The cortical deposition of Aβ is an event that occurs in Alzheimer’s disease, Down’s syndrome, head injury, and normal aging. Previously, in appraising the effects of different neurochemical factors that impact upon the solubility of Aβ, we observed that Zn²⁺ was the predominant bioessential metal to induce the aggregation of soluble Aβ at pH 7.4 in vitro and that this reaction is totally reversible with chelation. We now report that unlike other biometals tested at maximal biological concentrations, marked Cu²⁺-induced aggregation of Aβ₁–₄₀ emerged as the solution pH was lowered from 7.4 to 6.8 and that the reaction was completely reversible with either chelation or alkalinization. This interaction was comparable to the pH-dependent effect of Cu²⁺ on insulin aggregation but was not seen for aprotinin or albumin. Aβ₁–₄₀ bound three to four Cu²⁺ ions when precipitated at pH 7.0. Rapid, pH-sensitive aggregation occurred at low nanomolar concentrations of both Aβ₁–₄₀ and Aβ₁–₄₂ with submicromolar concentrations of Cu²⁺. Unlike Aβ₁–₄₀, Aβ₁–₄₂ was precipitated by submicromolar Cu²⁺ concentrations at pH 7.4. Rat Aβ₁–₄₀ and histidine-modified human Aβ₁–₄₀ were not aggregated by Zn²⁺, Cu²⁺, or Fe³⁺, indicating that histidine residues are essential for metal-mediated Aβ assembly. These results indicate that H⁺-induced conformational changes unmask a metal-binding site on Aβ that mediates reversible assembly of the peptide. Since a mildly acidic environment together with increased Zn²⁺ and Cu²⁺ are common features of inflammation, we propose that Aβ aggregation by these factors may be a response to local injury. Cu²⁺, Zn²⁺, and Fe³⁺ association with Aβ explains the recently reported enrichment of these metal ions in amyloid plaques in Alzheimer’s disease.

Aβ, a low molecular weight (39–43 amino acids) protein that is a proteolytic product derived from the larger amyloid precursor protein (1–3), is the major component of neocortical amyloid collections in Alzheimer’s disease (AD); see Refs. 4 and 5. As is the case with other amyloid proteins, Aβ originates as a normally soluble and constitutive protein found in biological fluids and tissue (6–15). Aβ also aggregates to form diffuse amorphous deposits in AD but also following head injury (16–18) and in healthy aged individuals (19). Combined with other inflammatory proteins such as proteoglycans (20), amyloid P-component (21), and apolipoprotein E (22, 23), Aβ is found in the brains of individuals affected by AD and Down’s syndrome (DS) as dense extracellular deposits of twisted β-sheet fibrils in the neuropil (senile plaques) and within cerebral blood vessels (amyloid congophilic angiopathy; see Refs. 4 and 5). The deposition of Aβ, however, is not confined to the brain parenchyma, having been detected in amyloid diseases of the muscle (24–26), blood vessels (27, 28), and in the kidneys, lungs, skin, subcutaneous tissue, and intestine of AD patients (29, 30).

Cerebral Aβ deposition occurs in other aged mammals that have the human Aβ sequence (31) but is not a feature of aged rats (32, 33). Although soluble Aβ₁–₄₀ is produced by rat neuronal tissue (34), it contains three amino acid substitutions (Arg → Gly, Tyr → Phe, and His → Arg at positions 5, 10, and 13, respectively (32)) that appear to alter the physicochemical properties of the peptide preventing it from precipitating in the neocortex.

Aβ accumulates in the brain in AD and DS in forms that can be resolubilized in water (35, 36) but also in forms that require harsher conditions to extract and exhibit associated SDS-resistant polymerization on polyacrylamide gel electrophoresis (35, 37). Aβ₁–₄₀ is the predominant soluble species in biological fluids, whereas Aβ₁–₄₂, a minor species of Aβ that is more insoluble in vitro, is the predominant species found in plaques and deposits associated with AD and DS (35–41).

We have pursued studies of the physicochemical properties of synthetic Aβ peptides in order to appraise the potential of various neurochemical environments to induce Aβ deposition. To this end, we had previously found that Aβ is strikingly precipitated by certain metals in vitro, in particular Zn²⁺ (42–45). This is important since zinc and other biometals are concentrated in the brain neocortical parenchyma. A recent study, using micro particle-induced x-ray emission analyses of the cortical and accessory basal nuclei of the amygdala, demonstrated that levels of Zn²⁺, Fe³⁺, and Cu²⁺ are significantly elevated within AD neuropil compared with control neuropil and that these metal ions are significantly further concentrated within the core and periphery of plaque deposits (46). We have also found that the extraction of Aβ deposits from brain tissue into aqueous buffers is increased in the presence of chelators of Zn²⁺ and Cu²⁺ (47), providing further evidence that these metal ions participate in the deposition of Aβ within amyloid plaques.
Copper-induced Aβ Aggregation Is Modulated by pH

In common with the appearance of amorphous and amyloid deposits is the observation that altered neuronal H+ homeostasis may accompany Alzheimer's disease and head injury. AD is complicated by cerebral acidosis (pH 6.6; see Ref. 48), which may be related to impaired glucose metabolism (49) or to the inflammatory response seen in AD-affected brain tissue (reviewed in Refs. 50 and 51). The release of metal ions such as Cu+ and Fe2+ from metalloproteins is induced by a mildly acidic environment (52–58). Therefore, we investigated the effect of several bioessential metal ions on their ability to bind and alter the solubility of Aβ40/42 under mildly acidic conditions (pH ≥ 6.6) to determine whether there are any unforeseen interactions that may help explain the propensity for Aβ to precipitate under the mildly acidic conditions anticipated within the metabolically diseased brain parenchyma. A striking and unexpected interaction between pH and Cu2+ was observed for human Aβ.

