is in excellent

agreement with the value (0.53 V) calculated based on the cumulative stability constants of A β -Fe(III)-NTA and A β -Fe(II)-NTA (*cf.* Table 1). We note that the redox potential of the complex is much higher than those of representative iron-containing proteins/complexes, such as transferrin and iron siderophore complexes.(43) In the presence of O₂, the reduction peak (red curve of Figure 4A) was found to increase at the expense of the oxidation peak, suggesting the catalytic nature of the reaction and the involvement of O₂.

A β -Fe(II) complex catalyzes the reduction of oxygen to hydrogen peroxide

As mentioned above, the reduced form of A β -Fe(III)-NTA complex can catalyze oxygen reduction, which could lead to the generation of either H₂O₂ or H₂O. To determine if the reduced complex can catalyze the reduction of oxygen to H₂O₂, we carried out spectrofluorometric detection of H₂O₂ from aliquots of an A β -Fe(III)-NTA solution that had been subject to controlled-potential electrolyses.

After electrolyses of these solutions at -0.05 V for different times, the solutions were analyzed for the amount of H₂O₂ generated. We chose -0.05 V because the electrocatalytic reduction peak of the complex is close to the maximum value (plateau) but the reduction of any unbound Fe(III)-NTA is relatively small. As shown in Figure 4, for the mixture of A β and Fe(III)-NTA, the fluorescence peaks increase with the electrolysis time and are significantly greater than those of the same mixture that had not been electrolyzed (black curve) and the Fe(III)-NTA only solution (magenta). It is worth mentioning that the fluorescence intensity measured from the Fe(III)-NTA solution electrolyzed for different times remained largely unchanged (data not shown), suggesting that Fe(II)-NTA was not generated or Fe(II)-NTA could react with O₂ via a different process (e.g., generation of superoxide radical(24)). All these observations indicate that it is the reduced A β -Fe(III)-NTA complex that is responsible for the H₂O₂ production. In fact, the amount of H₂O₂ produced is the highest when the initial solution contains A β -Fe(II)-NTA and O₂ (light blue curve in Figure 5).

Discussion

It is generally believed that the metal binding domain in A β is within the segment comprising residues 1-16, with Asp-1, Glu-3, His-6, Asp-7, Tyr-10, Glu-11, His-13 and His-14 as the possible binding sites.(50,51) Our MS study has demonstrated that A β is capable of binding Fe(III) in the presence of NTA, forming a ternary complex with the 1:1:1 stoichiometry. Although the exact binding sites to Fe(III) are not known, this represents the first study on an Fe(III)-containing A β complex. Our MS³ data (see Figure S1) suggest that A β possesses multiple binding sites towards Fe. In addition, nitrogen (especially that on the imidazole rings in the histidine residues) binds Fe(II) relatively stronger than oxygen in carboxylic group, and consequently nitrogen-coordinated iron complex should have a more positive redox potential than the corresponding oxygen-coordinated counterpart. Comparing the CVs of Fe-NTA and A β -Fe-NTA, we found the potential actually was indeed shifted positively. This further suggests that nitrogen-containing groups (from histidines) bind iron. *In vivo*, free Fe(III) ion is extremely low (in the order of 10⁻¹⁸M).(43) The cumulative dissociation constant measured by us is in the order of 10⁻²⁰ M², suggesting that the Fe(III) hydrolysis has been effectively prevented and the binding between A β and Fe(III) is indeed strong.

Similar to our previous study of the A β -Cu(II) complex, the reduction potential of A β -Fe(III)-NTA deduced from Figures 4 afforded a better understanding about the redox species that can reduce the A β -Fe(III) complex in cellular milieu. To reduce A β -Fe(III)-NTA, a species must have a reduction potential more negative (higher reducing power) than 0.03 V *vs.* Ag/AgCl or 0.23 V *vs.* normal hydrogen electrode (NHE). Since the Tyr-10 redox peak appears at ~0.78 V,(25) we conclude that Tyr-10 cannot reduce A β -Fe(III)-NTA. A Raman analysis of samples from deceased AD patients' brains indicated that Tyr-10 in A β was oxidized.(19) Thus, it can

be reasoned that the A β -Cu(II) and/or A β -Fe(III) complexes do(es) not directly oxidize the Tyr-10 residue, and other cellular species or processes must be involved.

