Amyloid-β peptide binds with heme to form a peroxidase: Relationship to the cytopathologies of Alzheimer's disease

Hani Atamna* and Kathleen Boyle

Nutrition and Metabolism Center, Children's Hospital Oakland Research Institute, Oakland, CA 94609

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Amyloid-β peptide (Aβ) is the toxic agent in Alzheimer's disease (AD), although the mechanism causing the neurodegeneration is not known. We previously proposed a mechanism in which excessive Aβ binds to regulatory heme, triggering functional heme deficiency (HD), causing the key cytopathologies of AD. We demonstrated that HD triggers the release of oxidants (e.g., H_2O_2) from mitochondria due to the loss of complex IV, which contains heme-*a*. Now we add more evidence that Aβ binding to regulatory heme *in vivo* is the mechanism by which Aβ causes HD. Heme binds to Aβ, thus preventing Aβ aggregation by forming an Aβ–heme complex in a cell-free system. We suggest that this complex depletes regulatory heme, which would explain the increase in heme synthesis and iron uptake we observe in human neuroblastoma cells. The Aβ–heme complex is shown to be a peroxidase, which catalyzes the oxidation of serotonin and 3,4-dihydroxyphenylalanine by H_2O_2. Curcumin, which lowers oxidative damage in the brain in a mouse model for AD, inhibits this peroxidase. The binding of Aβ to heme supports a unifying mechanism by which excessive Aβ induces HD, causes oxidative damage to macromolecules, and depletes specific neurotransmitters. The relevance of the binding of regulatory heme with excessive Aβ for mitochondrial dysfunction and neurotoxicity and other cytopathologies of AD is discussed.

curcumin | heme deficiency | mitochondria | regulatory heme | serotonin

Intracellular amyloid-β peptide (Aβ) and its aggregates are thought to be the neurotoxic agents in Alzheimer's disease (AD) (1–3). Important cytopathologies in AD include the accumulation of iron in cells; mitochondrial complex IV dysfunction; oxidative stress (4–7); and cholinergic, dopaminergic, and serotonergic dysfunctions. These cytopathologies play a role in subsequent cognitive impairment (8). Although the molecular link between Aβ and the important cytopathologies seen in AD is not fully understood, our earlier work produced strong evidence that heme may play a key role in the molecular link between Aβ and these cytopathologies. We found that heme metabolism is altered in AD brain (9); that the complex phenotype of heme deficiency (HD) (10, 11) overlaps with important cytopathologies in AD (10, 11); and that heme binds with Aβ, forming an Aβ–heme complex (9). We proposed a model in which excessive Aβ in AD brain binds to regulatory heme, creating functional HD (9, 12).

Heme plays an important role in cellular metabolism and gene regulation. All nucleated mammalian cells synthesize heme. The synthesis of the organic moiety of heme (protoporphyrin IX) starts in the mitochondrial matrix. δ-Aminolevulinic acid synthase (ALAS) catalyzes the condensation of succinyl-CoA with glycine, producing ALA (13), which is the first and rate-limiting step in heme synthesis. Ferrochelatase, located in the inner membrane of the mitochondria, inserts iron into protoporphyrin IX to produce heme (14, 15), which then reaches the cytosol to form the regulatory heme. Regulatory heme binds to the heme-regulatory motif in specific proteins. Heme-regulatory motif is different from the heme pockets in heme proteins, which bind heme with higher affinity. Regulatory heme controls the mitochondrial–cytosolic distribution of ALAS (16), iron uptake (17), specific neuronal signaling pathways (18), and transcription factors (19); regulatory heme also is the precursor for heme-*a*. Heme-*a* matures via several biochemical modifications to regulatory heme (20), exists only in complex IV in the mitochondria, and is rate-limiting for its assembly (21). Complex IV decreases in AD (5, 6), which causes release of oxidants during mitochondrial electron transport.

In this study, we confirm our earlier finding that heme readily complexes with Aβ. We conclude that this binding causes a decrease in heme-*a*, which accounts for the decrease in complex IV (10, 11), a release of oxidants (11), and the compensatory induction of heme synthesis and iron uptake. We also show that the Aβ–heme complex is a peroxidase, that serotonin is a substrate, and that curcumin is an inhibitor.

Results

Aβ Binds to Heme to Form an Aβ–Heme Complex. We tested the effect of heme on the aggregation of Aβ monomers and the dismantling of Aβ aggregates in solution. The fluorescence of thioflavin-T (TfT) is high after binding Aβ aggregates (Fig. 1*A*). When heme was added to Aβ aggregates, the fluorescence of TfT declined, indicating a dismantling of Aβ aggregates. When heme was added to Aβ monomers, it blocked the increase in TfT fluorescence, indicating inhibition of Aβ aggregation (Fig. 1*B*). Heme has no effect on the fluorescence of TfT in the absence of Aβ aggregates.