MATERIALS AND METHODS

Reagents and Preparation—Human Aβ1–40 peptide was synthesized, purified, and characterized by high pressure liquid chromatography analysis (HPLC), amino acid analysis, and mass spectrometry by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). Rat Aβ1–42 was obtained from Quality Control Biochemicals, Inc. (Hopkington, MA). The HPLC elution profiles of both peptides in these working batches were identified as a single peak. Amino acid analysis of the synthetic peptides indicated no apparent chemical modifications in the amino acid residues. Synthetic Aβ peptide solutions were dissolved in trifluoroethanol (30% in Milli-Q water (Millipore Corp., Milford, MA)) or 20 mM Hepes (pH 8.5) at a concentration of 0.5–1.0 mg/ml, centrifuged for 20 min at 10,000 × g, and the supernatants (stock Aβ) used for subsequent aggregation assays on the day of the experiment. Prior to use all buffers and stock solutions of metal ions were filtered though a 0.22-μm filter (Gelman Sciences, Ann Arbor, MI) to remove any particulate matter. All metal ions were the chloride salt, except lead nitrate.

The concentration of stock Aβ peptides, bovine serum albumin (BSA; Pierce), aprotinin (Sigma), and insulin (Sigma) was determined by spectrophotometric absorbance at 214 nm (against calibrated standard curves) or by Micro BCA protein assay (Pierce). The Micro BCA assay was performed by diluting 10 μM of stock Aβ (or BSA standard) in distilled, deionized water (140 μl) and then adding BCA reagent (150 μl) to a 96-well plate and measuring the absorbance at 562 nm the validity of this assay was previously confirmed by amino acid analysis. Aggregation Assays—To determine the centrifugation time required to completely sediment aggregated Aβ (2.5 μM) in 150 mM NaCl and 20 mM Hepes (pH 7.4) was incubated for 30 min at 37 °C with no metal, and under aggregating conditions of Zn2+ (100 μM), Cu2+ (100 μM), or pH 5.5. Reaction mixtures were centrifuged at 10,000 × g for different times or ultracentrifuged at 100,000 × g for 1 h. Centrifugation for >10 min at 10,000 × g was sufficient to completely sediment Zn2+, Cu2+, and pH-inducement aggregates of Aβ40-42 when compared with ultracentrifugation (100,000 × g for 1 h). Therefore, centrifugation for 10–20 min at 10,000 × g was used in subsequent assays to sediment aggregated particles.

To quantitate the effects of different metals and pH on protein solubility, synthetic Aβ stock, and BSA, aprotinin and insulin stocks were diluted to 2.5 μM in 150 mM NaCl in either 20 mM glycine (pH 3.0–4.5), MES (pH 5.0–6.2), or Hepes (pH 6.4–8.8) and then incubated (37°C) with or without metal ions for 30 min. The resultant aggregated particles were sedimented by centrifugation (20 min, 10,000 × g), and soluble protein was measured in the supernatant by the Micro BCA protein assay.

Stoichiometry and Binding Analyses of Cu for Aβ-Cu2+—Concentrations were determined using the spectrophotometric method of Matsum and Takahashi (59), adapted to microtiter plate volumes. Sample Cu2+ concentrations were determined by measuring the absorbance of a standard calibration curve (0–100 μM in 20 mM ammonium acetate, 150 mM NaCl (pH 7.4)). Cu2+-induced spectral changes in Aβ were monitored by incubating Aβ40 or Aβ42 (5 μM) in 20 mM ammonium acetate buffer, 150 mM NaCl (pH 7.4), with and without various concentrations of Cu2+ for 5 min prior to loading onto a quartz microtiter plate. Absorbance was scanned between 200 and 800 nm on a Spectramax Plus Spectrophotometer (Molecular Devices, Sunnyvale, CA). Incubation of Cu2+ with Aβ induced a change in the absorbance profile of the peptide which was maximal at 208 nm. However, subsequent analyses were performed at 214 nm where the spectral shift was still large but the background signal was lower.

By using Aβ aggregation as an end point for Cu2+ binding, the stoichiometry of Cu2+ binding to Aβ1–42 was determined by incubating Aβ1–42 (10 μM) in 20 mM ammonium acetate, 150 mM NaCl (pH 7.4) for 30 min at 37°C with Cu2+ or Cu2+-(glycine)2 (50 μM) (60). Reaction mixtures were centrifuged at 10,000 × g for 20 min, and the ratio of Cu2+ binding to Aβ was estimated by determining the concentrations of soluble Aβ and Cu2+ remaining in the supernatant. It was determined that precipitation of Aβ did not affect the assay.