Similar to the electrochemistry of A β -Cu(II) in solution,(25) the A β -Fe(III)-NTA complex can be reduced to A β -Fe(II)-NTA, once the cathodic scan has passed ~ 0.030 V:

The A β -Fe(II)-NTA complex or its analog can subsequently react with O₂ in solution, producing H₂O₂:

It is possible that eq 8 may have proceeded in a two-step reaction through the superoxide radical O₂^{•-}, another important ROS:

which subsequently reacts with protons on the A β molecule or a cellular species to produce H₂O₂:

O₂^{•-} is extremely reactive and cannot be detected voltammetrically. However, recent theoretical work by Hewitt and Rouk has shown that the above mechanism, similar to that occurs to the superoxide dismutases in converting O₂^{•-} to H₂O₂,(24) is possible. Although the elucidation of the detailed mechanism in producing H₂O₂ and/or O₂^{•-} awaits further theoretical and experimental work, the measurement of the redox potential of a Fe(III)-containing A β species has provided vital information about the feasibility of these reactions.

The above reactions are responsible for the O₂-dependent reduction peaks exhibited in Figures 4. As the potential of the A β -Fe(III)-NTA/A β -Fe(II)-NTA couple (0.23 V vs. NHE) is lower than that of O₂/H₂O₂ (0.295 V vs. NHE),(52) eq 8 is thermodynamically allowed. We should point out that the present study cannot rule out the possibility that some O₂ might be reduced to H₂O, via the reaction O₂ + 4H⁺ + 4e⁻ = 2H₂O. However, based on the amount of H₂O₂ detected, the reactions outlined above should constitute at least an important process.

From our spectrofluorometric measurements (Figure 5), it is clear that a prerequisite for H₂O₂ generation is the conversion of A β -Fe(III)-NTA to A β -Fe(II)-NTA. The amount of H₂O₂ generated must be dependent on all reactants (i.e., A β , Fe(III), and O₂). Since in senile plaques the content of A β and concentration of Fe(III) are both high and the function of brains requires a constant supply of O₂,(53) one would expect that H₂O₂ concentration be substantially high in AD brain. Regarding the biological relevance, it is interesting to note that mice do not develop AD(54) and their A β is different than the human variant at two of the potential metal binding sites, viz., His-13 and Tyr-10 (replaced by Arg and Phe, respectively). The decreased binding affinity towards metal ions may not inhibit the hydrolysis of Fe(III) to form redox inactive iron oxide or hydroxide. In the case of familial AD, A β is point-mutated, but none of the mutations occurs in the metal binding region. These facts strongly suggest a possible role of iron binding by A β in producing H₂O₂ and other ROS to subsequently inflict oxidative stress in AD.

Given the relatively high reduction potential of the A β -Fe(III)-NTA/A β -Fe(II)-NTA couple (relatively high reducibility compared with iron siderophore complex), a number of extracellular (e.g., ascorbic acid whose redox potential is 0.051 V vs. NHE(55)), intracellular (e.g., glutathione whose redox potential is -0.228 V vs. NHE(38)), and membrane species (e.g., the NADH dehydrogenase complex is -0.320 V vs. NHE(38)) should be able to reduce A β -Fe(III)-NTA or its analog. A comprehensive list of biological reductants and their reduction potentials has been provided by our previous paper.(25) In addition to H₂O₂ generation and the multitude of deleterious consequences (e.g., production of hydroxyl radical through iron- or copper-facilitated “Fenton-like” reaction, and the subsequent damages of DNA, protein, and lipid molecules(56)), the catalytic nature of reactions shown in eqs. 7 and 8 causes even a small amount of A β -Fe(III) to cycle multiple times. Consequently the levels of cellular antioxidants are diminished. Given the close proximity of the redox potentials between the A β -Cu(II) and A β -Fe(III)-NTA complexes, the redox reactions that can be initiated by the A β -Cu(II) complex (25) are also applicable to those involving the A β -Fe(III)-NTA complex. We should point out that the iron content in brain (18.5 mg/100 g wet tissue(57)) is more abundant than the quantity of copper (~0.5 mg/100 g brain mass(58)). It is therefore likely that iron contributes to metal-initiated oxidative stress in AD more than copper. In fact, the concentration of iron (0.9 mM) (4) is higher than copper (0.4 mM)(4) in senile plaques of AD patients, suggesting that both redox-active metals are involved and the extent of involvement is related to the overall metal loads in brain.