We previously showed that heme binds with Aβ, forming an Aβ–heme complex (9). Here, we investigate the formation of Aβ–heme by monitoring the spectrum of heme and by electrophoresis at the completion of experiments identical to those shown in Fig. 1 *A* and *B*. A red shift in the Soret band of heme was evident, indicating that an Aβ–heme complex was formed when Aβ aggregates were mixed with heme (Fig. 2). Additionally, an increase in the absorbance of heme at 530 nm was always observed in the spectrum of Aβ–heme (Fig. 2). SDS/PAGE and Western blotting were used to determine the change in the aggregation level of Aβ, using the specific antibody 6E10. A high-molecular-weight band was observed at the origin of the wells, indicating the presence of SDS-stable Aβ aggregates (Fig. 1*C*). This band has been shown to represent immobilized Aβ aggregates rather than a defined high-molecular-weight species (22). After incubation with heme, smaller Aβ aggregates (130–

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Abbreviations: Aβ, amyloid-β peptide; AβPP, Aβ protein precursor; AD, Alzheimer's disease; ALAS, δ-aminolevulinic acid synthase; DOPA, 3,4-dihydroxyphenylalanine; HD, heme deficiency; TfT, thioflavin-T; TMB, 3,3′,5,5′-tetramethylbenzidine.

*To whom correspondence should be addressed at: Nutrition and Metabolism Center, Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA, 94609-1673. E-mail: hatamna@chori.org.

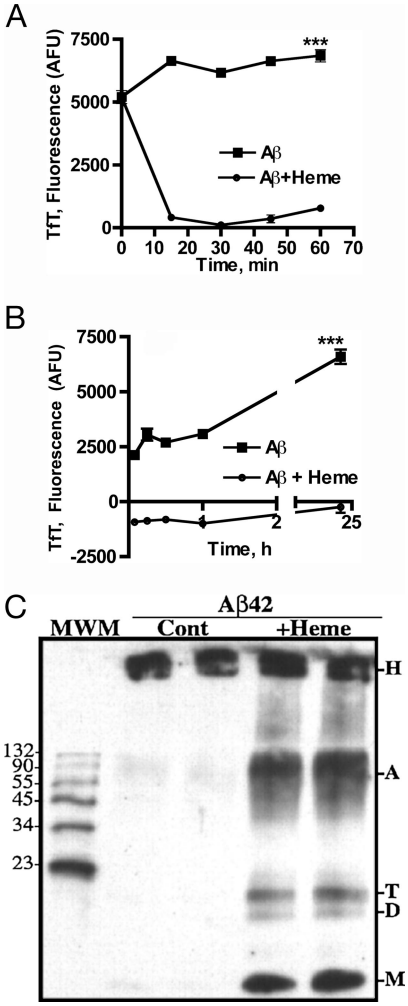

Fig. 1. Heme dismantles Aβ aggregates and inhibits Aβ aggregation. Changes to the fluorescence of TfT were used to determine the level of Aβ aggregation as described in *Materials and Methods*. (*A*) The high fluorescence of TfT [expressed in arbitrary fluorescence units (AFU)] resulting from binding to Aβ aggregates declines upon incubation with heme. (*B*) The increase in the fluorescence of TfT resulting from spontaneous aggregation of freshly prepared Aβ monomers is depressed by heme. See text for an explanation for the negative fluorescence. Heme has no effect on the fluorescence of TfT in the absence of Aβ aggregates (data not shown). Data shown are means ± SD of triplicates of one representative example of four experiments. The last points of each curve are compared. ***, $P < 0.001$. (*C*) Oligomers (A), tetramers (T), trimers (D), and monomers (M) of Aβ resulting from incubation with heme were determined at the end of the experiment in *A* by SDS/PAGE and Western blotting as described in *Material and Methods*. Controls (Cont) contain high-molecular-weight species (H) that did not enter the gel and a lack of monomeric forms of Aβ. Aβ oligomers, tetramers, trimers, and monomers appear by treatment with heme (+Heme). Duplicates are shown for each treatment. Shown is one representative example of four experiments.

140 kDa), tetramers, trimers, and a large amount of monomers appeared (Fig. 1*C*). The SDS-stable Aβ aggregates remaining after treatment with heme are likely products of the reaggregation of Aβ during preparation for separation on the gel. These observations indicate that heme binding with Aβ forms a stable Aβ–heme complex. Similar effects of Aβ–heme complexing were also observed in experiments that measured the inhibition of Aβ aggregation (Fig. 1*B*).

The effect of heme on Aβ aggregates is rapid, occurring within 15 min of incubation with Aβ (Fig. 1*A*). The kinetics of heme binding with Aβ, binding constants, and various biological

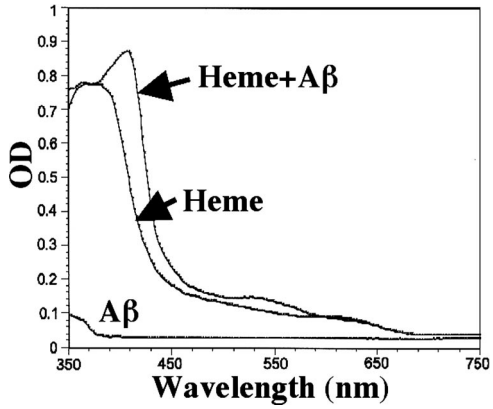

Fig. 2. The Aβ–heme complex is formed during the interaction of heme with Aβ. The spectrum of heme between 350 and 750 nm was measured at the end of the incubation in experiments identical to those described for Fig. 1 *A* and *B*. The red shift, an increase in the absorbance of the Soret band, and an increase in OD at 530 nm were always observed after heme binding to Aβ. Shown is one representative example of four experiments.

consequences of the Aβ–heme should be studied. The slightly negative values seen in Fig. 1*B* could be a result of interaction between TfT and the heme–Aβ complex.