Turbidimetric Assays—Turbidity measurements, as an assay for aggregation, were performed in a flat-bottomed 96-well microtiter plate (Corning Costar Corp.), and absorbances (405 nm) were measured using a Spectramax Plus spectrophotometric microplate reader (Molecular Devices, Sunnyvale, CA). Automatic 30-s plate agitation mode was selected for the plate reader to evenly suspend the aggregates in the wells before all readings. Aβ1–40 stock was brought to 10 μM (300 μl) in 20 mM Hepes buffer, 150 mM NaCl (pH 6.6, 6.8, or 7.4) ± metal ions prior to incubation (30 min, 37 °C) and absorbance measurement.

To investigate the reversibility of Cu2+-induced Aβ aggregation, 25 μM Aβ1–40 and 25 μM Cu2+ were mixed in 67 mM phosphate buffer containing 150 mM NaCl (pH 7.4), to a final volume of 200 μl. Turbidity measurements were taken at 4-min intervals. Subsequently, 20-μl aliquots of 10 mM EDTA or 10 mM Cu2+ were added to the wells alternatively, and following a 2-min delay, a further four readings were taken at 1-min intervals. After the final EDTA addition and turbidity reading, the mixtures were incubated for an additional 30 min before taking final readings.

To investigate the reversibility of pH-mediated Cu2+-induced Aβ1–40 aggregation, 10 μM Aβ1–40 and 30 μM Cu2+ were mixed in 67 mM phosphate buffer containing 150 mM NaCl (pH 7.4), and an initial turbidity measurement was taken. Subsequently, the pH of the solution was successively decreased to 6.6 with 0.1 M HCl and then increased back to 7.4 with 0.1 M NaOH. The pH of the reaction was monitored with a microprobe (Lazar Research Laboratories Inc., Los Angeles, CA) and the turbidity read at 5-min intervals for up to 30 min. This cycle was repeated three times.

Modification of Aβ1–40—Blockage of histidine residues was achieved by incubating Aβ1–40 (1.5 mg/ml dissolved in distilled, deionized H2O, following centrifugation (10,000 × g for 20 min) to remove undissolved peptide) with 5 mM diethyl pyrocarbonate (DEPC) in 25 mM phosphate buffer (pH 7.4) for 30 min at 4°C (61). Formation of N-carbethoxyhistidine residues was followed by the characteristic characteristic color change at 240 nm (62). The modified protein was dialyzed extensively to remove DEPC, and its ability to aggregate at pH 7.4 and 6.6 with and without Cu2+, Fe3+, and Zn2+ was tested as described above. Subsequently, reversal of the histidine modification was achieved by incubating the modified protein with 0.1 M hydroxylamine hydrochloride in 25 mM phosphate buffer (pH 7.4) for 30 min at 4°C. The restored peptide was then dialyzed extensively to remove excess hydroxylamine hydrochloride, and its ability to aggregate at pH 7.4 and 6.6 with and without Cu2+, Fe3+, and Zn2+ was retested.

Western Blot Analysis of the Aggregation of Nanomolar Concentrations of Aβ—Stock Aβ1–40 or Aβ40-42 (1 mg/ml) was diluted to a final concentration of 200 ng in 1 ml (44 mM) of buffer containing 150 mM NaCl, 20 mM Tris (pH 7.4 or 6.6). 0.1 μM BSA and CuCl2 (0, 0.1, 0.5, 1 or 5 μM), and the samples were mixed and then incubated (30 min, 37°C). The reaction mixtures were then centrifuged at 12,000 × g for 10 min in a fixed angle rotor centrifuge, and the supernatant was carefully removed leaving a pellet of aggregated Aβ on the side of the tube. Sample buffer (30 μl; containing 4% SDS, 5% β-mercaptoethanol) was added to the tubes which were vigorously mixed, then heated to 95°C for 5 min, and spun briefly. The SDS-extracted samples and a standard containing 50 ng of Aβ in sample buffer were loaded and analyzed by polycrylamide gel electrophoresis (Tricine gels, 10–20%; Novex, San Diego, CA), transferred to polyvinylidene difluoride membranes (Bio-Rad), fixed, blocked, and then probed with the anti-Aβ monoclonal antibody 6E10 (Senetek, Maryland Heights, MI) overnight at 4°C. The blotted peptides were detected using horseradish peroxidase-conjugate (Pierce) for 2 h at 22°C and incubated (5 min) with Supersignal Ultra (Pierce) according to the manufacturer’s instructions. The chemiluminescent signal was captured for 10 min at maximum sensitivity using the Fluoro-S Image Analysis System (Bio-Rad), and the electronic images were analyzed using Multi-Analyzer Software (Bio-Rad). This chemiluminescent image analysis system is linear over 2 orders of magnitude and has comparable sensitivity to film.
Immunofiltration Detection of Nanomolar Concentrations of Aβ Aggregate—Aβ<sub>1–40</sub> or Aβ<sub>1–42</sub> (1 mg/ml) stock solutions were diluted to 20 nM in buffer containing 150 mM NaCl, 20 mM Tris (pH 7.4 or 6.6), 0.1 mM BSA and CuCl<sub>2</sub> (0, 0.1, 0.2, 0.3, 0.5, 1, or 2 mM) added, and the samples were incubated (15 min, 37 °C). The reaction mixtures (200 μl) were then placed into the 96-well Easy-Titer ELIFA system (Pierce) and filtered through a 0.22-μm cellulose acetate filter (MSI, Westboro, MA). Aggregated particles were fixed to the membrane (0.1% glutaraldehyde, 15 min), washed thoroughly with Tris-buffered saline, and then probed with the anti-Aβ monoclonal antibody 6E10 (Senetek, Maryland Heights, MI) overnight at 4 °C. Blots were then processed as described above.