A major characteristic of proteins that chaperone redox metal ions is their ability of significantly modulating redox potentials of the corresponding free metal ions. In terms of potentiating the redox potential of free Fe(III), A β , together with a small chelator (e.g., NTA), appears to possess such a property. In fact, it has been suggested that A β and its precursor, APP, might participate in sequestering and transporting metal ions.(21) It has also been observed that cleavage of APP is dependent on the cellular iron content.(59) However, the modulation of the redox activity of the Fe(III)/Fe(II) couple via A β complexation does not appear to be sufficient (from 0.77 V to 0.23 V vs. NHE) to completely prevent the complex from interacting with other biological redox species. Binding of iron by A β renders Fe(III) in a soluble form and the Fe(III) center in a complex is still accessible to a range of cellular reductants. As a consequence, reactions such as those shown in eqs. 7 and 8 or other redox processes can happen. This insufficient redox potentiation is in sharp contrast to that performed by other commonly known Fe-containing proteins. For example, the high content of iron stored by ferritin is in the redox-inactive oxide forms, and iron in transferrin has a redox potential of -0.40 V (vs. NHE),(60) which is too negative (less reducible) for the Fe(III) center(s) to undergo electron transfer reactions with the aforementioned cellular reductants. Similarly, the iron center in hemoglobin is essentially redox inaccessible to many cellular reductants and the redox potential of myoglobin is also mild (0.06 V vs. NHE(61)). By the same token, the primary requirement for an iron chelator used in chelation therapy for treating iron overload diseases(62) is that the resultant complex must possess rather low (or negative) redox potential (low oxidizing power) to avoid toxicity that might arise from redox recycling of iron.

Another major difference between A β and transferrin is that the former has a much greater affinity towards Fe(II). It is generally believed that the Fe(III) center(s) in transferrin is(are) reduced to Fe(II) upon being internalized into cytosol. The binding affinity of transferrin to Fe(II) declines precipitously (by almost 17 orders of magnitude (60)), allowing Fe(II) to be sequestered by other cytosolic species. The rather strong binding of A β to Fe(II) (*cf.* Table 1) suggests that, even if A β (or APP) acts as a metal chaperone, its function and property are unconventional. Such a relatively strong binding, aided by a small chelator, withholds Fe(II) and disrupts the transport and homeostasis of iron. This might be at least part of the reason why iron coexists with A β aggregates in senile plaques. In addition, A β -Fe(II) appears to be able to prevent the free Fe(II) from participating in Fenton reaction to produce hydroxyl radical.

A recent study by Fischer and coworker showed that the Fe(II)-catalyzed generation of free radicals from H_2O_2 is halted by addition of A β .(16) By measuring dissociation constants of the complexes formed between A β and Fe(III)-NTA or Fe(II)-NTA, insight can be gained into the role of A β in iron sequestration and the possible oxidative stress induced by iron. We note that the measured binding affinity ($K_d = 6.3 \times 10^{-21}$) is close to those of transferrin (2.5×10^{-21} and 4.0×10^{-20} for the first and second iron respectively)(33). Such a comparability implies that A β together with other small chelating ligands can compete for Fe(III) from transferrin. The competition is particularly viable for Fe(III) bound in the c-lobe of transferrin. (33) As a consequence, the iron content in these proteins will be decreased and their normal functions are altered. The up-regulation of iron-responsive proteins(63) in AD brain may be associated with the loss of iron to A β . Equally detrimental is that the accelerated aggregation of A β in the presence of metal ions. In short, sequestration of iron by A β sidetracks the normal homeostasis of iron and the proteolysis of A β .

A β may also accumulate iron from the “labile iron pool”. It is now commonly accepted that within the cytosol there exists a transit labile iron pool where iron is complexed by small organic chelators such as phosphates, citrates, carboxylates, polypeptides, and species at the membrane (e.g., phospholipid head groups).(21,34) Iron in such a labile pool is transitory and will eventually become bound in metalloproteins. Thus, it is biologically relevant to study the properties of ternary complexes formed among A β , an organic chelator, and Fe(III) or Fe(II), since the small organic chelator not only provides additional stability to the transit iron, but also further modulates the redox potential of the Fe(III)/Fe(II) couple. APP is known to be rapidly internalized into cytosol(64) within which complexation with iron in the labile iron pool and competitive replacement of iron in a metalloprotein can take place. Although presently it is not known whether iron complexation precedes or follows the APP cleavage, the fact that A β can bind strongly to Fe(III) and Fe(II) to form redox-active complexes highlights the necessity of more extensive investigations on the properties of A β -Fe(III) or A β -Fe(II) complexes and their roles in triggering oxidative stress in AD.