The Aβ–Heme Complex Possesses Peroxidase Activity. We examined the biochemical activity of Aβ–heme. We found strong evidence for peroxidase activity of the Aβ–heme complex. As shown in Fig. 3, 3,3′,5,5′-tetramethylbenzidine (TMB), which is a classic substrate for peroxidases, was oxidized by H_2O_2 only when Aβ–heme complex was added. Interestingly, the antibody 6E10 did not affect the spectrum of Aβ–heme or inhibit its peroxidase activity even after 24 h of incubation at concentrations 5-fold higher than the Aβ–heme concentration (data not shown).

The neurotransmitters serotonin and 3,4-dihydroxyphenylalanine (DOPA) were very efficient in inhibiting TMB oxidation by Aβ–heme, suggesting they may be substrates for Aβ–heme. The mean (± SD) inhibition by serotonin was 89 ± 4% ($P = 0.002$, $n = 3$), whereas inhibition by DOPA was 66 ± 5% ($P = 0.01$, $n = 3$). We confirmed that serotonin is a substrate for Aβ–heme by demonstrating a rapid decrease in the 280-nm absorbance of serotonin and the appearance of new oxidation products upon adding Aβ–heme to a mixture of H_2O_2 and serotonin (Fig. 4). Adding Aβ–heme or H_2O_2 alone to serotonin did not have an

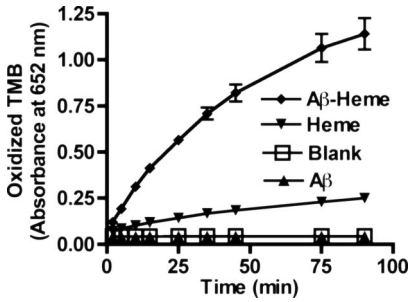

Fig. 3. Peroxidase activity of the Aβ–heme complex. The oxidation of TMB by Aβ–heme peroxidase was followed by an increase in the absorbance of 652 nm. Aβ–heme, Aβ, heme, or PBS (Blank) was added to a 200-μl TMB-substrate kit specific for peroxidases (all tubes have H_2O_2). Aβ–heme was very efficient in the oxidation of TMB compared with heme. No oxidation of TMB by H_2O_2 occurs without heme in the absence (Blank) or presence of Aβ. The Aβ–heme complex was prepared as described in *Materials and Methods*. Shown is one representative example of five experiments.

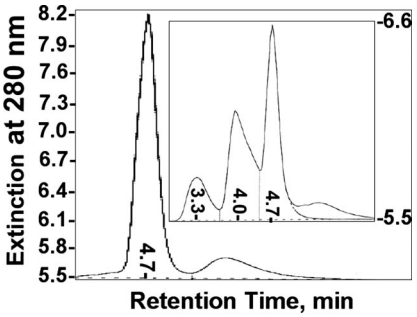

Fig. 4. Oxidation of serotonin by Aβ–heme and H_2O_2. An HPLC-UV detector set at 280 nm was used to measure the oxidation of serotonin catalyzed by Aβ–heme and H_2O_2 as described in *Materials and Methods*. The retention time of serotonin is 4.7 min, which was unchanged in the presence of H_2O_2. (*Inset*) Adding Aβ–heme to serotonin plus H_2O_2 decreases the serotonin peak after a 60-min reaction and leads to the formation of two oxidation products. The chromatogram shown is one representative example of five experiments.

effect; Aβ–heme with H_2O_2, however, induced catalytic oxidation of serotonin (Fig. 4 *Inset*). 5,7-Dihydroxyserotonin was not among the oxidation products of serotonin. The chemical identity of the oxidation products is an important topic for further study.

Curcumin lowers oxidative stress in a mouse model for AD. Therefore, we tested whether curcumin inhibits Aβ–heme-dependent peroxidase. Curcumin significantly inhibits the peroxidase activity of Aβ–heme at concentrations as low as 1 μM (Fig. 5).

Aβ Induces Heme Synthesis and Iron Uptake in Human Neuroblastoma Cells. The effect of Aβ on heme synthesis was used to test the biological effect of the Aβ and heme interaction. We expected an increase in heme synthesis due to depletion of regulatory heme if the binding between heme and Aβ occurs in the cell. Human neuroblastoma (SHSY5Y) cells synthesized 0.32 $\pm$ 0.1 and 0.84 $\pm$ 0.088 ng of heme per mg of protein after 1 and 2 h of incubation, respectively, in full medium, (mean $\pm$ SEM of six independent experiments, $P < 0.006$). Aβ at concentrations of 0.1, 1, and 10 μM significantly increased the synthesis of heme (Fig. 6*A* and *B*). A 40% increase in heme synthesis was seen after 2 h incubation with 0.1 μM Aβ (Fig. 6*A*). A 4-fold and 13-fold increase in heme synthesis was seen after 1 h of incubation with 1 and 10 μM Aβ, respectively. A 2.5-fold and 18-fold increase in heme synthesis was seen after 2 h of incubation with 1 and 10 μM Aβ, respectively (Fig. 6*B*).

An increase in the uptake of iron is necessary to support heme

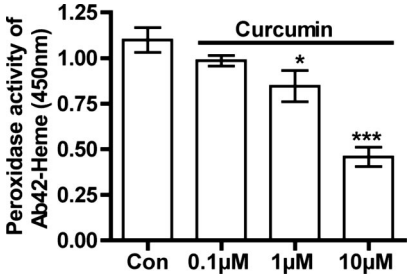

Fig. 5. Curcumin inhibits the peroxidase activity of Aβ–heme. The effect of curcumin (in 0.1 M NaOH) on Aβ–heme peroxidase activity was tested with TMB and H_2O_2 as described in *Material and Methods*. The addition of the 1–10 μl of curcumin solution did not affect the pH. The oxidation product of TMB was determined at 450 nm after acidification by sulfuric acid. Data shown are means $\pm$ SD ($n = 3$), which were compared with the control. *, $P < 0.016$ by unpaired *t* test; ***, $P < 0.0002$ by unpaired *t* test.