RESULTS

To determine the effect of mild acidity on the behavior of Aβ in the presence of different metals, Aβ<sub>1–40</sub> was incubated with different bioessential metal ions at pH 6.6, 6.8, and 7.4 at total metal ion concentrations observed in serum (Fig. 1A and B). Peptide aggregation was measured by sedimentation (Fig. 1A) and turbidimetry (Fig. 1B), which generally gave confirmatory results. Incubation of Aβ in the absence of metal ions induced no detectable aggregation over the pH range tested. Only incubation with Zn<sup>2+</sup> or Cu<sup>2+</sup> induced >30% aggregation under these conditions (Fig. 1A). As the [H<sup>+</sup>] was increased, all other metal ions induced an observable increase in sedimented Aβ (Fig. 1A), but Cu<sup>2+</sup> induced the most striking increase in Aβ aggregation as measured by both sedimentation and turbidimetry. Fe<sup>3+</sup> induced a marked increase in turbidity (Fig. 1B), but this aggregate was not as readily sedimented as the Cu<sup>2+</sup>- or Zn<sup>2+</sup>-induced Aβ aggregates (Fig. 1A), probably due to decreased density of the assembly. As expected, Zn<sup>2+</sup> induced ~50% of the soluble peptide to sediment over the pH range tested, but increasing the [H<sup>+</sup>] caused a decrease in Zn<sup>2+</sup>-induced aggregation (Fig. 1C), probably due to an alteration in the size or light scattering properties of the Zn<sup>2+</sup>-induced aggregates. Incubation of the mixtures for 16 h did not induce any appreciable change in the turbidometry readings compared with the original readings (data not shown), suggesting that the aggregation reactions had reached equilibrium within 30 min.

The effects of higher metal ion concentrations were also compared at equimolar (30 μM) concentrations (Fig. 1C). Under these conditions, as the [H<sup>+</sup>] increased, Ni<sup>2+</sup>-induced Aβ aggregation (~85%) which was comparable to the effect of incubation with the same concentration of Cu<sup>2+</sup>. Co<sup>2+</sup> induced a comparable level of Aβ aggregation to Zn<sup>2+</sup> (~70%) and, like...
Fig. 2. Cu²⁺-induced spectral changes in Aβ. Data points are means ± S.D., n = 3. The figures are each representative of two experiments. A, the change in the absorbance at 214 nm of Aβ₁₋₄₀ (10 μM) in 20 mM ammonium acetate buffer and 150 mM NaCl (pH 7.4), with increasing Cu²⁺ concentrations. B, the change in the absorbance at 214 nm of Aβ₁₋₄₀ (10 μM) in 20 mM ammonium acetate buffer and 150 mM NaCl (pH 7.4), with increasing Cu²⁺ concentrations.

Zn²⁺, Co²⁺-induced aggregation was independent of [H⁺]. At 30 μM, Fe²⁺, Al³⁺, and Pb²⁺ were observed to induce partial (~30%) Aβ aggregation at pH 6.6 but no significant aggregation at pH 7.4.

To quantify the binding affinity of Cu²⁺ for Aβ, spectral analysis was performed. The half-maximal binding of Cu²⁺ for Aβ₁₋₄₀ and Aβ₁₋₄₂ based upon shift in absorbance was estimated as 4.0 and 0.3 μM, respectively (Fig. 2, A and B). The stoichiometry of Cu²⁺ binding to Aβ₁₋₄₀ in the Cu²⁺-induced Aβ aggregate was 3.4 and 3.0 at pH 7.0 and 6.6, respectively.

The stoichiometry of Cu²⁺ binding to Aβ₁₋₄₀ required for precipitation is in agreement with that required for the saturation of the spectral shift (Fig. 2A). Cu²⁺ supplied as a Cu²⁺-(glycine)₂ complex did not alter the amount of Aβ aggregation or binding stoichiometry compared with that induced by non-complexed Cu²⁺ in the same buffer (data not shown).

Next, we tested other proteins to determine whether Cu²⁺-induced protein aggregation under mildly acidic conditions was specific for Aβ (Fig. 3A). In the absence of metal ions, BSA aggregation was low (<15%) at both pH 7.4 and 6.6. However, in contrast to Aβ₁₋₄₀, the amount of BSA aggregation increased only marginally in the presence of Cu²⁺ at both pH 7.4 and 6.6. Aprotinin was significantly aggregated (~30%) by mildly acidic conditions, but unlike Aβ its aggregation at pH 6.6 was not potentiated by the presence of Cu²⁺. In contrast, insulin was not aggregated by pH 6.6 or by Cu²⁺ at pH 7.4. However, there was a marked increase in insulin aggregation at pH 6.6 in the presence of Cu²⁺, resembling that seen with Aβ₁₋₄₀.

Since higher concentrations of Zn²⁺ are required to induce the aggregation of rat Aβ₁₋₄₀ compared with human Aβ₁₋₄₀, we examined whether rat Aβ₁₋₄₀ also was resistant to Cu²⁺-induced aggregation (Fig. 3B). At pH 7.4, there was a similar slight increase in the aggregation of both human Aβ₁₋₄₀ and rat Aβ₁₋₄₀ as the [Cu²⁺] increased. However, the amount of aggregation of rat Aβ₁₋₄₀ as [Cu²⁺] increased at pH 6.6 was less than half that of human Aβ₁₋₄₀ under the same conditions.