Conclusions

We have, for the first time, observed the formation of ternary complexes among A β , NTA, and Fe(III) or Fe(II). The cumulative dissociation constants of the ternary complexes with Fe(III) and Fe(II) were determined to be $6.3 \times 10^{-21} \text{ M}^2$ and $5.0 \times 10^{-12} \text{ M}^2$, respectively. The binding affinity of A β to Fe(III) is comparable to that of transferrin, a strong iron-binding protein in brain, implying that A β together with a small chelator has the ability of competing for Fe(III) against a host of iron-containing proteins. Although A β binds to Fe(II) less strongly than to Fe(III), the affinity to Fe(II) is still considerably high (about eight orders of magnitude greater than transferrin!). Thus, Fe(II) converted from the Fe(III) center in the A β complex by cellular redox reactions is still withheld by A β . Using voltammetry, the redox potential of the complex was determined to be 0.03 V vs. Ag/AgCl, representing a 0.54 V shift in the cathodic (negative) direction from the redox potential of the free Fe(III)/Fe(II) redox couple. Despite such a large modulation, the potentiation of the Fe(III) redox power by A β is still not adequate. As a result, the Fe(III) center can participate in a range of redox reactions. We have demonstrated that H_2O_2 can be generated by O_2 reduction catalyzed by the electrogenerated A β -Fe(II)-NTA complex. Taken together, the A β -Fe(III)-NTA/A β -Fe(II)-NTA couple behaves analogously to the well-studied A β -Cu(II)/A β -Cu(I) couple. Therefore, deleterious redox cycles of iron and copper could both occur in brain, facilitating the production of ROS and depleting essential cellular redox-buffering species and antioxidants. The net outcome is that the complexation sidetracks the normal clearance of A β and the homeostasis of iron. The presence of the large quantities of iron and A β , as found in senile plaques, further exacerbates oxidative stress and accelerates the formation of other pernicious species such as the A β oligomers/fibrils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AD, Alzheimer's disease
 NTA, Nitrolotriatic acid
 ROS, Reactive oxygen species
 Met-35, Methionine at position 35
 Asp-1, Aspartic acid at position 1
 His-6, Histidine at position 6
 Glu-11, Glutamic acid at position 11
 His-14, Histidine at position 14
 ESI-MS, Electrospray ionization mass spectrometry
 HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
 A β , β -amyloid
 APP, Amyloid precursor protein
 ET, Electron transfer
 Tyr-10, Tyrosine at position 10
 Glu-3, Glutamic acid at position 3
 Asp-7, Aspartic acid at position 1
 His-13, Histidine at position 13
 CV, Cyclic voltammetry

Reference

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Figure 1. Positive-ion ESI-MS of Aβ(1-16)/Fe(III)-NTA mixed at 1:1 molar ratio. Inset shows the MS of the triply charged Aβ(1-16)-Fe(III)-NTA collected from a high-resolution ultrazoom scan.

Figure 2.
Product-ion spectrum of the triply charged ion of A β (1-16)-Fe(III)-NTA (m/z 733.9).

Figure 3.

Fluorescence spectra of 100 μM A β (1-16) in 0.02 M HEPES buffer (pH 7.0, squares) and 100 μM A β (1-16) solutions that contained 10 (solid circles), 70 (triangles) and 210 μM (open circles) Fe(III)-NTA. The inset shows the determination of K_{dl} using the tyrosine fluorescence quenched by Fe(III)-NTA. The open squares are experimental data, and the curve is the fit using eq1 with K_{dl} of 3.2×10^{-5} M ($r^2 = 0.999$).

Figure 4.

Cyclic voltammograms of (A) deaerated 400 μM Fe(III)-NTA in the presence (black curve) and absence (blue) of 1 mM A β (1-16) and (B) the same as (A) except that Fe(III)-NTA was replaced with Fe(II)-NTA of the same concentration. The red and green curves in both panels correspond to air-saturated solutions of Fe(III)-NTA with and without A β (1-16), respectively. All solutions were prepared with a buffer containing 20 mM HEPES, 20 mM acetate, and 0.2 M NaNO₃ (pH 7.0). Inset in panel (A) depicts a CV of 1 mM FeCl₃ prepared in 1 M HCl and 1 M CaCl₂ and acquired at an electrochemically treated glassy carbon electrode. The scan rate used for all voltammograms was 2 mV/s.

Figure 5.

Fluorescence spectra of resorufin for detecting H_2O_2 in solutions of $\text{A}\beta$ -Fe(III)-NTA before (black curve) and after electrolyses for different times (red, green, and dark blue curves), $\text{A}\beta$ -Fe(II)-NTA (light blue) and Fe(III)-NTA (purple). The times for electrolysis at -0.05 V are 0.5 (red curve), 2 (green) and 8 h (dark blue). The concentration of $\text{A}\beta$ in all sample solutions was 1 mM, whereas those of Fe(III)-NTA and Fe(II)-NTA were 400 μM .

Table 1

Apparent dissociation constants of A β -Fe(III)-NTA and A β -Fe(II)-NTA complexes measured at different NTA concentrations

Initial [NTA] (mM)	0.0	1.0	2.0
K_{dl} (M, A β -Fe(III)-NTA)	$(3.2 \pm 0.1) \times 10^{-5}$	$(5.5 \pm 0.4) \times 10^{-5}$	$(5.3 \pm 0.3) \times 10^{-5}$
K_{dl} (M, A β -Fe(II)-NTA)	$(3.4 \pm 0.1) \times 10^{-4}$	$(4.2 \pm 0.3) \times 10^{-4}$	$(2.9 \pm 0.2) \times 10^{-4}$