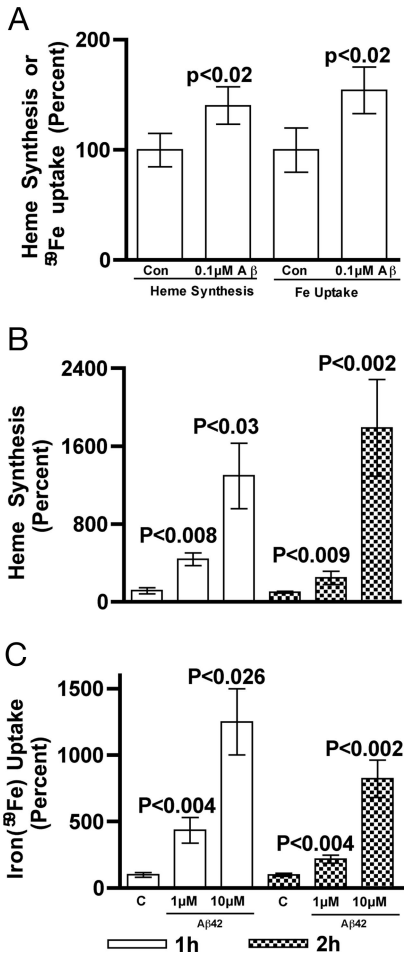

Fig. 6. Aβ induces heme synthesis and iron uptake in human neuroblastoma (SHSY5Y) cells. Heme synthesis and ^{59}Fe uptake by SHSY5Y cells were measured as described in *Materials and Methods*. Three concentrations of Aβ [0.1 μM (*A*), 1 μM (*B*), and 10 μM (*C*)] increased heme synthesis and iron uptake above the controls (mean $\pm$ SEM of the percent of the increase in heme or iron uptake). (*A*) Data are presented for only the 2-h incubations. (*B*) Heme synthesis in the control cells was 0.32 $\pm$ 0.1 and 0.84 $\pm$ 0.088 ng of heme per mg of protein after 1 and 2 h, respectively (mean $\pm$ SEM of six independent experiments, $P < 0.006$, nonparametric Mann–Whitney test). (*C*) Iron uptake was 2.44 $\pm$ 0.43 and 4.1 $\pm$ 0.46 ng of ^{59}Fe per mg of protein after 1 and 2 h, respectively (mean $\pm$ SEM of six independent experiments, $P < 0.03$, nonparametric Mann–Whitney test).

synthesis. Therefore, we measured total iron uptake in response to Aβ (Fig. 6 *A* and *C*). SHSY5Y cells accumulated 2.44 $\pm$ 0.43 and 4.1 $\pm$ 0.46 ng of ^{59}Fe per mg of protein after 1 and 2 h of incubation, respectively, in full medium (mean $\pm$ SEM of six independent experiments, $P < 0.03$). Aβ at concentrations of 0.1, 1, and 10 μM significantly increased the intracellular iron (Fig. 6 *A* and *C*). A 55% increase in iron was seen after 2 h of incubation with 0.1 μM Aβ (Fig. 6*A*). A 4-fold and 12.5-fold increase in iron uptake was seen after 1 h of incubation with 1 and 10 μM Aβ, respectively. A 2-fold and 8-fold increase in iron uptake was seen after 2 h of incubation with 1 and 10 μM Aβ, respectively (Fig. 6*C*).

The exposure of SHSY5Y cells to Aβ was not toxic as measured by trypan blue exclusion tests and compared with controls. The percentages of trypan blue-positive cells were 2.9 $\pm$ 2.2%, 4.3 $\pm$ 1.8%, and 3.5 $\pm$ 2% for control, 1 μM Aβ, and 10 μM Aβ, respectively, at the end of the 2-h incubation (mean $\pm$ SD, $n = 3$).

Discussion

Several lines of evidence support the role of intracellular Aβ in the cytopathology of AD, whereas direct and indirect experimental evidence strongly suggests that heme metabolism is also involved in AD (9, 23, 24). We show that the binding of heme with Aβ to form a complex (Aβ–heme) may be the molecular mechanism for the disparate cytopathologies seen in AD and accumulation of Aβ.

We provide experimental evidence of heme's prevention of Aβ aggregation (Fig. 1) by tightly binding with Aβ. We show that heme rapidly binds with Aβ, causing a red shift to the spectrum of heme in the Soret band (Fig. 2), indicating that the heme iron is involved in this binding. His is known to bind with heme in several heme proteins (25), suggesting that at least one of the three His residues in Aβ is likely to bind with heme iron. A shift in the Soret band of free heme has been reported recently in several heme-binding proteins (16, 18, 26), indicative of specific binding of heme to these proteins. Additionally, the amino acid sequence of Aβ contains hydrophobic amino acids (two Leu residues and three Ile residues) and the amino acids Asn and Gln, which are usually found in heme-binding pockets of heme proteins. One of the His residues in Aβ was shown to bind to Zn or Cu, enhancing its aggregation (27). We suggest that heme out-competes Zn or Cu ions in binding to Aβ, inhibiting Aβ aggregation (9).