We next proceeded to elaborate the effects of [H⁺] upon Cu²⁺-mediated precipitation of Aβ over a broader pH range (Fig. 4A). [H⁺] alone was sufficient to precipitate Aβ₁₋₄₀ (2.5 μM) dramatically once the pH was brought below 6.3 (Fig. 4A). At pH 5.0, 80% of the peptide was precipitated, but the peptide was less aggregated by acidic environments below pH 5.0, in agreement with previous reports on the effect of pH on Aβ solubility (reviewed in Ref. 63). Zn²⁺ (30 μM) induced a constant level (~50%) of aggregation between pH 6.2 and 8.5, whereas below pH 6.0, aggregation could be explained predominantly by the effect of [H⁺].

In the presence of Cu²⁺ (30 μM), a decrease in pH from 8.8 to 7.4 induced a marked drop in Aβ₁₋₄₀ solubility, whereas a slight decrease in pH below pH 7.4 strikingly potentiated the effect of Cu²⁺ on the aggregation of the peptide (Fig. 4A). Surprisingly, Cu²⁺ caused >85% of the available peptide to aggregate by pH 6.8, a pH which plausibly represents a mildly acidic environment. We hypothesize that conformational changes in Aβ brought about by small increases in [H⁺] result in the unmasking of a second metal interaction site that leads to its rapid Cu²⁺-dependent aggregation. Below pH 5.0, the ability of both Zn²⁺ and Cu²⁺ to aggregate Aβ was diminished, consistent with the observation that Zn²⁺ binding to Aβ is abolished below pH 6.0 (42), probably due to protonation of histidine residues.

Further elaboration of the relationship between pH and Cu²⁺ on Aβ₁₋₄₀ solubility (Fig. 4B) confirmed that there was potentiation between [H⁺] and [Cu²⁺] in producing Aβ aggregation; as the pH fell, less Cu²⁺ was required to induce the same level of aggregation, suggesting that [H⁺] controls Cu²⁺-induced Aβ₁₋₄₀ aggregation. At pH 7.4, Cu²⁺-induced Aβ aggregation was far less than that induced by Zn²⁺ at similar concentrations, consistent with our earlier report (43).

To determine if this reaction occurred at lower concentrations of Aβ and to compare the effects of Cu²⁺-induced aggregation of Aβ₁₋₄₀ with Aβ₁₋₄₂ (which is not stable in solution at micromolar concentrations in aqueous buffers), we incubated both peptides (50 nM) with various Cu²⁺ concentrations (0–5 μM), sedimented the aggregated peptide, resolubilized the Aβ pellets with SDS, and visualized the Aβ on Western blots.
Increased aggregation of Aβ1–40 was apparent at Cu²⁺ concentrations as low as 500 nM (Fig. 5A). As previously observed at higher Aβ1–40 concentrations, a decrease in pH from 7.4 to 6.6 potentiated the effect of Cu²⁺ on the aggregation of Aβ1–40 in this system. Aβ1–42 aggregation followed a similar pattern to that of Aβ1–40, but 500 nM Cu²⁺ appeared to induce more aggregation of Aβ1–42 than Aβ1–40 at pH 7.4 (Fig. 5B).

To study this reaction at even lower concentrations of Aβ1–40, Aβ1–42 (20 nM), and Cu²⁺ (≤1 μM), such as those found in cerebrospinal fluid (6, 11, 13, 14), we employed a novel filtration immunodetection system² (Fig. 6, A and B). This sensitive technique confirmed that a decrease in pH from 7.4 to 6.6 potentiated the effect of Cu²⁺ (≥200 nM) on the aggregation of Aβ1–40 (Fig. 6A). An increase in Aβ1–42 aggregation also was observed with increasing [Cu²⁺] which also was potentiated by pH 6.6 (Fig. 6B). At pH 7.4, Aβ1–42 was more sensitive than Aβ1–40 to aggregation induced by increasing Cu²⁺ concentrations in this range.

Since transition metals often coordinate to histidine residues, and since rat Aβ1–40 (His → Arg substitution at position 13) exhibits attenuated aggregation by both Cu²⁺ and Zn²⁺, we tested whether modifying histidine residues with DEPC (61) affected metal ion-induced aggregation of human Aβ1–40. Treatment of Aβ1–40 with DEPC resulted in an increase in absorbance at 240 nm compared with the unmodified protein, confirming the formation of N-carboxethyhistidine residues (data not shown), whereas the formation of O-carboxethytyrosine, characterized by a decrease in the absorption spectrum at 278 nm, was not observed. N-Carboxethyhistidine modification completely abolished Aβ aggregation in the presence of Zn²⁺ (Fig. 7). Reversal of the modifications with hydroxylamine resulted in almost complete restoration of Zn²⁺-induced aggregation of Aβ, indicating the absolute requirement for the coordination of Zn²⁺ to the histidine(s) of Aβ1–40 in order to induce aggregation. Likewise, the requirement for Fe³⁺-histidine coordination for Aβ aggregation was demonstrated by the abolition of Fe³⁺-induced Aβ aggregation at pH 6.6 following N-carboxethyhistidine modification. As with Zn²⁺-mediated Aβ aggregation, reversal of the modifications restored Fe³⁺-mediated Aβ aggregation (Fig. 7). A marked decrease in Cu²⁺-induced aggregation of the modified protein at both pH 7.4 and 6.6 also was observed, and reversal of the modifications restored Cu²⁺-induced aggregation, indicating that Cu²⁺-histidine coordination also was required for Aβ1–40 aggregation (Fig. 7).

We have recently reported that Zn²⁺-mediated Aβ1–40 aggregation is totally reversible by chelation, whereas Aβ1–40 aggregation induced by pH 5.5 is irreversible with alkalinization (45). Therefore, using turbidometry, we examined whether Cu²⁺/pH-mediated Aβ1–40 aggregation was also reversible. We observed that Cu²⁺-induced Aβ1–40 aggregation at pH 7.4 was reversible following EDTA chelation, although for each new aggregation cycle, complete resolubilization of the aggregates required a longer incubation (Fig. 8A).

The reversibility of pH-potentiated, Cu²⁺-induced Aβ1–40 aggregation in buffers cycled between pH 7.4 and 6.6 was studied by turbidometry (Fig. 8B). Unlike the irreversible aggregation of Aβ1–40 observed at pH 5.5 (45), Cu²⁺-induced Aβ1–40 aggregation was fully reversible as the pH oscillated between pH 7.4 and 6.6.

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²R. D. Moir, A. I. Bush, D. M. Romano, C. S. Atwood, X. Huang, and R. E. Tanzi, personal communication.
expressed as a percentage of starting peptide (2.5 μM) incubation (30 min, 37 °C) at pH 3.0–8.8 in buffered saline with no metal (○), Cu2+ (30 μM, ●) or Cu2+ (30 μM, ▲), and centrifugation (10,000 × g, 20 min), expressed as a percentage of starting peptide (2.5 μM). Data points are means ± S.D., n = 3. B, proportion of aggregated Aβ1–40, following incubation (30 min, 37 °C) at pH 5.4–7.8 with different Cu2+ concentrations (0, 5, 10, 20, 30 μM), and centrifugation (10,000 × g, 20 min), expressed as a percentage of starting peptide (2.5 μM). Data points are means ± S.D., n = 3. ○, 0 μM; ●, 5 μM; ▲, 10 μM; ▲, 20 μM; ▲, 30 μM.

**FIG. 4. Effect of pH on Zn2+ - and Cu2+ -induced Aβ1–40 aggregation.** A, proportion of aggregated Aβ1–40 following incubation (30 min, 37 °C) at pH 3.0–8.8 in buffered saline with no metal (○), Zn2+ (30 μM, ●) or Cu2+ (30 μM, ▲), and centrifugation (10,000 × g, 20 min), expressed as a percentage of starting peptide (2.5 μM). Data points are means ± S.D., n = 3. B, proportion of aggregated Aβ1–40, following incubation (30 min, 37 °C) at pH 5.4–7.8 with different Cu2+ concentrations (0, 5, 10, 20, 30 μM), and centrifugation (10,000 × g, 20 min), expressed as a percentage of starting peptide (2.5 μM). Data points are means ± S.D., n = 3. ○, 0 μM; ●, 5 μM; ▲, 10 μM; ▲, 20 μM; ▲, 30 μM.

**DISCUSSION**

These results indicate that subtle conformational changes mediated by [H+] alter the interaction of Aβ with transition metal ions, modulating peptide aggregation induced particularly by Cu2+ and Ni2+ over a narrow pH range (5.6–4.8). This reaction resembles the pH-potentiated, metal ion-induced aggregation described here. Therefore, we suspect that the displacement of 65Zn2+ by Cu2+ binding to Aβ described by Clements et al. (71) reflects competition for the less specific, lower affinity Zn2+ -binding site on the peptide. We have previously shown that high affinity Zn2+ binding to Aβ1–40, appreciated when Mn2+ is used to create stringent conditions that eliminate lower affinity interactions (42), cannot be displaced by other transition metals. Since Cu2+ has been shown to compete for low affinity metal binding under conditions where high affinity Zn2+ binding would be preserved, we suspect that Aβ1–40 may be capable of binding Cu2+ and Zn2+ simultaneously, a possibility that may be contingent upon the peptide subunits dimerizing in solution, as we have previously observed (45, 72).

Our results support the likelihood of at least two classes of metal-binding sites on Aβ as follows: the Cu2+/Ni2+ -site that is responsible for significant Aβ assembly only under mildly acidic (pH 6.6–7.0) conditions, and the Zn2+ /Co2+ site that mediates significant Aβ assembly at pH 7.4 (Fig. 1). The finding that Aβ1–40 associates with Co2+ in a similar manner to Zn2+ is of interest since Co2+, unlike Zn2+, is paramagnetic and therefore might be used to substitute for Zn2+ in structural biology studies (75). The interactions of Ni2+ and Co2+ with Aβ were achieved with metal ion concentrations that are supra-physiological for these metal ions, although they are physiologically possible for Cu2+ and Zn2+. Therefore, we believe that Cu2+ and Zn2+ would be the most relevant interacting metal ions with Aβ in biological systems.