Aβ appears to bind with heme with high affinity. The antibody 6E10 recognizes amino acids 1–17 of Aβ, which include the three His residues (His-6, Hi-13, and His-14) that are proposed to bind with heme iron. The antibody did not reverse the Aβ–heme spectrum back to heme or inhibit the peroxidase. These observations indicate that the antibody 6E10 does not displace heme from Aβ–heme, suggesting high-affinity binding of Aβ to heme and alteration to the conformation of amino acids 1–17 of Aβ. Interestingly, the aggregates of Aβ were resistant to SDS detergent (Fig. 1), suggesting that the minor detergent action of heme plays little role in preventing Aβ aggregation. Additionally, γ-globulin, which binds many substances nonspecifically, did not change the Soret band of heme or acquire a peroxidase activity upon mixing with heme (data not shown).

The complex Aβ–heme is a peroxidase (Fig. 3). In our experiment, the ratios of the substrates TMB (832 μM) and H_2O_2 (3 mM) to the complex Aβ–heme (0.1 μM) were 8,320:1 and 300,000:1, respectively, indicating a catalytic activity of the complex Aβ–heme (Fig. 3). The amino acid sequence of Aβ contains Arg, His, and Phe, which participate in the H_2O_2 binding and catalyze the heterolytic split of O—O bond of H_2O_2 by peroxidases (28). A structural study of the complex Aβ–heme to identify the mechanism of catalysis is needed to confirm this mechanism.

The Aβ–heme peroxidase was efficient in oxidizing serotonin (Fig. 4) and DOPA in addition to TMB. We suggest that the oxidation of serotonin by Aβ–heme in the presence of H_2O_2, which would be produced after the decrease in complex IV (10), is a possible molecular link between Aβ and the abnormal neurotransmitters and oxidative damage seen in AD brain. Peroxidases usually oxidize several organic substrates with minimal selectivity. Therefore, we predict that additional biomolecules also are oxidized by the peroxidase activity of excessive Aβ–heme complexes. Consistently, serotonin and DOPA can be oxidized by myeloperoxidase (29), further supporting the finding that both neurotransmitters are substrates for the Aβ–heme-dependent peroxidase. The peroxidase activity of Aβ–heme was inhibited by curcumin (Fig. 5); curcumin lowers oxidative damage in the brain of a mouse model for AD (30), possibly by chelating Cu and iron (31). Redox-active iron was proposed to play a role in the oxidative damage of AD (32, 33). We propose that inhibiting Aβ–heme peroxidase with curcumin may prevent part of the oxidative damage in AD.

Our data suggests that excessive Aβ–heme peroxidase may play a role in the oxidative damage of AD. Frey and coworkers (23) demonstrated the inactivation of muscarinic acetylcholine receptor by oxidative damage catalyzed by a low-molecular-weight substance enriched from AD brain. Heme was proposed to be involved in this inactivation. Aβ–heme may account, in part, for the results observed by Frey and colleagues (23).

Aβ induces heme synthesis and iron uptake in SHSY5Y cells (Fig. 6), which would follow from Aβ-decreasing regulatory heme. Aβ added to the growth medium may penetrate the cells by pinocytosis or endocytosis, or it may bind with cellular membranes, forming a sink that pulls the regulatory heme out of the cytosol. Regulatory heme is thought to be loosely associated with specific proteins (e.g., at a heme-regulatory motif site) (34). It is known that regulatory heme controls its own synthesis (16, 35) by binding to the heme-regulatory motif in ALAS and regulating its import into the mitochondria. Depletion of regulatory heme increases the import of premature ALAS from the cytosol to the mitochondrial matrix, where it is processed into active enzyme. The half-life of ALAS is between 20 and 60 min, indicating a short response time to changes in heme (36). Thus, the increase in heme synthesis induced by Aβ would be a response to the depletion of intracellular regulatory heme, which creates a functional HD.

An increase in heme synthesis due to depletion of regulatory heme causes an increase in iron uptake (37, 38), because iron uptake is influenced by the availability of heme. Regulatory heme enhances the degradation of iron regulatory protein-2 (IRP2) (17); thus, a depletion of regulatory heme by Aβ will stabilize IRP2, increasing the synthesis of transferrin receptor, and lead to increased iron uptake (Fig. 6). An abnormal localization of IRP2 in AD brain has been reported (39), indicating altered function.

The proposed HD induced by excessive Aβ would cause a shortage of heme-*a* (40), which then decreases complex IV (11), causing the release of H_2O_2 and other oxidants from the mitochondria (10). Complex IV is known to decrease in AD. Depleting the regulatory heme by Aβ may also influence additional metabolic functions in addition to those discussed above. The regulatory heme regulates the function of several transcription factors (19, 26), soluble guanylate cyclase (41), and metabolic pathways (10, 42, 43). Thus, it is likely that a decline in the regulatory heme also disturbs specific signaling pathways and gene expression.

We propose that heme and Aβ interact *in situ*. A likely site for this interaction might be the lysosomal–endocytic vesicles, the cell membrane, mitochondria, or the cytosol (Fig. 7). There is some hint that heme may also bind to Aβ protein precursor (AβPP), forming a AβPP–heme complex. We have demonstrated that HD causes dimerization of AβPP (10), suggesting that heme may be necessary to keep the monomer form of AβPP. The function of AβPP is not known but could involve heme. HD human neuroblastoma cells, which possess dimers of AβPP, failed to form axonal extensions after induction of differentiation as compared with heme-sufficient cells (10). The steady-state tissue concentrations of heme and Aβ *in vivo* are not known. It is generally estimated that regulatory heme concentration is <30 nM (34), whereas the concentration of free Aβ is estimated to be in the low nanomolar range (44).

We suggested that heme binding with Aβ is an endogenous mechanism that prevents Aβ aggregation (9). However, excessive production of Aβ, as is the case in AD, may have detrimental consequences if the Aβ–heme peroxidase is produced. Although low concentrations of Aβ–heme (e.g., in the normal brain) might have a physiologic role, we have constructed a model for AD pathology considering excessive levels of Aβ (Fig. 7). In this

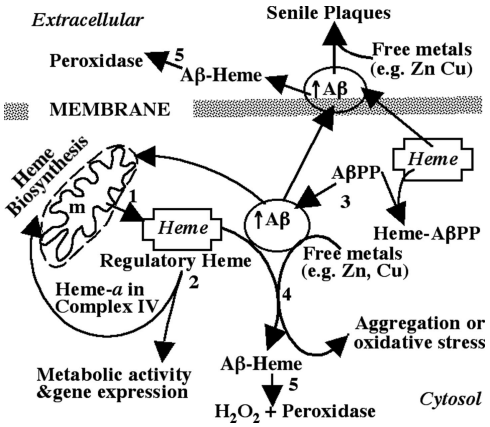

Fig. 7. Proposed consequences of excessive binding of heme with Aβ. Upon synthesis in the mitochondria (m), heme is exported to join the regulatory heme in the cytosol (step 1), where it serves in several metabolic activities (step 2). In AD brain, the production of Aβ increases ($\uparrow$ Aβ) from the processing of AβPP (step 3). Heme prevents Aβ aggregation (step 4). An excessive level of Aβ depletes the regulatory heme, creating HD and producing excess of Aβ–heme peroxidase (step 5), which contributes to oxidative stress in AD. Additional metabolic consequences are addressed in *Discussion*.

model, the binding of regulatory heme to elevated levels of Aβ in AD brain creates HD. HD causes a decline in complex IV, iron accumulation, mitochondrial dysfunction, and increased production of oxidants (e.g., H_2O_2) (9–11), which are seen in AD (5, 6). The Aβ–heme-dependent peroxidase activity, however, could explain the oxidative damage and the decline in specific neurotransmitters, such as serotonin and DOPA, in AD.

We propose that HD and excessive peroxidase activity from Aβ–heme create a vicious cycle that increases oxidative damage. HD leads to an increased production of H_2O_2, which could serve as a substrate for the peroxidase Aβ–heme. In contrast to the physiologic peroxidases that are usually compartmentalized and under strict metabolic regulation, the excessive Aβ–heme might escape physiologic regulation. This model also could explain the accelerated pathology of familial AD, in which excessive Aβ production occurs at young ages compared with sporadic AD.

We have discussed that heme synthesis requires the cofactors iron, copper, zinc, biotin, pantothenic acid, riboflavin, and lipoic acid (13, 45). Deficiencies of these in the U.S. population are very common, e.g., 25% of menstruating women ingest <50% of the recommended dietary allowance for iron. Deficiency leads to a decrease in heme and complex IV and an increase in oxidants; inadequate levels may limit the compensatory response to a shortage in heme and play a role in Aβ clearance and processing of AβPP (10)

The Aβ–heme complex opens new directions of research in AD and supports the view that changes in cellular heme metabolism might be an early pathologic sign of AD brain. Preventing excessive formation of Aβ–heme complex, inhibiting the Aβ–heme peroxidase, and counteracting HD may prevent AD.

Materials and Methods

Materials. Aβ (as Aβ42) was from American Peptide (Sunnyvale, CA); hemin (which is referred to as heme) was from Frontier Scientific (Logan, UT); butyl acetate, TfT, γ-globulin, trypan blue, 30% H_2O_2, and curcumin were from Sigma; mouse monoclonal antibody against amino acids 1–17 of Aβ (clone 6E10) was from Chemicon International (Temecula, CA); cell media (DMEM and MEM), nonessential amino acids, trypsin-EDTA, and Na-pyruvate were from Invitrogen; the protein quantification kit was from Bio-Rad; iron isotope ^{59}Fe (as 1 mCi/ml $^{59}FeCl_3$) (1 Ci = 37 GBq) was from PerkinElmer; liquid scintillation CytoScint was from ICN; and an immunoPure TMB substrate kit for peroxidases was from Pierce. The reagents used for SDS/PAGE and Western blotting and HPLC were of the highest grade available.

Preparation of Heme. A stock solution of heme was freshly prepared in 0.1 M NaOH in ultrapure water (NANOpure, Barnstead, Dubuque, IA) and stored in the dark. The stock of heme was gradually diluted at pH 9, followed by a second dilution to the desired concentration and a pH of 7.5.

Interaction of Heme and Aβ. Aβ was prepared at stock concentration of 600 μM in ultrapure water. Aggregates of Aβ were prepared by allowing spontaneous aggregation in an Aβ solution at 25°C for at least 24 h, which was then divided into aliquots and stored at ≈20°C. The aggregation of Aβ was measured by assaying the fluorescence of TfT, which increases upon binding to Aβ aggregates [excitation filter of 435 nm (bandwidth, 5 nm) and emission filter of 486 nm (bandwidth, 10 nm)] (46, 47).