Aβ exhibits spectral alterations in the presence of Cu2+ (Fig. 2), which appear to be due to saturable binding of the metal ion. Like the prion protein (74), we found that Aβ binds multiple copper ions. By using the half-maximal binding of Cu2+ for Aβ first, the absence of a competing transition metal ion in their assay system (Mn2+ in our assay (42)) to abolish low affinity metal binding, and second, the absence of physiological (150 mM) NaCl in their assays which is a factor that we have found profoundly alters the interaction of metal ions with Aβ1–40 (45). Therefore, we suspect that the displacement of 65Zn2+ by Cu2+ binding to Aβ described by Clements et al. (71) reflects competition for the less specific, lower affinity Zn2+ -binding site on the peptide. We have previously shown that high affinity Zn2+ binding to Aβ1–40, appreciated when Mn2+ is used to create stringent conditions that eliminate lower affinity interactions (42), cannot be displaced by other transition metals. Since Cu2+ has been shown to compete for low affinity metal binding under conditions where high affinity Zn2+ binding would be preserved, we suspect that Aβ1–40 may be capable of binding Cu2+ and Zn2+ simultaneously, a possibility that may be contingent upon the peptide subunits dimerizing in solution, as we have previously observed (45, 72).

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Copper-induced Aβ Aggregation Is Modulated by pH

![Graph A](image)

![Graph B](image)

**Fig. 6.** Immunofiltration detection of Aβ aggregation at sub-micromolar concentrations of Aβ and Cu²⁺. Aβ₁₋₄₀ (A) or Aβ₁₋₄₂ (B) (20 nM) were incubated (37°C, 30 min) in buffered saline (pH 7.4 (black box) or pH 6.6 (shaded box)) containing BSA (0.1 μM) and CuCl₂ (concentrations indicated). The reaction mixtures (200 μL) were then filtered through a 0.22-μm cellulose acetate filter, and aggregated particles were fixed to the membrane and probed with the anti-Aβ monoclonal antibody 6E10. The signals from the retained peptide aggregates were quantified by image capture analysis. Blots were then processed as described previously. These data are representative of four experiments.

As an indicator of affinity, we found that Aβ₁₋₄₂ has a higher affinity for Cu²⁺ than Aβ₁₋₄₀, which is in agreement with our current findings that Aβ₁₋₄₂ is more readily precipitated by Cu²⁺ at pH 7.4 (Fig. 5). Although the spectral alterations that we observed saturated in response to micromolar Cu²⁺ concentrations, these changes may correspond to relatively low affinity Cu²⁺ binding. Since three to four Cu²⁺ ions bind to each Aβ subunit when the peptide is precipitated by Cu²⁺, it is possible that Cu²⁺-binding sites of submicromolar affinity exist but could not be appreciated by the current aggregation and spectral assays. Total Cu²⁺ has been measured at ~15 μM in the synaptic cleft and up to 100 μM in the normal neocortex (46, 75–78). However, the concentrations of Cu²⁺, Fe³⁺, and Zn²⁺ are each more than doubled (~300, ~700, and ~790 μM, respectively) in the neuropil of the cortical and accessory basal nuclei of the amygdala of AD brains compared with the neuropil of normal age-matched brains (46). These metal ions are even further concentrated within the core and periphery of senile plaque deposits (~400, ~950, and ~1100 μM, respectively) (46). Our present and previous findings would explain a basis for Cu²⁺, Fe³⁺, and Zn²⁺ enrichment in cerebral Aβ deposits, which would, in turn, become a sink for these metal ions.

The lower levels of Aβ₁₋₄₀ turbidity seen at pH 7.4 in the presence of all metals combined (Fig. 1B) compared with that induced by Zn²⁺ alone may reflect a protective effect of one of the metal ions in the mixture, possibly Mg²⁺, which is the only metal ion not to exhibit a precipitating effect even at millimolar concentrations. This protective effect is, however, not evident in incubations performed at lower pH, indicating that Mg²⁺ may block the pH-insensitive binding site for Zn²⁺/Cu²⁺. This possibility awaits experimental verification.

We have previously described the rapid aggregation of Aβ₁₋₄₀ by Zn²⁺ (42–45), as well as the abolition of zinc binding to Aβ at pH <6.0, consistent with histidine-mediated interaction. In the current study, modification of Aβ with DEPC abolished Zn²⁺-induced Aβ aggregation (Fig. 7), supporting the possibility that the aggregation of Aβ₁₋₄₀ by Zn²⁺ is dependent upon coordination with histidine. Although Fe³⁺ induced considerably less aggregation of Aβ than Zn²⁺ or Cu²⁺, even this aggregation reaction appeared to be coordinated by the histidine residues of Aβ, suggesting that the association of redox active iron with senile plaques from AD brains recently reported by Smith et al. (79) may be due to the metal ion binding directly to Aβ.

Histidine modification did not completely abolish Cu²⁺-induced Aβ aggregation, suggesting that a proportion of that form of aggregate is assembled by interaction with non-histidine residues on the peptide. Histidine modification of human Aβ₁₋₄₀ reduced the amount of Cu²⁺-induced Aβ aggregation to that observed following the incubation of rat Aβ₁₋₄₀ with the same Cu²⁺ concentrations. Taken together, these data indicate that Cu²⁺ coordination to histidine at position 13 and tyrosine at position 10 may coordinate the aggregation of Aβ₁₋₄₀. Access to these residues by Cu²⁺ therefore appears to be restricted at pH 7.4.