Dismantling the previously prepared aggregates of Aβ with heme was measured by monitoring the decrease in TfT fluorescence. Aβ aggregates (equivalent to 6 μM monomers) were mixed with heme (6 μM) in 100 μl of PBS. The ratio of heme to Aβ was maintained stoichiometrically at 1:1 to be able to detect the spectral changes to heme.

Spontaneous aggregation of Aβ was followed by diluting freshly prepared Aβ stock concentration to 6 μM in 100 μl of PBS, pH 7.4, and incubating at 37°C with or without 12 or 6 μM heme. At different time intervals, aliquots of 10 μl were removed and mixed with 20 μM TfT in 200 μl of PBS, and the fluorescence was measured. The spectrum of heme between 350 and 750 nm was measured at the end of each experiment. Aβ aggregates, treated or not treated with heme, were resolved by 15% SDS/PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibody 6E10.

Peroxidase Activity of the Aβ–Heme Complex. The peroxidase activity of Aβ–heme was measured by the oxidation of TMB by H_2O_2 (ImmunoPure TMB kit) by following the increase in absorbance at 652 nm, which allows continuous monitoring of the TMB oxidation product. Alternatively, the reaction was terminated by 4 M sulfuric acid, which shifts the absorbance peak of the TMB oxidation product to 450 nm. The peroxidase activity of Aβ–heme was tested in the concentration range of 100–700 nM of the complex for 15–30 min at room temperature. The effect of potential inhibitors (curcumin) or substrates (serotonin and DOPA) on Aβ–heme peroxidase activity was measured by the TMB assay or by HPLC. For the HPLC assay, 100 μM serotonin was incubated with 3 mM H_2O_2 and 500 nM Aβ–heme in 50 mM Hepes, pH 7.2. At specific time intervals, the oxidation products of serotonin were analyzed by injecting 23.5 μl from the reaction mixture into RP-HPLC on a 300- × 3.9-mm column of Bond-Clone-C18 (Phenomenex) by using as mobile-phase 10% methanol, pH 3 (48). Serotonin or oxidation products were analyzed with a UV-online detector set at 280 nm.

Effect of Aβ on Heme Synthesis and Iron Uptake. Heme synthesis by human neuroblastoma cells (SHSY5Y) was measured by using radioisotope ^{59}Fe. The cells were maintained as described in ref. 10. The cells were harvested by trypsinization and counted, and 2×10^6 cells per milliliter were incubated in iron-free MEM/10% FBS/20 mM Hepes supplemented with 0.25 μCi/ml $^{59}FeCl_3$. The amount of iron in the serum used for these experiments was 150 ng/ml. Heme synthesis was tested at 37°C with constant gentle mixing at three Aβ concentrations: 0.1, 1, and 10 μM. Control cells did not receive Aβ. At different time intervals, cells from a 1-ml aliquot were collected by centrifugation, washed three times (3 min each) with ice-cold PBS supplemented with 50 μM of deferroxamine, and lysed into 100

μl of PBS/1% Tween 20. Then 400 μl of 5 M HCl was added followed by 1.2 ml of butyl acetate (49). This procedure extracts only heme into butyl acetate, leaving the non-heme iron in the aqueous phase. The radioactivity was measured in both phases. Deferroxamine was included in the washes to remove residual iron that might bind to the cell surface. Cell viability of control and Aβ-treated cells at 60 and 120 min of incubation was measured by trypan blue exclusion.

Statistical Analysis. Unpaired Student's two-tailed t tests or nonparametric Mann–Whitney tests were performed by using PRISM 4 (GraphPad, San Diego). Significance was defined as $P < 0.05$.