The relationship of Aβ₁₋₄₀ solubility with pH alone mirrors the conformational changes observed by CD spectra within the N-terminal fragment (residues 1–28) of Aβ (63) as follows: α-helical and soluble between pH 1–4 and >7, but β-sheet and aggregated between pH 4–7. Thus, the altered interaction of Cu²⁺ with soluble Aβ below pH 7.0 may be due to changes in peptide conformation. Aβ₁₋₄₀ aggregation by Cu²⁺ was observed to be rapidly reversible by alkalinization. Since increasing pH above 7.0 promotes the α-helical conformation (63), this conformation may be unfavorable for the Cu²⁺ coordination that mediates peptide assembly.

Cu²⁺-induced Aβ₁₋₄₀ aggregation at pH 7.4 also was reversible by chelation; however, less peptide was resolubilized during each cycle perhaps because a denser aggregate was formed during each aggregation cycle which retarded chelator access to the peptide mass. This possibility is supported by the observation that complete resolubilization of the Cu²⁺-induced Aβ aggregates by EDTA eventually occurred with additional incubation time. The modulation of Aβ solubility by Cu²⁺ through a number of aggregation/resolubilization cycles suggests that its interaction does not induce the amyloid β-sheet conformation, which is not easily reversible.

Although the pathogenic nature of Aβ in AD is well described (4, 5), the function of Aβ remains unclear. Taken together, our results emphasize three physiologically plausible environments that could aggregate Aβ as follows: lowered pH (Fig. 4; Refs. 80–86), elevated [Zn²⁺] (Figs. 1, 4, and 7; Refs. 42–45 and 86), and under mildly acidic conditions, elevated [Cu²⁺] (Figs. 1–7). Inflammation generally induces locally acidic conditions (87, 88) and the mobilization of both Zn²⁺ and Cu²⁺ (89–94), which are both found in high concentrations in the neocortex (Zn²⁺ ~150 μM, Ref. 95; Cu²⁺ ~100 μM, Refs. 77 and 78). Inflammatory mechanisms also have been suggested as participating in the pathophysiology of AD (96), and acute phase inflammatory proteins such as α1-antichymotrypsin and C-re-
active protein, elements of the complement system, and activated microglial and astroglial cells are observed in AD-affected brains (see Refs. 50 and 51 for reviews). A sustained increase in regional Zn\(^{2+}\), Cu\(^{2+}\), and H\(^+\) concentrations induced by a cortical inflammatory response is predicted by our data to contribute to the deposition of A\(\beta\) in AD.

Systemic amyloidoses are often associated with chronic inflammation (97–99). An example is the amyloidosis caused by serum amyloid A, an acute phase-reactant protein (100). Serum copper levels increase during inflammation, associated with increases in ceruloplasmin, a Cu\(^{2+}\)-transporting protein that transfers Cu\(^{2+}\) to enzymes active in processes of basic metabolism and wound healing such as cytochrome oxidase and lysyl oxidase (101, 102), and whose metabolism is abnormal in AD-affected neocortex (103). Whereas the neocortex contains high levels of Cu\(^{2+}\), the availability of Cu\(^{2+}\) is normally restricted by its binding to metalloproteins such as albumin and ceruloplasmin (104). However, the release of reduced copper from ceruloplasmin is greatly facilitated by acidic environments (52), and periods of mild acidosis may promote an environment of increased “free” Cu\(^{2+}\) and therefore promote its exchange to other regional proteins. Such an exchange of Cu\(^{2+}\) at low pH has been described as mediating the binding of serum amyloid P component, an acute phase reactant that also forms an amyloid, to the cell wall polysaccharide zymosan (105). A similar exchange of Cu\(^{2+}\) within a low pH milieu may be responsible for precipitating a fraction of the brain collections of A\(\beta\) in AD.

A further mechanism by which Zn\(^{2+}\), Cu\(^{2+}\), and H\(^+\) levels may be elevated in the cortical interstitium is through abnormal energy metabolism. Intracellular concentrations of Zn\(^{2+}\) and Cu\(^{2+}\) are approximately 1000- and 100-fold higher than extracellular concentrations, respectively (77, 78, 95). This large gradient between intracellular and extracellular compartments is sustained by highly energy-dependent mechanisms (95, 106). Therefore, alterations in energy metabolism, such as those seen in AD (49), trauma, or vascular compromise, may affect the compartmentalization of these metal ions and possibly promote their pooling in the A\(\beta\) compartment. A\(\beta\) precipitation by Cu\(^{2+}\) in this case may be potentiated by the acidosis that is associated with abnormal energy metabolism. These mechanisms may contribute to the rapid appearance of A\(\beta\) deposits reported to occur following head injury (16, 107). The reversible aggregation properties of A\(\beta\) at local sites of injury may, in this context, be compatible with a role for the peptide in maintaining regional structural integrity.

If the involvement of Cu\(^{2+}\) in A\(\beta\) deposition in AD is confirmed, then the reversibility of this pH-mediated, Cu\(^{2+}\)-induced interaction presents the potential for therapeutic intervention. Thus, cerebral alkalinization or metal ion chelation may be explored as potential therapies for the reversal of A\(\beta\) deposition in vivo.

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Copper-induced Aβ Aggregation Is Modulated by pH

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