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1. Billings, L. M., Oddo, S., Green, K. N., McGaugh, J. L. & Laferla, F. M. (2005) *Neuron* **45,** 675–688.
2. Skovronsky, D. M., Doms, R. W. & Lee, V. M. (1998) *J. Cell Biol.* **141,** 1031–1039.
3. Walsh, D. M. & Selkoe, D. J. (2004) *Protein Pept. Lett.* **11,** 213–228.
4. Smith, M. A., Nunomura, A., Zhu, X., Takeda, A. & Perry, G. (2000) *Antioxid. Redox. Signal* **2,** 413–420.
5. Parker, W. D., Jr., Parks, J., Filley, C. M. & Kleinschmidt-DeMasters, B. K. (1994) *Neurology* **44,** 1090–1096.
6. Mutisya, E. M., Bowling, A. C. & Beal, M. F. (1994) *J. Neurochem.* **63,** 2179–2184.
7. Connor, J. R., Snyder, B. S., Beard, J. L., Fine, R. E. & Mufson, E. J. (1992) *J. Neurosci. Res.* **31,** 327–335.
8. Gsell, W., Jungkunz, G. & Riederer, P. (2004) *Curr. Pharm. Des.* **10,** 265–293.
9. Atamna, H. & Frey, W. H., II (2004) *Proc. Natl. Acad. Sci. USA* **101,** 11153–11158.
10. Atamna, H., Killilea, D. W., Killilea, A. N. & Ames, B. N. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14807–14812.
11. Atamna, H., Liu, J. & Ames, B. N. (2001) *J. Biol. Chem.* **276,** 48410–48416.
12. Atamna, H. (2006) *J. Alzheimer's Dis.*, in press.
13. Atamna, H. (2004) *Aging Res. Rev.* **3,** 303–318.
14. Sellers, V. M., Wu, C. K., Dailey, T. A. & Dailey, H. A. (2001) *Biochemistry* **40,** 9821–9827.
15. Ponka, P., Borova, J. & Neuwirt, J. (1973) *Biochim. Biophys. Acta* **304,** 715–718.
16. Goodfellow, B. J., Dias, J. S., Ferreira, G. C., Henklein, P., Wray, V. & Macedo, A. L. (2001) *FEBS Lett.* **505,** 325–331.
17. Goessling, L. S., Mascotti, D. P. & Thach, R. E. (1998) *J. Biol. Chem.* **273,** 12555–12557.
18. Dioum, E. M., Rutter, J., Tuckerman, J. R., Gonzalez, G., Gilles-Gonzalez, M. A. & McKnight, S. L. (2002) *Science* **298,** 2385–2387.
19. Dhakshinamoorthy, S., Jain, A. K., Bloom, D. A. & Jaiswal, A. K. (2005) *J. Biol. Chem.* **280,** 16891–16900.
20. Brown, K. R., Allan, B. M., Do, P. & Hegg, E. L. (2002) *Biochemistry* **41,** 10906–10913.
21. Wielburski, A. & Nelson, B. D. (1983) *Biochem. J.* **212,** 829–834.
22. Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C. & Glabe, C. (1992) *J. Biol. Chem.* **267,** 546–554.
23. Fawcett, J. R., Bordayo, E. Z., Jackson, K., Liu, H., Peterson, J., Svitak, A. & Frey, W. H., II (2002) *Brain Res.* **950,** 10–20.
24. Howlett, D., Cutler, P., Heales, S. & Camilleri, P. (1997) *FEBS Lett.* **417,** 249–251.
25. Paoli, M., Marles-Wright, J. & Smith, A. (2002) *DNA Cell. Biol.* **21,** 271–280.
26. Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M., Shibahara, S., Fujita, H. & Igarashi, K. (2001) *EMBO J.* **20,** 2835–2843.
27. Bush, A. I. (2003) *Trends Neurosci.* **26,** 207–214.
28. Fukuyama, K., Kunishima, N., Amada, F., Kubota, T. & Matsubara, H. (1995) *J. Biol. Chem.* **270,** 21884–21892.
29. Dunford, H. B. & Hsuanyu, Y. (1999) *Biochem. Cell Biol.* **77,** 449–457.
30. Lim, G. P., Chu, T., Yang, F., Beech, W., Frautschy, S. A. & Cole, G. M. (2001) *J. Neurosci.* **21,** 8370–8377.
31. Baum, L. & Ng, A. (2004) *J. Alzheimer's Dis.* **6,** 367–377, and discussion **6,** 443–449.
32. Sayre, L. M., Perry, G., Harris, P. L., Liu, Y., Schubert, K. A. & Smith, M. A. (2000) *J. Neurochem.* **74,** 270–279.
33. Dwyer, B. E., Takeda, A., Zhu, X., Perry, G. & Smith, M. A. (2005) *Curr. Neurovasc. Res.* **2,** 261–267.
34. Sassa, S. (2004) *Antioxid. Redox. Signal* **6,** 819–824.
35. Scholnick, P. L., Hammaker, L. E. & Marver, H. S. (1972) *J. Biol. Chem.* **247,** 4132–4137.
36. Granick, S. & Sassa, S. (1971) *δ-Aminolevulinic Acid Synthetase and the Control of Heme and Chlorophyll Synthesis* (Academic, New York).
37. Muller-Eberhard, U., Liem, H. H., Grasso, J. A., Giffhorn-Katz, S., DeFalco, M. G. & Katz, N. R. (1988) *J. Biol. Chem.* **263,** 14753–14756.
38. Iacopetta, B. & Morgan, E. (1984) *Biochim. Biophys. Acta* **805,** 211–216.
39. Smith, M. A., Wehr, K., Harris, P. L., Siedlak, S. L., Connor, J. R. & Perry, G. (1998) *Brain Res.* **788,** 232–236.
40. Atamna, H., Walter, P. B. & Ames, B. N. (2002) *Arch. Biochem. Biophys.* **397,** 345–353.
41. Ignarro, L. J., Ballot, B. & Wood, K. S. (1984) *J. Biol. Chem.* **259,** 6201–6207.
42. Grinstead, G. F., Trzaskos, J. M., Billheimer, J. T. & Gaylor, J. L. (1983) *Biochim. Biophys. Acta* **751,** 41–51.
43. Litman, D. A. & Correia, M. A. (1985) *J. Pharmacol. Exp. Ther.* **232,** 337–345.
44. Fukuyama, R., Mizuno, T., Mori, S., Nakajima, K., Fushiki, S. & Yanagisawa, K. (2000) *Eur. Neurol.* **43,** 155–160.
45. Ames, B. N., Atamna, H. & Killilea, D. W. (2005) *Mol. Aspects. Med.* **26,** 363–378.
46. Ban, T., Hamada, D., Hasegawa, K., Naiki, H. & Goto, Y. (2003) *J. Biol. Chem.* **278,** 16462–16465.
47. Lockhart, A., Ye, L., Judd, D. B., Merritt, A. T., Lowe, P. N., Morgenstern, J. L., Hong, G., Gee, A. D. & Brown, J. (2005) *J. Biol. Chem.* **280,** 7677–7684.
48. Huether, G., Fettkotter, I., Keilhoff, G. & Wolf, G. (1997) *J. Neurochem.* **69,** 2096–2101.
49. Lange, H., Kispal, G. & Lill, R. (1999) *J. Biol. Chem.* **274,** 18989–18996.